

# HETEROGENEITY OF TUMORIGENICITY PHENOTYPE IN MURINE TUMORS

## I. Characterization of Regressor and Progressor Clones Isolated from a Nonmutagenized Ultraviolet Regressor Tumor\*

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Tumor cell heterogeneity is an inadequately explored aspect of tumor biology, especially with respect to the complexity of the host-tumor interaction. The concept of heterogeneity is not a new one, and many studies have focused on single phenotypic traits of intratumor variants. Heterogeneity has been demonstrated for several physical and biochemical criteria, including morphology, karyotype, growth kinetics, anchorage independence, hormone sensitivity, drug resistance, and metastatic potential (1–7). Fluctuation analysis of cloned tumor variants offers convincing evidence that this heterogeneity exists *in vivo* and is not merely an artifact of *in vitro* culture (8). In these studies, however, cells were often used that had been derived from the venerable survivors of tumors propagated *in vitro* for years, even decades. In addition, many of these sublines had been coaxed from cultured “tumor” lines by disruptive intervention into the normal cultural life histories of the cells. One must therefore approach the interpretation of the results of these investigations with caution, because the cell lines used were sometimes of questionable biological relevance.

Systems are known in which manipulation of cells in tissue culture results both in tumorigenic variants that grow in normal hosts, and also in nontumorigenic variants that, when placed in the proper environment, can and will grow progressively to the death of the host (9–11). Boon and Kellermann (9) have isolated cloned tumor variants after *in vitro* mutagenesis of a malignant teratocarcinoma cell line with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. These variants differ in tumorigenicity phenotype ( $\text{tum}^+$  and  $\text{tum}^-$  variants) and are immunologically cross-reactive. Collins, Patek, and Cohn (10) have isolated similar variants after viral or chemical treatment of normal cells. Ultraviolet (UV)<sup>1</sup> carcinogenesis also results in the emergence *in vivo* of tumors of both phenotypes (regressors and progressors), possessing the same common tumor-associated antigens (TAA), in addition to unique tumor-specific transplantation antigens (TSTA; 11–14). The ability to induce both tumorigenic and nontumorigenic variants in cultured cells, and the existence of both regressor and progressor

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<sup>1</sup> Abbreviations used in this paper: B:T, blocker:target ratio; CTL, cytotoxic T lymphocytes; DLN, draining lymph node cells; E:T, effector:target ratio; MEM, minimal essential medium; TAA, tumor-associated antigens; T<sub>s</sub>, suppressor T cells; TSTA, tumor-specific transplantation antigens; UV, ultraviolet.

UV tumors suggested to us that heterogeneity of tumorigenicity phenotype might preexist within tumors and may in fact be an important influence on the growth of a neoplasm in a primary host. The isolation and characterization of clonal outgrowths of these subpopulations might then provide a suitable model for the investigation of cellular interactions, both within tumors and within the tumor-bearing host.

We have cloned, without prior mutagenesis, a murine UV fibrosarcoma, RD-1024, in soft agarose, and have isolated and characterized its daughter clones by several criteria. Although the parent tumor is a regressor, which is rejected after transplantation into normal syngeneic mice, half of the stable clones isolated from RD-1024 possess a progressor phenotype and are capable of growth in normal immunocompetent hosts. If one assumes that these progressor cell subpopulations existed within the parent regressor tumor, then interesting questions arise concerning their function during tumor emergence and progression: (a) Why is the regressor phenotype retained by RD-1024, or in other words, why doesn't a progressor cell population become dominant? (b) Is there any influence of the regressor on the progressor phenotype, or of the progressor on the regressor phenotype? (c) Can the progressor clones be implicated in the tumorigenicity of RD-1024 in the autochthonous host, and if so, what is their role in tumorigenesis? In this report we shall explore some of the implications of heterogeneity of tumorigenicity phenotype, confining ourselves to the well-characterized UV tumor model system.

### Materials and Methods

*Animals.* 4-6-wk-old C3H/HeN female mice, obtained from Charles River Breeding Laboratories, Wilmington, Mass., were housed at a density of five to six animals per standard 7 × 11-inch cage and maintained on Wayne Lab Blox, Sterilizable (Allied Mills, Chicago, Ill.) and acidified water ad libitum.

