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A multicellular tumor spheroid model of cellular immunity against head and neck cancer*

Peter G. Sacks[1, 2], Dorothy L. Taylor[1], Tamas Racz[1, 3], Tracey Vasey[2], Victon Oke[2], and Stimson P. Schantz[1]

[1] Departments of Head and Neck Surgery, and [2] Tumor Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas, USA
[3] Department of Head and Neck Surgery, National Institute of Oncology, Budapest, Hungary

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Summary. A multicellular tumor spheroid (MTS) model for head and neck cancers has been used to examine the immune function of fresh and 6-day interleukin-2(IL-2)-activated peripheral blood lymphocytes (PBL). MTS are individually cultured in the presence of effector cells, and the spheroids' growth is monitored by sizing them under an inverted microscope. Dose/response studies for IL-2 (0–100 U/ml) alone and for fresh unstimulated PBL (0–10^5 cells/MTS) showed no effects on MTS growth. IL-2-activated PBL (0–10^5 cells/MTS), in contrast, modulated MTS growth in a multiphasic pattern: MTS growth was unperturbed for the first 3 days and then growth inhibition occurred, followed by MTS disintegration. Histological analysis showed that intact MTS histoarchitecture correlated with unperturbed growth, and increasing cell sloughing and MTS dissolution and replacement by activated PBL correlated with growth inhibition and disintegration. Flow-cytometric sorting of lymphocyte subset populations indicated that it was the Leu19$^+$CD3$^-$ cells that produced these growth-modulatory effects. In contrast to the initial LAK cell resistance of MTS, single-cell suspensions demonstrated significant lysis in standard 4-h chromium-release assays. Differences between single cells and MTS suggest a potential for tissue-like organization as a factor in lymphokine-activated killing.

Introduction

An important immunological effector mechanism within head and neck cancer patients may reside within the natural killer (NK) cell. NK cells have been characterized morphologically as large granular lymphocytes and functionally by their ability to lyse tumor targets without major histocompatibility complex (MHC) class I restriction [23]. Previous efforts have noted that deficient NK cell function, as measured in vitro against K562 target cells, can be identified within the head and neck cancer population.

Furthermore, such quantitative measures of natural immune status have prognostic implication. Those patients with deficient NK cell function, as expressed by peripheral blood lymphocytes (PBL), are at increased risk of death from uncontrolled metastatic disease [16, 17]. Additional support for this association between deficient NK cell function and metastatic disease was provided by Mickel et al. [8], who noted that deficient NK cell function was expressed by lymphocytes within regional lymph nodes of head and neck cancer patients. Those lymph nodes that contained microscopic foci of metastatic disease contained lymphocytes with the most deficient cytotoxic activity.

These results emphasize the potential biological significance of the interaction between non-MHC-restricted killer cells and squamous cell carcinoma (SCC) of the upper aerodigestive tract. However, such interaction can only be defined by inference because of the limitations inherent in the nature of existing in vitro methods. The standard in vitro cytotoxicity assay, i.e., the chromium-release assay against allogeneic targets, may have only limited relationship to in vivo circumstances. In vivo, lymphocytes interact with tumor masses over a protracted period. In all likelihood, during such a prolonged interval, the host immunological status would not be constant. Likewise, the microenvironment within a neoplastic growth undergoes changes. Thus, actual tumor cell/lymphocyte interactions in vivo may bear no relationship to biological inferences drawn from standard cytotoxicity assays.

A potentially valuable method of analyzing host/tumor interactions may reside in the multicellular tumor spheroid (MTS) growth model. As compared with monolayer cultures and single-cell tumor suspensions, the MTS more closely approximate the three-dimensional in vivo growth of solid tumors [9, 20]. Using MTS it is possible to study

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Offprint requests to: S. P. Schantz, Department of Head and Neck Surgery, Box 69, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030, USA

interactions between lymphocytes and aggregate tumor structures. Such interactions may more meaningfully model the cell-to-cell interactions within solid tumors. We describe here an MTS tumor model for squamous cell carcinoma of the upper aerodigestive tract.

