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Immunology

Mitogenic stimulation of human tumor-infiltrating lymphocytes by secreted factor(s) from human tumor cell lines

(cytokines/immunotherapy)

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ABSTRACT **Tumor-infiltrating lymphocytes (TILs) have shown *in vivo* antitumor efficacy in both animal and human studies. Functions thought necessary for antitumor activity include cytolysis, homing, and proliferation at tumor sites. TILs, which are T lymphocytes grown *ex vivo* directly from tumors, bear interleukin 2 (IL-2) receptors capable of transducing the IL-2 mitogenic signal. However, IL-2 is not normally synthesized by solid tumor cells. This study was aimed at exploring the possible presence of T-cell mitogens of tumor origin. To this end four TIL lines derived from four melanoma patients were studied for their ability to use the environments of cultured tumor cell lines as mitogenic sources. The presence of four irradiated cultured human tumor cell lines, three of which were derived from the same melanoma patients as the TILs, were found to stimulate proliferation of human TILs in the absence of IL-2. Further investigation showed that the observed proliferative stimulation by the fourth tumor line was due to secreted factor(s) as mitogenic activity was present in the serum-free tumor cell supernatant. Both immunologic analyses of this medium and proliferative assays in which TILs were stimulated with recombinant lymphokine standards suggest the presence of a yet uncharacterized T-cell mitogen.**

The idea that the tumor environment is intrinsically immunogenic and, therefore, contains lymphocytes with the potential to induce tumor regression has led to the use of tumor-infiltrating lymphocytes (TILs) in clinical trials (1–14). In this procedure lymphocytes are grown *ex vivo* directly from tumors in the presence of the lymphokine interleukin 2 (IL-2); patients are reinfused with their TILs after the latter have been expanded in culture for several weeks. Although 60% of metastatic melanoma patients who had not previously received any immunotherapy showed objective responses in the largest clinical TIL trial to date (15), the characteristics of the tumor environment that produces TILs *in vivo* remain unclear.

Two classes of stimuli—one derived from cell–cell contact between tumor cells and immunocytes and the other from soluble factors—may influence T-cell homing and proliferation. IL-2 is a potent mitogen for T cells, the cell type of TILs used (Table 1); however, its biosynthesis appears to be confined to immunocytes (16). This fact is consistent with the current belief that immunologically competent cells such as lymphocytes and monocytes represent the major sources of secreted mitogenic stimulation for lymphocytes. In this study the subject of soluble factors secreted by tumor cell lines as sources of stimuli for lymphocyte proliferation was addressed.

As a starting point for this work, the issue of whether a tumor cell line established from a melanoma tumor could stimulate the growth of TILs derived from melanoma masses was examined. After confirming the amplification potential of three melanoma cell lines, a nonmelanoma line was tested for the same bioactivity and found to be at least as potent a mitogenic source; tumor-derived mitogenic activity for TILs could be ascribed to secreted factor(s) as mitogenic activity for TILs was found in its serum-free supernatant. Immunologic and proliferative assays indicate nonidentity of at least one secreted factor with any previously characterized lymphokine.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM) (4.5 g of glucose per liter) and Ham's F-12 medium were purchased from Flow Laboratories; AIM-V and RPMI 1640 media and fetal calf serum (FCS) were from GIBCO; insulin/transferrin/sodium selenite medium supplement was from Sigma; human albumin (Albutein) was from Alpha Therapeutic (Los Angeles); and [^{3}H]thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. Recombinant human interleukins 4 and 6 (IL-4 and IL-6) and tumor necrosis factor α (TNF-α) as well as neutralizing murine monoclonal antibody against human IL-6 (catalog 40028) were from Genzyme; rabbit anti-human IL-2 IgG antibody (catalog 40012) was from Collaborative Research; recombinant human transforming growth factor β (TGF-β) was from R and D Systems; and IL-2 came from Cetus. Fluorescein- and phycoerythrin-labeled antibodies (anti-CD3, -CD4, and -CD8) were from Becton Dickinson.