*Generation and Maintenance of UV Tumor RD-1024.* The induction of RD-1024 by UV irradiation of shaved C3H/HeN mice has been described in detail previously (15). This tumor has been classified histologically as a spindle cell fibrosarcoma. It is not transplantable into normal syngeneic mice, but it will grow progressively in mice treated with subcarcinogenic doses of UV light (30-50 half-hour exposures). The UV light source is a bank of six FS-40 Westinghouse fluorescent sun lamps (Westinghouse Electric Corp., Pittsburgh, Pa.) emitting principally 280-320 nm with a total energy output of  $1.79 \times 10^3$  ergs/cm<sup>2</sup>/sec. RD-1024 is also maintained in vitro in alpha modified minimal essential medium (MEM; Flow Laboratories, Inc., Rockville, Md.) supplemented with 100 U of penicillin/ml, 100 µg of streptomycin/ml, 2 mM glutamine (Microbiological Associates, Walkersville, Md.) and 5-10% fetal or newborn calf serum (Flow Laboratories, Inc.; complete alpha MEM). Over a period of several years, RD-1024 has retained its morphological characteristics and exhibits stability with regard to its tumorigenicity. Hereafter, the mice in which this tumor and all regressor tumors are passaged will be called UV-treated mice.

*Cloning of RD-1024.* RD-1024 growing in a UV-treated animal was excised and finely minced before dissociation in a solution of 0.1% collagenase plus 0.1 dispase in alpha MEM (~1 g of tissue/20 ml, at 37°C for 30 min with stirring). 10 million viable cells were seeded into 75-cm<sup>2</sup> plastic gelatin-coated tissue culture flasks (T-75; Corning Glass Works, Corning, N. Y.) and allowed to adhere for 48 h at 37°C in 6.5% CO<sub>2</sub>. After washing three times with phosphate-buffered saline, cells were trypsinized with a dilute trypsin solution (0.25%), washed, and counted. Cells were pipetted vigorously to ensure a fine single cell suspension, and suspended in a 0.33% agarose solution (Seaplaque; FMC Corp., Homer City, Pa.) in complete alpha MEM, and held at 42°C; final cell densities were 10<sup>4</sup>, 10<sup>3</sup>, and 10<sup>2</sup> cells/ml. 10 ml of these agarose cell suspensions were seeded onto the bottoms of 75-cm<sup>2</sup> tissue culture flasks over 0.66% agarose-alpha MEM sublayers. After gelification at room temperature, cultures were held at 37°C in 6.5% CO<sub>2</sub> and were fed every 5-7 d with 10 ml of the warm 0.33% agarose-alpha

MEM solution. Microscopically visible clumps appeared after 3 d and became visible to the unaided eye within 2 wk. At 16 d, the flask seeded at  $10^2$  cells/ml contained 166 visible clones, the flask seeded at  $10^3$  cells/ml contained 506 clones, and the flask seeded at  $10^4$  cells/ml contained too many to count. 12 well-isolated clones were picked at random by suction with a bent Pasteur pipette and each was placed into a well of a 24-well Costar plate (Corning Glass Works) in 1.5 ml of complete alpha MEM. After 7–13 d, when ~50% confluency had been reached, the wells were trypsinized and the cells were transferred first to 25-cm<sup>2</sup>, then into 75-cm<sup>2</sup> tissue culture flasks. 50 million cells of each stable clone were frozen in alpha MEM containing 10% dimethyl sulfoxide and 20–50% fetal bovine serum.

*Phenotypic Characterization of Clones.* Clones were evaluated for tumorigenicity as described in the legend to Fig. 1. In addition to the parent regressor tumor, RD-1024, one stable regressor, Cl 8, and two progressor clones, Cl 4 and Cl 9, were chosen for further characterization.

Cell doubling times for the various clones in vitro were determined by plating the tumor lines in 75-cm<sup>2</sup> flasks at  $2 \times 10^5$ /ml and incubating at 37°C in 6.5% CO<sub>2</sub> for various lengths of time. After careful washing three times with phosphate-buffered saline, the remaining cells were trypsinized and viable cells were counted. The number of doublings during a particular time period, *t*, was determined as the difference in the number of cells at time *t* minus the number of cells at the time of plating, expressed as a function of log<sub>2</sub>. Doubling time is expressed as the time in culture divided by the number of doublings occurring during that period.