Materials and methods

Culture medium and reagents. We used RPMI-1640 medium (Gibco, Grand Island, N. Y.) supplemented with 10% fetal calf serum (Irvine, Santa Ana, Calif.), 1% glutamine, and 2.5% gentamicin, termed supplemental culture medium (SCM). Medium used for culturing squamous cell carcinoma cell lines was a 1:1 mixture of Dulbecco's minimal essential medium (Gibco) and Ham's F12 medium (Gibco) supplemented with 10% fetal calf serum and antibiotics, termed tumor cell medium. Hanks' balanced salt solution (HBSS), used in washing cells, as well as Dulbecco's phosphate-buffered solution, used in monoclonal antibody labeling for flow cytometry, were obtained from Gibco.

Preparation of peripheral blood lymphocytes. Effector cells (PBL) were prepared as previously described [17]. Briefly, lymphocyte-rich mononuclear cells were isolated by centrifugation at 400 *g* for 50 min on a Ficoll/sodium diatrizoate density gradient (density 1.077 g/cm^3, Litton Bionetics, Charleston, S. C.). Cells at the interface were collected, washed twice in HBSS, and resuspended in SCM. Monocytes were removed from the mononuclear cell suspensions by incubation for 1 h in 15 ml SCM on 100×15-mm plastic petri dishes at 37° C. Nonadherent cells were recovered by repeated washings with SCM and resuspended in 20 ml SCM. Recovered cells were then stored at 4° C for 18 h in each instance before cytotoxicity testing, unless cultured in interleukin-2 (IL-2) as described below.

IL-2 stimulation of PBL. PBL, as isolated above, were incubated in 24-well plastic dishes (each well measuring 16×20 mm) (Costar, Cambridge, Mass.) at 2×10^6 cells/well. Cells were incubated for 6 days at 37° C, in a 5% CO_2 atmosphere in a total volume of 2 ml SCM per well, as previously reported [18]. Included in the media were various concentrations of human recombinant IL-2 (Roussel UCLAF, Romainville, France) (1.3×10^6U BRMP). The biological activity of this recombinant IL-2 in our culture concentrations has been previously described [18]. At 6 days, these lymphokine-activated killer (LAK) cells were harvested and washed in SCM. Viability upon completion of IL-2 culture was 95%.

Target cell preparation. Target cell populations used in cytotoxicity assays included K562, an erythroleukemia cell line, and squamous cell cancer (SCC) lines MDA 686-Ln and MDA 886-Ln. As previously described, MDA 686-Ln was developed from a moderate-to-poorly differentiated SCC metastatic to a regional lymph node from a primary cancer at the base of the tongue [18] and MDA 886-Ln from a moderately differentiated laryngeal lymph node metastasis [11]. Both SCC lines were established by explant outgrowth techniques as previously described [11, 13]. SCC lines were routinely grown in tumor cell medium, passaged weekly using 0.05% or 0.125% trypsin in 2 mM EDTA, and resuspended at split ratios of 1:3 or 1:6 for MDA 686-Ln and MDA 886-Ln, respectively. Prior to labeling with chromium for cytotoxicity assays, SCC lines were detached from culture dishes using 0.05% trypsin and 2 mM EDTA for 10 min. All remaining handling of SCC cell line and K562 targets for chromium labeling was as previously described [18]. The K562 cell line was maintained in SCM. When used as single-cell targets in the chromium-release assay, MTS were dissociated into a single-cell suspension using a collagenase/pronase/DNase enzyme cocktail (0.2:0.5:0.2 mg/ml) plus mechanical shearing with a 23-gauge needle.

Cytotoxicity assay. Isolated PBL and chromium-labeled target cells were incubated at effector: target ratios varying from 6:1 to 100:1 as previously described [17]. After incubation and subsequent centrifugation, a 0.1-ml aliquot was removed and counted in a gamma spectrometer (Packard, Downers Grove, Ill). The percentage cytotoxicity was computed according to this formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100.$$

Maximum release (cpm) was determined by incubating target cells in 10% Triton X-100 for 6 h at room temperature.