The clone of A-431 cells used in this study was from J. E. DeLarco (Monsanto), dog smooth muscle cells were from T. Innerarity (Gladstone Foundation, University of California, San Francisco), Madin–Darby canine kidney (MDBK) cells were from the American Type Culture Collection, and the 618, 677, and 660 tumor lines were established from human melanoma tumors by culturing in RPMI 1640/10% human serum. Four T-lymphocyte lines, TILs 618, 641, 660, and 677, were established from four human melanoma tumors as described (17).

A bioreactor with a cartridge containing hollow fibers having a molecular-weight cutoff of 10 kDa [Cellco Advanced Bioreactors (Kensington, MD)] was used for the large-scale culture of A-431 cells.

ELISA kits for IL-2, IL-4, and IL-6 were from Genzyme; a second ELISA kit for IL-2 was from Collaborative Research.

METHODS

Culturing. All tumor lines were carried in DMEM 10% FCS, and the four TIL lines were in AIM-V supplemented with IL-2 at 1000 units/ml.

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Abbreviations: TIL, tumor infiltrating lymphocyte; IL-2, -4, and -6, interleukins 2, 4, and 6, respectively; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ECSM, extracapillary space medium; SFM, serum-free medium.

Table 1. Phenotypes of TILs measured by flow cytometry

TIL	$CD3^+$, %	$CD8^+$, %	$CD4^+$, %
618	97	83	12
677	99	93	3
641	97	19*	95
660	98	88	<3

*Nineteen percent of 641 TILs were double-positive for both CD8 and CD4; none was singly positive for CD8.

The serum-free medium (SFM) used for proliferation assays was a modification of a serum-free medium used for melanoma cells (18) with DMEM/Ham's F-12 at a 1:1 ratio substituting for DMEM alone.

The bioreactor cartridge was seeded with $\approx 5 \times 10^9$ A-431 cells in DMEM/10% FCS. After 3 weeks, the medium in the extracapillary space (ECSM) of the cartridge was switched to the SFM and the FCS concentration in the reservoir medium was lowered from 10% to 2%. The ECSM was drained daily from the cartridge, which was then filled with fresh SFM. The conditioned medium (ECSM) was then centrifuged, filtered (0.22 μm), and stored at 4°C.

Phenotyping. Staining was done at 4°C for 30–60 min in Hanks' balanced salt solution/10% FCS/0.02% NaN_3. Flow microfluorometric analyses were performed by using a Coulter EPICS flow cytometer.

Cellular Proliferation Assays. Tumor cells at 5×10^4 cells per well were plated in 96-well flat-bottomed plates. Confluent cultures of tumor cells were irradiated (3000 rads) (1 rad = 0.01 Gy) just before cocultivation with TILs. Lymphocytes were removed from IL-2-containing AIM-V and placed in the appropriate medium for bioassay 48 hr before commencement of proliferation assays. They were resuspended in SFM or DMEM/10% FCS at 1.25×10^5 lymphocytes per ml for cocultivation experiments and at 6×10^5 lymphocytes per ml for experiments in which soluble factors were being assayed; 200 μl of cell suspension was added per well. Cellular proliferation was determined in the presence of conditioned media and cytokines at the indicated concentrations. For experiments in which neutralization by an antibody was being measured, conditioned media were preincubated with the antibody at concentrations up to 10-fold the labeled neutralization capacity for 2.5 hr at 37°C before the addition of cells.

After 24- or 48-hr stimulation, the level of lymphocyte proliferation was assayed by adding 0.5 μCi of [^{3}H]thymidine to each well of a 96-well plate for 4 or 18 hr. A Skatron harvester was used to harvest the cells, and the radioactivity was counted by using an LKB β-plate reader. Each measurement was done in sextuplicate; each experiment was repeated at least three times.