Plating efficiency was performed in triplicate in complete alpha MEM at two dilutions, 200 and 400 cells/ml. This parameter is expressed as the average number of colonies visible after 10 d divided by the number of cells seeded ( $\times 100$ ).

*Draining Lymph Node Cell (DLN) Assay.* Cytotoxic lymphocytes were generated as described in detail previously (14). Briefly, mice were challenged in the hind footpads with tumor. 8 d later, the draining popliteal nodes were removed and put into culture. After 4 d, these effectors were cultured for 6 h with <sup>61</sup>Cr-labeled tumor cell targets. The supernate was then removed from each well and counted in a Beckman Biogamma counter (Beckman Instruments, Inc., Fullerton, Calif.). Percent specific lysis is expressed as:

$$\text{Percent specific lysis} = \frac{\text{experimental counts} - \text{control counts}}{\text{maximum releasable} - \text{spontaneous release}} \times 100.$$

For experiments in which unlabeled tumor cells were added as blockers of effector activity, an effector:target (E:T) ratio of 25:1 was used, with blocker:target (B:T) ratios of 4:1, 2:1, and 1:1. Percent inhibition is expressed as:

$$\text{Percent inhibition} = 100 - \frac{\text{percent lysis with blockers}}{\text{percent lysis without blockers}} \times 100.$$

*Gamma Irradiation of Mice.* Mice were irradiated with 500 rad of gamma irradiation in a Gammator (Isomedix, Parsippany, N. J.) containing a <sup>137</sup>Cs source. Mice were held in 50-ml plastic centrifuge tubes and exposed at an exposure rate of 625 rad/min. This dose has been shown to abolish the capacity of mice to mount a primary anti-tumor response, in that UV regressor tumors can grow progressively (12).

*Cross Immunization of Mice.* Mice were immunized by the subcutaneous injection of  $5 \times 10^5$  tumor cells in 0.1 ml of alpha MEM without serum. After 14–21 d, when the implants had been completely rejected, mice were challenged with either  $10^6$  progressor cells freshly trypsinized from tissue culture, or with a 1-mm<sup>3</sup> tumor fragment excised directly from a tumor-bearing animal. Tumor growth was evaluated weekly. Nonimmunized animals served as controls for the growth of progressor tumor.

*Double Challenge of Mice.* Two methods were employed for the simultaneous challenge of mice with both regressor and progressor cells. In the first, a mixed inoculum of viable, freshly trypsinized, single cells was inoculated subcutaneously on the shaved belly in 0.1 ml of alpha MEM without serum. Tumor growth was monitored weekly. The second method was by separate subcutaneous injection of regressor and progressor cells at two different sites on the belly. Both implants in each animal were evaluated weekly for tumor growth. During the

period of evaluation (4 wk) the separate implants remained isolated from one another, or one or both were rejected completely. Cell numbers used for challenge are supplied in the tables.

## Results

*Characterization of Tumor Clones.* The UV regressor tumor RD-1024 is not tumorigenic when transplanted into normal syngeneic mice. It was capable of progressive growth, however, in the autochthonous host and in animals immunosuppressed by other means. Because it has been previously shown (9) that one can isolate both tumorigenic and nontumorigenic clones from tumor cells exposed in vitro to mutagens, we reasoned that host immune pressures or other genetic or environmental factors might also be able to cause the generation of tumor cell heterogeneity. Consequently, a progressing tumor may contain subpopulations of tumor cells that differ from the parent in tumorigenic potential, as has been shown by Dexter et al. (16), and that perhaps clones can be isolated from a tumor that have increased tumorigenic potential as compared to the parent tumor.

To test this hypothesis, RD-1024, a UV regressor tumor, was excised from a UV-treated tumor-bearing animal and cloned in soft agarose. Thus, the survival of clones was dependent upon the property of anchorage independence, a property often accepted as a correlate of cell transformation and tumorigenicity (17). After growth to semiconfluency in liquid medium, each clone was injected into animals for an evaluation of tumorigenicity phenotype. After 5 wk, the clones were judged to be regressors or progressors. The regressor parent, RD-1024, grew in only one of the test animals; two of the test clones (Cl 5 and Cl 8) did not grow in any animals; and two clones (Cl 4 and Cl 9) grew progressively in the large majority of animals challenged with these tumors (Fig. 1).