Immunofluorescence labeling and cell sorting. PBL and tumor cells were labeled with mAb according to the manufacturer's specifications for flow-cytometric analysis. In each instance, 1×10^6 PBL were suspended in 50 µl Dulbecco's phosphate-buffered solution plus 0.2% sodium azide before the respective mAb were added. Isotype-matched fluorosceinisothiocyanate(FITC)- and phycoerythrin-conjugated antibodies were used as controls to exclude Fc-related binding; all procedures were performed at 4° C. All dilutions and washings were done with Dulbecco's phosphate-buffered solution plus 0.2% sodium azide. When fluorochrome-conjugated antibodies were not used, a second-step reagent consisting of FITC-conjugated goat anti-(mouse Ig) mAb was added after initial labeling and was incubated for 20 min. Typically, 10000 cells were analyzed for single- and dual-color immunofluorescence.

The mAb used for PBL labeling in this study included anti-Leu19 mAb (NK cells) (Becton-Dickinson, Mountain View, Calif.) and the anti-CD3 mAb (T cell receptor) (anti-Leu4) (Becton-Dickinson). For separation of Leu19$^+$ cells, 2×10^7 cells were stained and sorted as previously described [18]. Cell viability after sorting by trypan blue exclusion exceeded 95%. The purity of each fluorescence-stained positive population was nearly 90%. Less than 3% contamination of Leu19$^+$ cells existed within the negative population. Morphologically, more than 90% of Leu19$^+$ cells were large granular lymphocytes.

MTS culture procedures. For MTS initiation, monolayers of MDA 886-Ln cells were dissociated with trypsin, as above, and plated on petri dishes precoated with 1.25% agarose in normal medium [11]. After 3 days growth, small spheroidal aggregates were transferred to spinner flasks and grown at 37° C; media were changed as needed. For experimental use, MTS of uniform initial diameter were picked under a dissecting microscope and sized using an inverted microscope with a calibrated reticle [diameter = $(ab)^{1/2}$].

MTS growth assays. MTS growth studies were performed in agarose-coated 24- or 48-well cluster dishes, one MTS plus effector cells per well. Effector cells were varied between 500 and 500×10^3 cells/well. MTS were sized and media replenished three times per week. With IL-2-activated PBL, IL-2 at 100 U/ml was added to the growth medium.

Histological analysis. For histological analyses, MTS were fixed in 10% formalin and processed using standard histological techniques. Slides were stained with hematoxylin and eosin.

Results

SCC line MDA 886-Ln grows as a monolayer culture; when plated on a nonadherent surface, it will grow as multicellular tumor spheroids. The cytotoxicity of single-cell suspensions of erythroleukemia line K562, SCC cell lines MDA 686-Ln, and MDA 886-Ln, and dissociated MDA 886-Ln MTS was determined in standard 4-h chromium-release assays (Table 1). Fresh PBL or 6-day IL-2-activated PBL were used as effector cells. As we reported previously both K562 monolayers and the SCC cell line MDA 686-Ln can give reproducible cytoxicity values in 4-h cytotoxicity tests [18]. Cytotoxicity against single-cell suspensions of MDA 886-Ln was similar, although slightly lower. The uptake of ^{51}Cr was only 20% of that of

Table 1. Four-hour cytotoxicity mediated by interleukin-2(IL-2)-activated peripheral blood lymphocytes (PBL) against squamous cell populations

Target	Cytotoxicity[a] (%)							
	Fresh PBL				IL-2-activated PBL			
	50:1	25:1	12:1	6:1	50:1	25:1	12:1	6:1
K562	41.9±4.2	23.3±1.8	13.0±0.9	5.8±0.1	63 ±1.1	57.9± 8.3	47.6±7.1	28.5±9.3
MDA 686-Ln	4.3±0.2	3.7±0.5	2.3±0.7	2.1±1.1	59.4±4.9	53.9±10.3	40.9±8.2	26.9±8.3
MDA 886-Ln	4.9±2.5	3.8±5.4	3.1±4.4	2.0±2.8				
MDA 886-Ln MTS					45.2±4.9	39.1± 0.7	29.6±1.4	17.6±2.1
Cells[b]	–	–	–	–	–	–	–	–

[a] PBL were freshly isolated or incubated for 6 days in IL-2 (100 U/ml) and tested for cytotoxicity against the respective cell. Mean ± SD from two repetitions is shown

[b] Single-cell preparations from dissociated multicellular tumor spheroids (MTS) had spontaneous release of over 60%, precluding assessment of percentage cytotoxicity

K562 and MDA 686-Ln. In contrast, single-cell preparations produced by dissociating MDA 886-Ln MTS with a collagenase/pronase/DNase enzyme cocktail could not be assayed for sensitivity to lysis because their spontaneous release was over 60%.