RESULTS

Does the Presence of Tumor Cells Alter the Growth Characteristics of TILs? In Fig. 1*a* TIL line 618 cells are shown growing in large clusters in suspension. In contrast, in Fig. 1*b* these TILs in the presence of a monolayer of cell line 618 tumor cells are seen growing in tight apposition to the tumor cells; the absence of large clusters of lymphocytes suggests the predominance of cellular heterophilia over homophilia. Thus, tumor cells induced a striking change in the morphology of TIL–TIL interactions. The question of whether this morphologic alteration was due to an interchange of biologic signals between the two different cell types was addressed.

Do Tumor Cells Potentiate TIL Proliferation? To determine whether a tumor cell line can stimulate growth of TILs the cellular proliferation rates of TIL lines derived from four (618, 677, 641, and 660) patients were determined with and without an irradiated tumor cell line established from one of the patients (618). Because all four TIL lines had a culture history of IL-2 dependence, the initial set of experiments was done in the presence of IL-2 (1000 and 2 units/ml) as well as in its absence. As seen in Fig. 2*a* with IL-2 present at either 1000 or 2 units/ml, 618 tumor cells did not enhance by >2 SD [^{3}H]thymidine incorporation into DNA of any of the four TIL lines except for 677 at 2 units of IL-2 per ml; the latter was barely significant because the observed enhancement was by just 2 SD. In contrast, Fig. 2*b* shows that without IL-2, proliferation of both 618 and 660 TIL lines was dramatically increased when irradiated 618 tumor cells were present. The response of the 677 TIL line was again marginally significant. Presence of tumor cells had no effect on the proliferation of the 641 TILs, a cell line with a phenotype of 100% $CD4^+$. The three other TIL lines (618, 660, and 677) are composed of, at least, 80% $CD8^+$ cells. Hence, lymphocyte proliferation assays suggested the proliferative potentiation of tumor cells for $CD8^+$ lymphocytes in the absence of IL-2.

Is Serum Essential to the Tumor Potentiation? To determine whether tumor stimulation in the absence of IL-2 was inde-