Because the possibility existed that the two regressor clones did not grow for the simple reason that they were not tumors, Cl 8 was injected into UV-treated and into 500-rad-irradiated animals. It grew progressively in these immunocompromised hosts, establishing its categorization as a tumor (Fig. 2). (Cl 5 had unfortunately been lost previously in tissue culture.)

Three clones were evaluated in normal animals for possible dose-related tumorigenicity. Cl 8 did not grow in any animals at any dilutions tested ( $5 \times 10^2$ – $5 \times 10^7$ ). Cl 4 and Cl 9 both grew progressively to the death of the host in 100% of the test animals at  $10^6$  cells/mouse or greater (data not shown). In subsequent experiments with progressor clones, this dose ( $10^6$ ) was employed as a known tumorigenic dose.

Morphologically, all three daughter clones appear similar in vitro, consisting of long, thin, spindle-shaped cells that grow in a semi-ordered cordlike array until confluency, when they begin to pile up and form clusters on the monolayer surface. In this respect, they are all very much like the parent tumor, RD-1024. Cell doubling times are also similar among parent and clones: RD-1024, 27.5 h; Cl 4, 29.3 h; Cl 8, 22.6 h; Cl 9, 27.5 h. Plating efficiencies at low cell densities yielded disparate results among the cell lines: RD-1024, 11.6%; Cl 4, 12%; Cl 8, 6.6%; Cl 9, 22.2%. Although these figures show wide variation, no correlation with tumorigenicity phenotype can be drawn.

*Cross-Reactivity of Clones and Parent Tumor.* "Antigenicity" per se has long been touted as the cause of growth or rejection of transplanted tumors, the less antigenic tumors (i.e., little disparity between host and tumor) growing progressively in second-

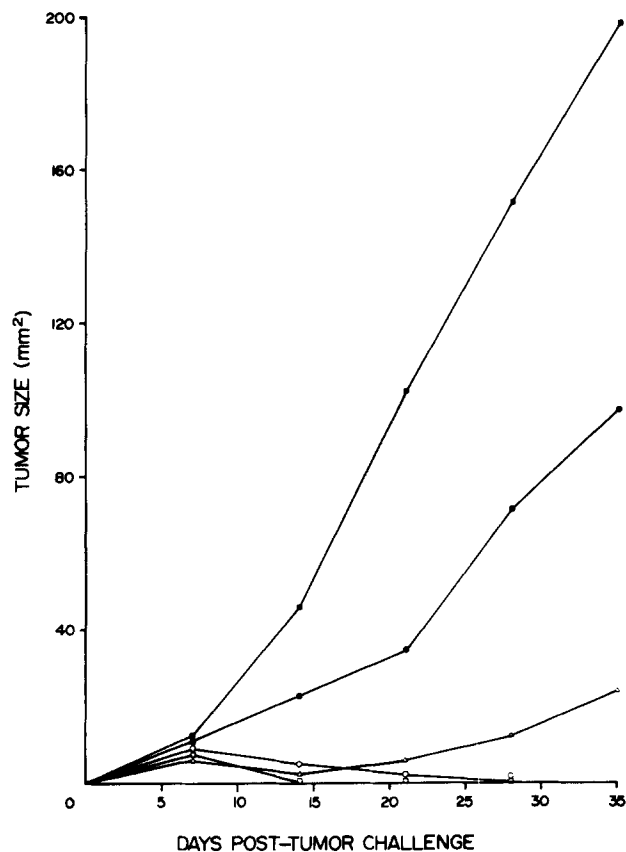


FIG. 1. Growth of RD-1024 and daughter clones in vivo. Parent tumor and clones were trypsinized at semi-confluency, washed twice in alpha MEM without serum, and resuspended at a cell density of  $5 \times 10^6$  viable cells/ml (by trypan blue exclusion). 0.1 ml of each tumor cell suspension was inoculated subcutaneously onto the shaved bellies of five C3H mice ( $5 \times 10^6$  cells/mouse). Tumor growth was measured weekly and is expressed as the mean of the product of two perpendicular diameters of a palpable tumor mass. ■ = Cl 9 (growth in 5/5 mice), △ = RD-1024 (1/5), ● = Cl 4 (4/5), □ = Cl 5 (0/5), ○ = Cl 8 (0/5).