Although cell suspensions derived from MDA 886-Ln MTS could not be used as targets in the chromium-release assay, we examined whether MTS growth studies could be used as a cytotoxicity assay for unstimulated or IL-2-activated PBL. Initial studies (data not shown) monitored the growth of individual MTS cultured in 24-well cluster dishes with different effector cell concentrations (500–500×10^3 effector cells/well). Although destruction of MTS could be found when interactions occurred between MTS and IL-2/effector cells, the surface area of individual wells was so large compared with that of the MTS that in many wells the MTS and effector cells were not in contact. With 48-well dishes, however, MTS/effector cell interactions were reproducible.

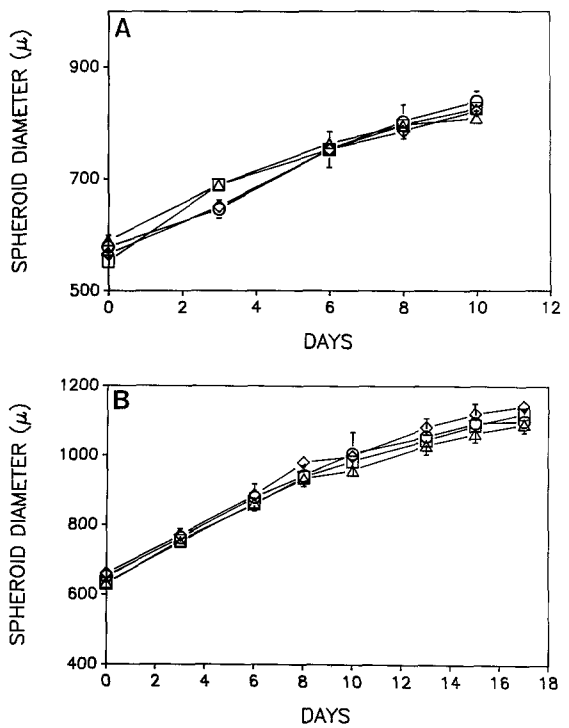

Fig. 1. A MTS growth study showing dose response for IL-2. Each point represents the average of six MTS, which were individually cultured in multiwell dishes. Medium was replenished three times per week, at which time diameters were measured. ○, Control; △, 100 U/ml IL-2; □, 10 U/ml IL-2; ◇, 1 U/ml IL-2. *Error bars* show SD. **B** MTS growth study showing dose response for effects of freshly isolated PBL. MTS were cultured with various concentrations of unstimulated PBL as described above. ○, Control, no PBL; △, 100 000 PBL; □, 50 000 PBL; ◇, 10 000k PBL

Dose/response studies for IL-2 (1, 10, 100 U/ml) were performed to examine the effects of IL-2 on MTS growth, since IL-2 is in the medium when activated cells are assayed. As shown in Fig. 1 A, no effect on MTS growth was evident. When MTS were cultured in the presence of various concentrations of unstimulated PBL, no effect on MTS growth was seen (Fig. 1 B). The increase in MTS diameter was linear over the first 8–10 days, and then the growth rate decreased as demonstrated by changes in slope.

In contrast to unstimulated PBL, 6-day IL-2-activated cells, inhibited MTS growth in culture, and disintegration of MTS occurred in a dose-dependent manner (Fig. 2, Table 2). MTS growth was unperturbed at all activated PBL effector:target ratios for the first 3 days. Growth inhibition occurred by day 6 for 100 000 and 50 000 LAK cells and by day 8 for 10 000 LAK cells (Fig. 2). Growth curves, as in Fig. 2, are derived from intact MTS and give no information on when disintegration occurs or how many MTS are left. Information on MTS disintegration is shown in Table 2. MTS integrity was maintained for 10 days, and disintegration occurred subsequently. By day 17, no intact MTS remained. The basic pattern presented in Fig. 2 [unperturbed MTS growth, inhibition (stasis), MTS disintegration] was reproducible with LAK cells derived from different donors.