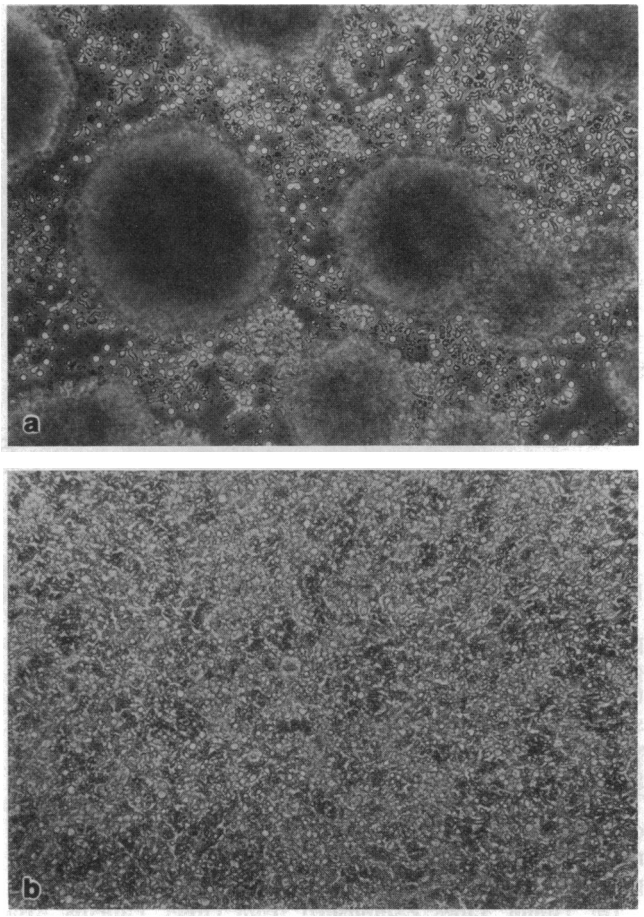

FIG. 1. TILs growing in the absence (*a*) and presence (*b*) of 618 tumor cells. Tumor cells were plated at 5×10^4 per 0.2 cm^2 in DMEM/10% FCS. After 1 day the medium was changed to SFM and left for at least 48 hr. TILs at 1×10^6 cells per ml were plated without and with a confluent monolayer of 618 tumor cells. Twenty-four hours later the cultures were examined and photographed. In the former, lymphocytes grow as large clusters, whereas in the latter cellular heterophilia—i.e., adhesion between lymphocytes and tumor cells—predominates over cellular homophilia—i.e., adhesion among lymphocytes. Although TILs 618, 660, and 677 could lyse autologous tumor cells that had been frozen but never cultured, they showed virtually no lytic capability against any of the cultured tumor lines in the configuration used in this study—i.e., as adherent monolayers. This sharply contrasts with lymphokine-activated killer cells, which effectively lysed all tumor lines used in this work. (×110.)

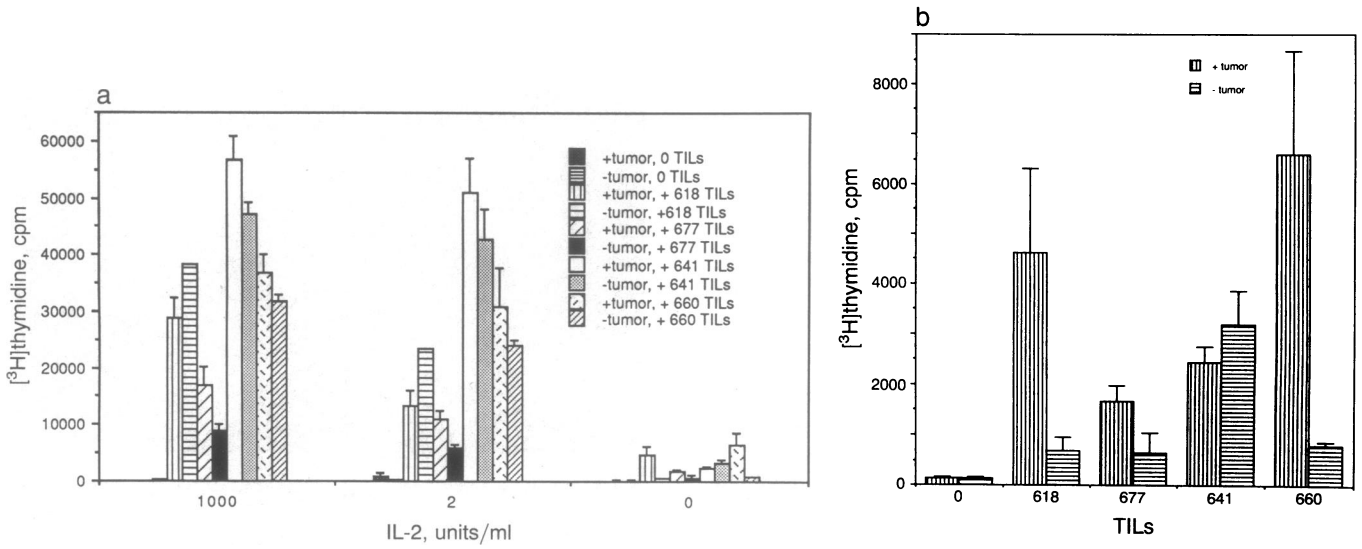

FIG. 2. Effect of irradiated 618 tumor cells on proliferation of four TIL lines at three IL-2 concentrations: 1000, 2, and 0 units/ml (*a*). (*b*) Expansion of right third of Fig. 2*a*: proliferation in the absence of IL-2.

pendent of serum factors, proliferation assays were done in parallel in SFM and DMEM/10% FCS. Fig. 3 indicates that the proliferation levels of both 618 and 660 TILs were significantly increased by 618 tumor cells whether or not serum was present. The proliferation of 677 TILs was marginally affected—i.e., by not >2 SD in either medium. 641 TILs showed statistically insignificant changes upon addition of tumor cells. Hence, the results suggested that the enhancement of proliferation of TILs by 618 tumor cells was independent of serum-derived factors.