ary hosts, the more antigenic tumors being immunologically rejected by secondary hosts (18). The expression of tumor antigens on the surface of neoplastic cells is presumably responsible for this phenomenon. Several mechanisms have been invoked to explain the appearance of these antigens: reexpression of fetal antigens (19), expression of altered or nonhistocompatible H-2 antigens (20), or of neoantigens arising from some unknown stimulus. UV tumors are known to express both TSTA, antigens unique to each tumor, and TAA, antigens expressed by all UV tumors in common (12). The TSTA seem to be the antigens against which the major rejection response is elicited, although TAA can also be demonstrated to function as rejection antigens (12). It thus seemed reasonable to test for the presence of tumor rejection antigens on our clones and for the functional specificity of the response raised against these antigens during tumor challenge. The possibility existed that one could find a difference between regressor and progressor antigenic expression, either qualitative or quantitative.

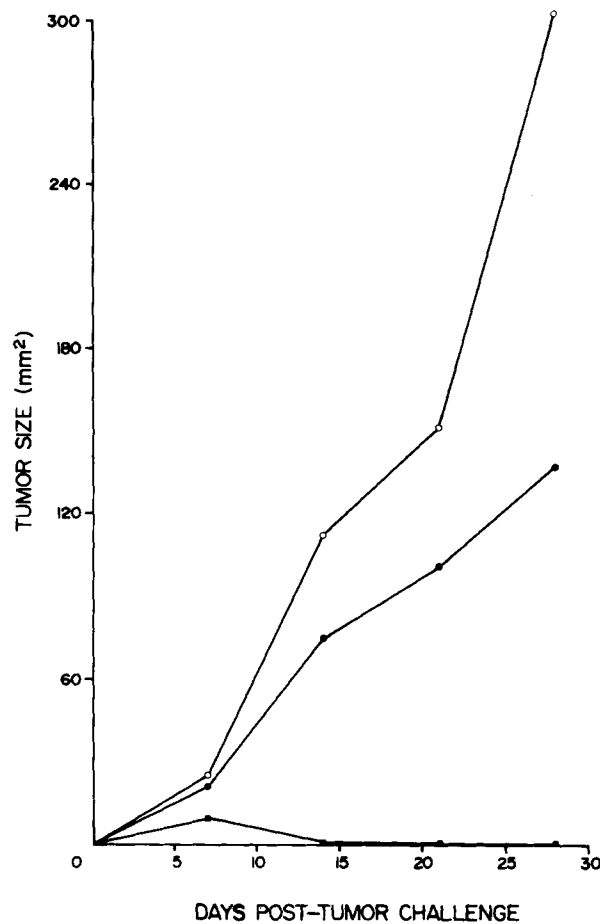


FIG. 2. Cl 8 in normal, 500 rad-, and UV-treated mice. Cl 8 was trypsinized at semi-confluency, washed twice in alpha MEM without serum, and resuspended at a cell density of  $10^7$  viable cells/ml. 0.1 ml of this suspension was inoculated subcutaneously onto the bellies of five normal, five 500-rad-irradiated, and five UV-treated C3H mice ( $10^6$  cells/mouse). Tumor area is expressed as the mean of the product of two perpendicular diameters of tumor growth as measured weekly for 4 wk. ■ = normal animals, ● = 500-rad-irradiated animals, ○ = UV-treated animals.

It was found that prior immunization with either the parent regressor tumor, RD-1024, or with Cl 8, the regressor clone, protected against subsequent challenge with either progressor clone, Cl 4 or Cl 9 (Figs. 3 and 4; data shown for Cl 4). These results suggested to us that the immune apparatus recognized cell surface antigens on the challenge tumor similar to those present on the immunizing tumor. Had the parent been a heterogeneous mixture of different subpopulations expressing different antigens, one might have expected less protection afforded by the parent than by Cl 8, on purely statistical grounds. If the clones had possessed different rejection antigens, the challenge tumor would have progressed in spite of prior immunization with regressor. Although these clones are phenotypically different by the criterion of tumorigenicity, they nevertheless appear to possess similar or even the same TSTA as one another and as the parent tumor.