IL-2 effects were further studied by culturing MTS with freshly isolated PBL plus IL-2 (100 U/ml) (Fig. 3). This study used PBL cells derived from two normal controls; although the duration of the stationary phase differs (12 vs 19 days), the pattern of unperturbed growth, stasis, and MTS disintegration is evident. The initial period of unperturbed growth is similar to that found when activated effector cells are used (Fig. 2), as is the timing of MTS

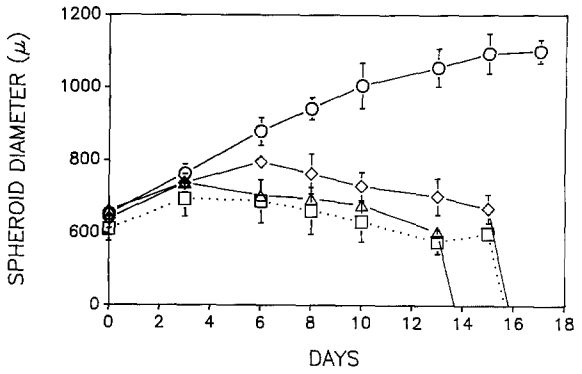

Fig. 2. Effects of varying concentrations of 6-day IL-2-activated PBL on MTS growth. PBL were activated and MTS grown in the presence of 100 U/ml IL-2. ○, Control, no PBL; △, 100000 activated PBL; □, 50000 activated PBL; ◇, 10000 activated PBL. The growth study performed as described in the legend to Fig. 1

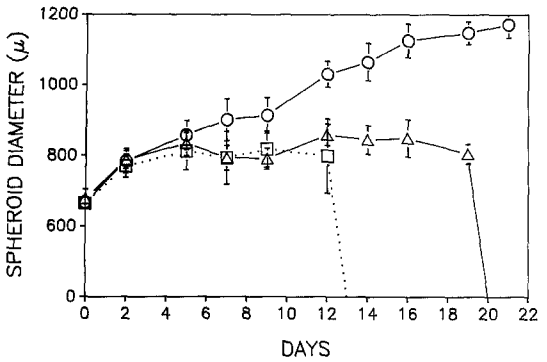

Fig. 3. MTS growth study using freshly isolated PBL (10000/well) obtained from two normal individuals (△, □) is compared to control MTS growth with no PBL (○). This growth study was performed in the presence of 100 U/ml IL-2, as described in the legend to Fig. 1

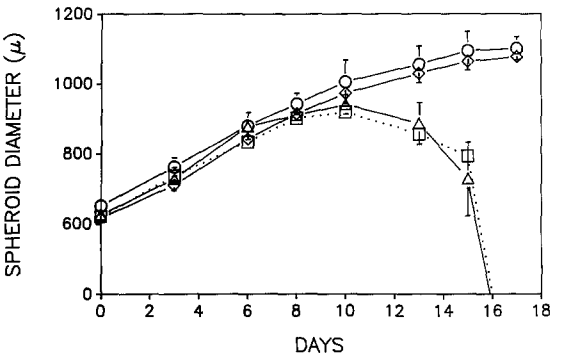

Fig. 4. MTS growth study performed using PBL obtained from a cancer patient on in vivo IL-2 therapy. Control MTS growth, no PBL (○), was compared with that in cultures with freshly isolated PBL, either unsorted (△) or sorted into Leu19$^+$ CD3$^-$ (□) or Leu19$^-$ CD3$^+$ (◇) (10000 PBL/well). The growth study was performed as described in the legend to Fig. 1

disintegration. With IL-2 in the medium, the assay produces similar results whether the PBL are freshly isolated or have been previously IL-2-activated.

The assay can utilize PBL derived from healthy individuals and from cancer patients. Figure 4 shows results