Do Tumor Lines Established from Other Patients Also Enhance TIL Proliferation? Proliferation of TIL 660, the best responder to tumor cell-enhanced proliferation, was measured in the presence of two additional melanoma lines, 660 and 677, both established from the respective TIL patients. Data listed in Table 2 show a similar 3- to 4-fold increase in the proliferative response of 660 TILs to the presence of irradiated 660 or 677 tumor lines, seen with the irradiated 618 tumor line.

Is Tumor Cell-Induced Potentiation Specific to Cells of Melanoma Origin? Because melanomas derive from the neural crest, cells of a line not derived from this embryologic origin—i.e., the A-431 cell line, which was originally derived from an epidermal carcinoma, were tested for ability to potentiate TIL proliferation in both serum-containing and serum-free media. Fig. 3 shows that irradiated A-431 cells potentiated proliferation of all four TIL lines in serum (Fig. 3*a*) and showed effects similar to 618 tumor cells in SFM (Fig. 3*b*).

Is Potentiation Specific to Transformed Cell Lines? In contrast to the melanoma and A-431 cell lines tested, irradiated dog smooth muscle cells and MDBK cells, two untransformed cell lines, did not potentiate TIL proliferation (data not shown). Although this fact, along with stimulation by the murine melanoma B16 line, supports the concept of TIL stimulation by transformed and not normal cells, it would be premature to make this generalization, as all cell lines by virtue of their ability to survive *ex vivo* for extended times have acquired some traits of a transformed phenotype. Validation of tumor specificity awaits determination of the presence or absence of a purified factor in fresh uncultured cells.

Is Proliferative Activity Due to Secreted Factors? A scanning electron micrograph shows A-431 cells in the hollow fiber

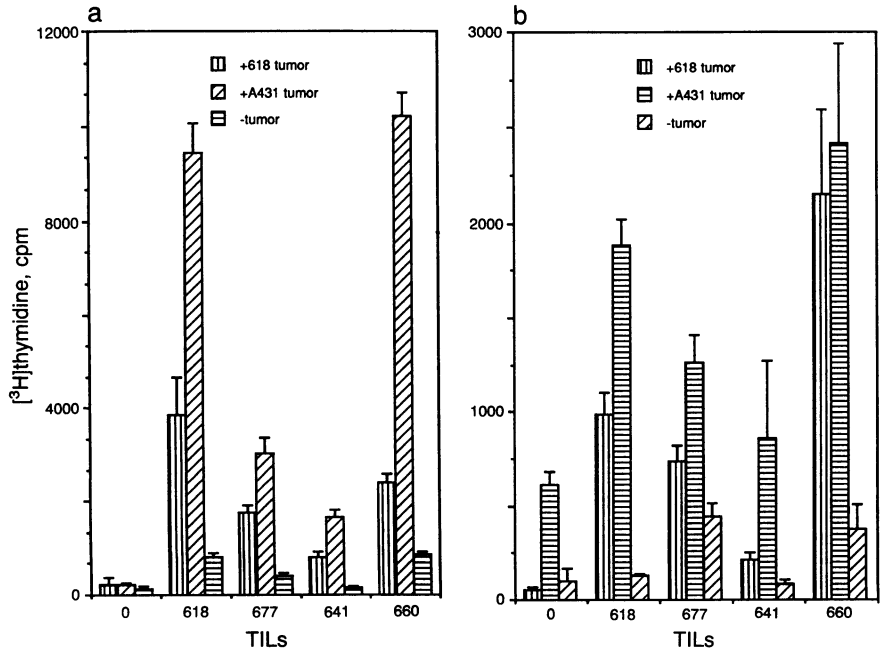

FIG. 3. Effect of serum on tumor-potentiated TIL proliferation. Comparison of enhanced TIL proliferation due to irradiated 618 tumor cells with that from irradiated A-431 cells in both serum-containing (*a*) and serum-free (*b*) media. A higher level of radioresistance of the A-431 cells resulted in a higher background for the assays in SFM.