*Susceptibility to Lysis by Cytotoxic T Lymphocytes (CTL) Generated against Cl 8.* Because

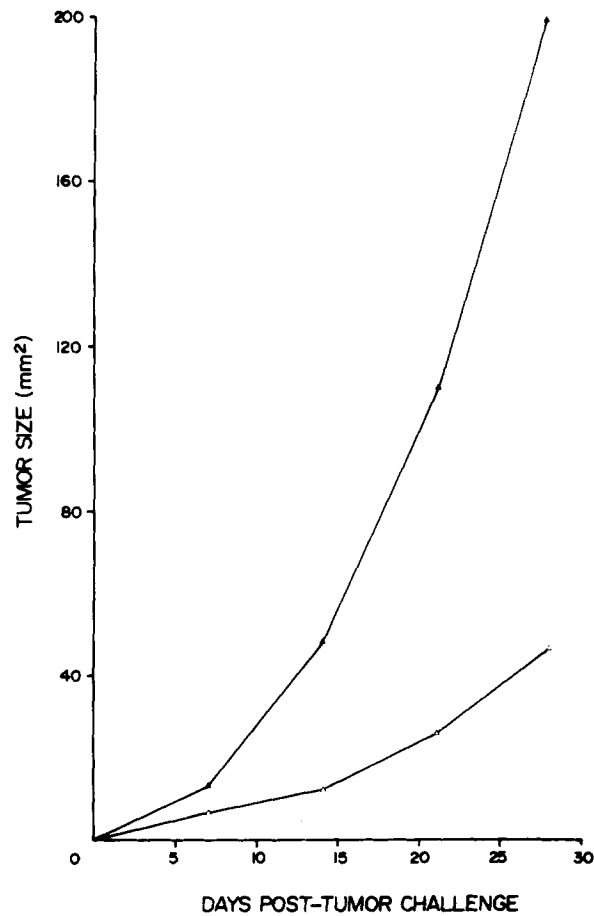


FIG. 3. Parent RD-1024 protects against challenge with progressor. C3H mice were immunized with RD-1024 cells, then challenged with Cl 4 cells as described in Materials and Methods. Nonimmunized animals challenged with Cl 4 served as controls. ▲ = Cl 4 (6/6), △ = Cl 4 in RD 1024 immune mice (2/6).

immune recognition seemed to be operative in the preceding immunization-challenge experiments, and only cross-reactive antigens could be discerned, a second, indirect, approach to the problem of antigen expression was attempted in the expectation that one might in some way be able to discriminate between progressor and regressor cell surfaces.

In vivo experiments in our lab have demonstrated that effector cells that will recognize and cause the destruction of tumor in vivo can be generated by a combination of in vivo and in vitro manipulations (R. A. Daynes, unpublished data). When CTL generated against a particular progressor tumor are injected simultaneously with that tumor into a test animal or intravenously at the same time as a subcutaneous tumor challenge, the CTL activity will result in the rejection of the tumor cells. The tumor will grow normally in control animals.

Similarly, by using in vitro analysis, there exist means to assess the susceptibility of tumor cells to lysis by effector cells (CTL) generated against a particular tumor. If