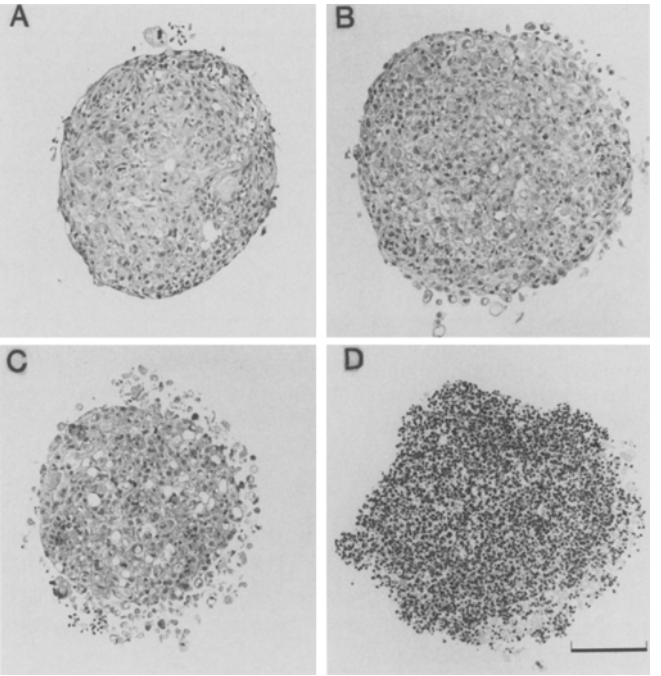

Fig. 5. A – D. Histological study of MTS cultured in presence of 100000 activated PBL. **A** Two-day MTS growth; **B** 5-day MTS growth. Note cellular disorganization and cell sloughing is apparent; **C** 7-day MTS composed primarily of tumor cells; **D** 7-day MTS replacement of tumor cells by PBL is evident. Bar = 100 μm

using PBL from a patient receiving therapy with recombinant IL-2 by continuous infusion. The details of this therapy will be reported soon (Schantz et al. unpublished results). PBL were further separated into Leu19$^+$CD3$^-$ and Leu19$^-$CD3$^+$ populations by fluorescence-activated cell sorting. Results of a standard chromium-release assay of respective populations from this individual at one clinical time point are shown in Table 3. Although unsorted and Leu19$^+$ cells have the ability to lyse K562 and to a lesser extent MDA 686-Ln cells in the 4-h assay (Table 3), MTS growth is initially unaffected. In this experiment, growth was unperturbed for 8 days, stasis occurred through day 13, after which MTS disintegrated. Leu19$^-$CD3$^+$ lymphocytes failed to mediate significant anti-tumor effects in either the chromium-release or the MTS assay.

In correlation with MTS growth studies, the morphological basis of growth studies was analyzed by sequentially fixing and histologically examining MTS that were being cultured in the presence of 100000 activated LAK cells (Fig. 5). At day 2, MTS were morphologically intact and, occasionally, serial sections showed effector cells adjacent to the MTS (Fig. 5 A). Day-5 MTS were irregular, with loosened cell-to-cell organization and an appearance of cell sloughing (Fig. 5 B). This dissolution of the MTS increased with time. MTS became fragile and difficult to process for histological studies as they fell apart. On day 7, two types of MTS were found. The first type displayed increased cell sloughing and dissolution (Fig. 5 C). The second MTS structure had been almost totally replaced by LAK cells (Fig. 5 D).

Table 2. Intact MTS in growth studies

Day	No. MTS[a]			
	Control	10×10^4 LAK	5×10^4 LAK	1×10^4 LAK
0	6	6	6	6
3	6	6	6	6
6	6	6	6	6
8	6	6	6	6
10	6	6	6	6
13	6	3	4	6
16	6	0	1	4
17	6	0	0	0

[a] The number of MTS remaining in each group identified in Fig. 2 at the indicated interval in days

Discussion

The immune function of unstimulated and IL-2-activated PBL is normally studied in short-term cytotoxicity assays using appropriate cell lines that show sensitivity to lysis. Standard cells used for natural killer activity include K562, an erythroleukemia cell line, while IL-2-activated function studies commonly use the Daudi cell line [3, 4]. We [18] and others (reviewed by Trinchireri [23]) have examined the potential of cell lines derived from specific tumor types to be used as target cells in standard chromium-release assays. In studies aimed at understanding natural killer cell function for tumors of the upper aerodigestive system, we have used a series of SCC cell lines in addition to the standard K562 target, as results produced with such cells may be more clinically relevant [17, 18].