Table 2. Proliferation of 660 TILs in SFM in the absence and presence of irradiated melanoma cell lines

Tumor cell line	[^{3}H]Thymidine, cpm
None	355 ± 54
618	1661 ± 57
677	1289 ± 52
660	1182 ± 154

Background counts for tumor alone have been subtracted.

cartridge in cross section (Fig. 4). Tumor cells were only seen in the space outside the hollow fibers. The membrane lining these fibers had a molecular mass cutoff of 10 kDa; hence, any factors secreted by the tumor cells with a molecular mass >10 kDa were retained in the ECSM. The medium that circulated inside the hollow fibers was composed of DMEM/2% FCS and was designated as the reservoir. The ECSM was drained daily and assayed for bioactivity.

Rates of [^{3}H]thymidine incorporation into both 618 and 660 TILs as a function of increasing concentrations of ECSM are plotted in Fig. 5*a*. There is a dose-dependent increase in proliferative activity up to the assay composition of 50% ECSM for both 618 and 660 TILs. Above this level both curves fall off. These declines may be due to the absence of nutrients in the spent ECSM or the presence of inhibitory factor(s). Additionally, these curves may also represent behavior similar to that of other known mitogens, for which at high concentrations proliferative levels are submaximal. In support of the latter hypothesis—when partially purified material was used as the stimulus, the dose–response curve was U-shaped with the maximum observed at ≈1 mg of protein per ml (data not shown).

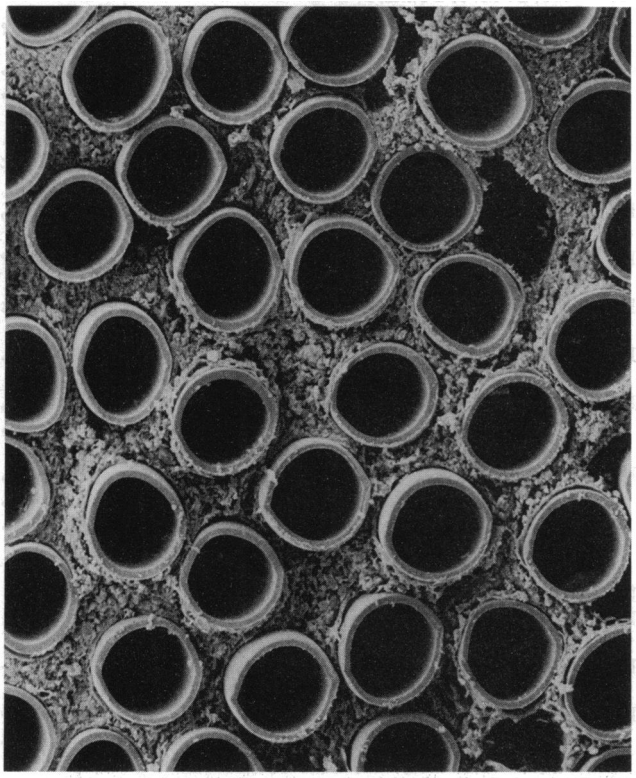

FIG. 4. Scanning electron micrograph of A-431 cells grown in hollow fiber bioreactor. Dark insides of hollow fibers that contain reservoir medium—i.e., DMEM/2% FCS—are separated from extracapillary space containing A-431 cells by a membrane with a 10-kDa cutoff. (×60.)

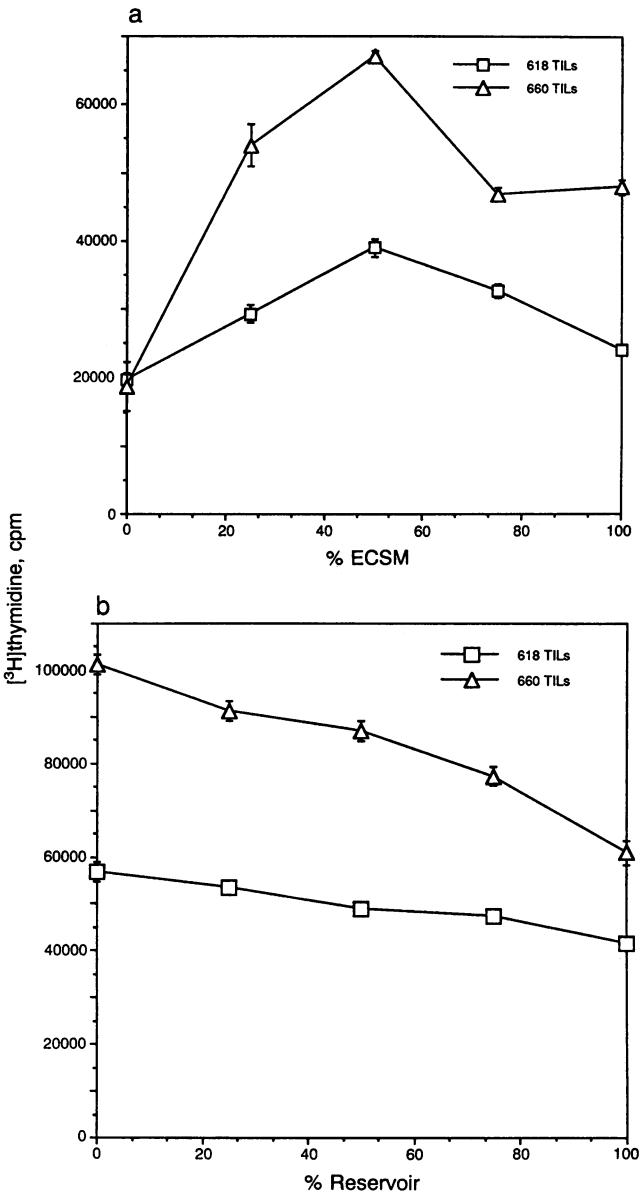

FIG. 5. Proliferation assays with serum-free supernatants from bioreactor ECSM secreted by A-431 cells (*a*) and serum-containing medium of reservoir—i.e., dark insides of hollow fibers shown in Fig. 4 (*b*).

In Fig. 5*b* rates of [^{3}H]thymidine incorporation into both 618 and 660 TILs as a function of increasing concentrations of reservoir medium are plotted. No stimulatory activity was detectable. Thus, molecular mass of the mitogenic factor(s) must be, at least, 10 kDa as no mitogenic activity was associated with factor(s) secreted into the reservoir.

Is the Secreted Activity Due to Known T-Cell Mitogens? To determine whether the mitogenic activity present in ECSM was due to factors that have previously been shown to have direct mitogenic activity for T cells, three ECSM samples collected at 2-week intervals were tested for IL-2, IL-4, and IL-6 by ELISA.

The presence of neither IL-2 (using kits from the two indicated companies) nor IL-4 was measurable at or above detection levels for these two lymphokines—i.e., 0.4 Cetus unit per ml and 90 pg/ml, respectively. At these two respective concentration levels no significant proliferative activity above background was detected. However, IL-6 was found to be present at 2 ng/ml; the presence of IL-6 in A-431 cell supernatants has been reported (19).

To ascertain whether IL-6 could be a mitogen for TILs under the conditions used in this study [^{3}H]thymidine incorporation was measured after culturing 660 TILs for 24 hr in the presence of IL-6 at concentrations ranging from 0.01 to 20 ng/ml; no [^{3}H]thymidine incorporation above background was measured. Furthermore, the presence of a neutralizing monoclonal antibody against IL-6 had no effect on the stimulation of [^{3}H]thymidine incorporation induced by ECSM, whereas mitogenic stimulation by IL-2 standards up to 100 units/ml was completely inhibited by the anti-IL-2 antibody used.