An MTS model for squamous carcinoma of the head and neck has recently been established [11, 14, 15]. MTS have an organizational complexity intermediate between monolayer cultures and in vivo tumors. Their three-dimensional structure has strong analogy to in vivo tumors. Gradients in proliferation occur as in vivo, and their histoarchitecture is similar, with intercellular junctions, extracellular matrices, cell differentiation, and central zones of necrosis (reviewed by Mueller-Klieser) [9]. MTS have been used with murine models to study tumor/lymphocyte interactions in both in vitro and in vivo assays [6, 7, 19, 21]. A human glioma spheroid system has recently examined LAK-induced killing using chromium release from intact MTS [5]. In MDA 886-Ln MTS, spontaneous release is too high for this type of assay (unpublished results). In addition, the cloning efficiency of dissociated MTS is too low for the type of clonogenicity studies performed in murine models [6, 7, 19]. Low cloning efficiency is a general characteristic of squamous cell carcinomas [10]. It is also recognized that not all MTS systems can be successfully dissociated into single cells and it is believed that extracellular matrices and strong cell-cell junctions are responsible [1, 22]. In this context, single-cell preparations from dissociated MTS are also too fragile to be used in chromium-release assays, i.e., spontaneous release is too high, indicating cell damage. This probably results from disruption of numerous epithelial junctional complexes during the enzymatic and mechanical dissociation steps.

Using MTS growth assays, we showed that reproducible effects on MTS size and integrity can be produced. These effects are specific for IL-2-activated cells; when PBL were cultured with MTS in the absence of IL-2 (Fig. 1 B), no deleterious effects on MTS growth or histology were found. With activated effector cells, the reproducible response could be divided into three phases: a period of no effect on MTS growth, inhibition of MTS growth, and MTS disintegration. These responses were dependent on effector cell concentration (Fig. 2). There was not, however, an obvious correlation with the initial cytotoxicity values produced in a 4-h chromium-release assay. The response was similar whether the assay used activated cells, which have high 4-h cytotoxicity values, or unstimulated PBL, which have lower initial 4-h cytotoxicity values, as long as the growth studies were performed in the presence of IL-2.

The assay measures MTS size, is reproducible with respect to growth, growth inhibition, and MTS disintegration, but does not give information on tumor cell number or viability of cells within MTS. Similar results have been shown in EMT_6 spheroids; no correlation existed between spheroid size and cell number, primarily because of infiltration of lymphocytes [6, 7]. Our histological studies have shown that although an MTS can be measured, the histological composition of that structure requires specific examination. Two types of 7-day MTS were found (Fig. 5 C, D), one showing cell sloughing and dissolution but primarily composed of tumor cells, and the other composed mostly of effector cells.

In contrast to the 4-h chromium-release assay results, the interactions within the MTS growth assay occur over a

Table 3. Four-hour cytotoxicity results for in vivo IL-2-treated PBL[a]

Effector cell population[b]	Cytotoxicity (%)							
	K562				MDA 686-Ln			
	25:1	12:1	6:1	3:1	25:1	12:1	6:1	3:1
Unseparated	54±2	27±2	18±3	8±1	4±2	6±2	7±1	1±1
$Leu19^+CD3^-$	ND	34±2	19±2	8±1	ND	12±2	10±2	4±0.4
$Leu19^-CD3^+$	7±1	6±1	4±1	0	0	0	0	0

[a] Percentage cytotoxicity ± SD in a 4-h chromium-release assay for the given effector : target ratios. Effector : target ratios were performed in triplicate. ND, not done

[b] PBL were incubated for 6 days in IL-2 and tested either without further separation or following separation into $Leu19^+$ or $Leu19^-$ populations by fluorescence-activated cell sorting

prolonged time. Since monolayers of MDA 886-Ln can be lysed in the 4-h assay, the basis for this increased time span is at present unknown. Damage does not become evident for several days and this is probably related to the three dimensional structure of the MTS. Differences in cell-surface components, their topographical localization within membranes, and/or glycocalyxes could hinder initial LAK recognition. Individual MTS cells are joined by numerous cell-cell junctions (unpublished observations), which could also inhibit LAK penetration. An increased resistance, compared with monolayer cultures, has been noted for cells within spheroids exposed to mixed leukocyte cultures in a murine model [21]. Similarly, cells within MTS are known to exhibit increased resistance when exposed to ionizing and nonionizing radiation [2, 12]. In contrast to our model, in a glioma MTS model, effects on MTS size occurred within 24 h [5]. The increased time span of our model adds an attractive feature. Modulations in the standard chromium-release assay are limited, whereas the MTS growth assay has two periods in which target/effector cell interactions can be manipulated: the initial growth phase and the inhibitory phase prior to disintegration.

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