Lack of mitogenic activity for 660 TILs was also seen for TGF-β ($1 \times 10^{-7,-8,-9,-10,-11}$, and $^{-12}$ M) and TNF-α (1, 10, and 100 ng/ml). In all experiments IL-2 and ECSM served as positive controls.

DISCUSSION

The idea that the tumor environment contains lymphocytes that may exhibit antitumor activity was the basis for using lymphocytes expanded in culture as therapeutic agents (1–14). Much work supports the concept that an immune response is initiated against tumors via direct tumor cell–lymphocyte contact, followed by the secretion of factors mitogenic for lymphocytes from several immunocyte sources. However, the possibility of tumor cells providing a mitogenic stimulus for lymphocytes in the form of secreted factors has not been well explored. Thus, the objective of this work was to determine whether tumor cells could secrete factor(s) mitogenic for lymphocytes.

Fig. 1*a*, which shows TILs growing in the presence of IL-2, is typical of cultures of activated lymphocytes—i.e., clusters of cells in suspension. In contrast, in the presence of monolayers of tumor cells TILs have a spread appearance (Fig. 1*b*). To determine whether the observed apposition between TILs and tumor cells was part of an immunostimulatory environment, proliferation of four TIL lines was compared in the presence and absence of irradiated tumor cells. In the presence of IL-2 addition of irradiated culture 618 tumor cells did not significantly affect mitosis of the four TIL lines tested (Fig. 2*a*). However, in the absence of IL-2 (Fig. 2*b*) these tumor cells dramatically increased the proliferation rates of two TIL lines, 618 and 660, and increased less significantly the rate of a third, 677. Thus, cultured tumor cells can support T-cell mitogenesis independently of IL-2. This fact may be essential in understanding the observed disparity in phenotypic profiles of TIL cultures at the stage of reinfusion into patients with those of the starting populations (15, 20). That is, because receptors for IL-2 are present on virtually all activated T cells (21), the T-cell population that will grow *ex vivo* in the constant presence of this lymphokine is strongly biased toward those cells capable of transducing and using the IL-2 signal most efficiently. Hence, factors from the tumor environment, which may be essential for maintenance of lymphocytes with antitumor activity, may not be retained in long-term cultures in which all tumor cells have died and the only exogenous cytokine is the pan-T-cell mitogen IL-2.

In the present study the observed mitogenic stimulation of TILs by irradiated tumor cells was shown to be of cellular origin and not from the serum, as indicated by Fig. 3. In addition to the tumor line established from patient 618, data in Table 1 show that tumor lines from two other melanoma patients, 677 and 660, were similar in their ability to enhance proliferation of 660 TILs in SFM. Thus, the presently reported mitogenic T-cell stimulation by tumor cell lines, all of which were derived from unrelated patients, is clearly not major histocompatibility complex-restricted.

To determine whether tumor cell-induced lymphocyte mitogenicity is characteristic of tumors of a specific embryonic origin, such as the neural crest, which includes melanomas as well as neuroblastomas, a cell line derived from a tumor of epidermal carcinoma origin, the A-431 line was used as a source of potentiation of TIL proliferation. Data indicating that A-431 cells can serve as a superior mitogenic source for TILs in serum (Fig. 3*a*) and equivalently to the 618 melanoma line in SFM (Fig. 3*b*) suggest that this type of immunocyte stimulation may be broadly prevalent.

Proof that activity mitogenic for TILs is secreted by A-431 cells was confirmed by the activity in the ECSM (Fig. 5*a*). These dose–response curves indicate the presence of cytokines that exhibit behavior similar to that of other known mitogens, where submaximal proliferative levels have been measured at high concentrations. The absence of IL-2 and IL-4 by ELISA and the lack of proliferative stimuli from IL-6, TNF-α, and TGF-β under the experimental conditions used suggest the presence of a direct T-cell mitogen not previously characterized.

In conclusion, tumor cell lines can increase the proliferative potential of TILs in the absence of IL-2. The molecule(s) responsible for this activity may be essential components of the biochemical basis of immunogenicity observed *in vivo* and require further characterization.

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