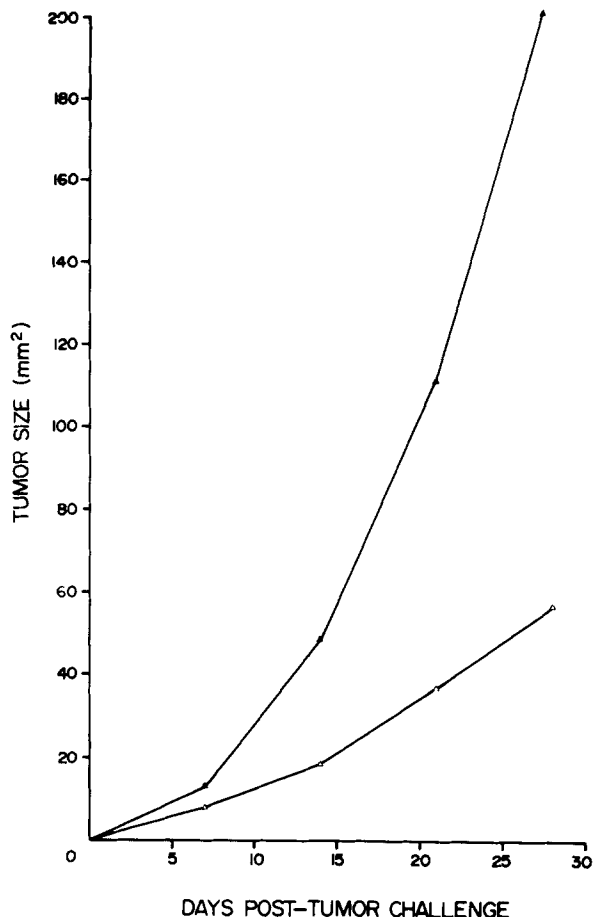


FIG. 4. Regressor Cl 8 protects against challenge with progressor. C3H mice were immunized with Cl 8 cells, then challenged with Cl 4 cells as described in Materials and Methods. ▲ = Cl 4 (6/6), △ = Cl 4 in Cl 8 immune mice (2/5).

target epitopes on progressor and regressor cells differ, either qualitatively or quantitatively, a standard  $^{51}\text{Cr}$  release assay can be used to detect these differences, and to establish whether equivalent sensitivity to lysis by effector lymphocytes exists among progressor and regressor clones. Footpad challenge of mice with Cl 8 tumor cells results 8 d later in DLN that will differentiate into CTL during an additional 4 d of *in vitro* culture. The amount of specific lysis of labeled target tumor cells by these CTL can be expressed as a function of  $^{51}\text{Cr}$  released upon 6-h incubation of cultured DLN with  $^{51}\text{Cr}$ -labeled tumor targets. Using this assay, the parent tumor and all clones were susceptible to lysis to the same degree, again suggesting the presence of a similar target determinant on all clones, which is recognized by CTL with functional specificity for Cl 8 only (Table I).

Epitope density was also considered to be a possibly significant parameter for discrimination between regressor and progressor clones. Target determinants for CTL can be titrated by employing a variation on the basic  $^{51}\text{Cr}$  release assay, with the addition of unlabeled tumor cell blockers at several dilutions to compete with the



TABLE I  
Lysis of RD-1024 and Daughter Clones by Anti-CI 8 DLN

Effector cells*	Target cells‡	E:T ratio	Percent specific lysis§
C3H anti-CI 8	CI 8	50:1	70.7
		25:1	52.1
		12.5:1	24.0
C3H anti-CI 8	RD-1024	50:1	65.6
		25:1	57.1
		12.5:1	29.1
C3H anti-CI 8	CI 4	50:1	88.2
		25:1	72.9
		12.5:1	51.1
C3H anti-CI 8	CI 9	50:1	60.9
		25:1	42.8
		12.5:1	37.2

\* 4-d-old cultured popliteal lymph node cells from 8-d immunized C3H mice.  
‡  $10^4$   $^{51}\text{Cr}$ -labeled cells per microtiter well in a total volume of 200 ml of complete alpha MEM.

§ Percent specific lysis of  $^{51}\text{Cr}$ -labeled targets was calculated as described in Materials and Methods.

TABLE II  
Cold Cell Inhibition of Lysis of  $^{51}\text{Cr}$ -labeled CI 8 Targets

Effector cells*	Target cells‡	Blocker cells§	Percent specific lysis	Percent inhibition of lysis
C3H anti-CI 8	CI 8	—	53.3	—
C3H anti-CI 8	CI 8	CI 8		
		4:1	26.9	49
		2:1	39.8	25
		1:1	42.0	22
C3H anti-CI 8	CI 8	RD-1024		
		4:1	18.8	65
		2:1	27.4	49
		1:1	37.7	29
C3H anti-CI 8	CI 8	CI 4		
		4:1	15.7	70
		2:1	25.4	52
		1:1	37.8	29
C3H anti-CI 8	CI 8	CI 9		
		4:1	19.3	64
		2:1	35.6	33
		1:1	44.4	17

\* 4-d-old cultured popliteal lymph node cells from 8-d immunized C3H mice.  
E:T ratio, 25:1.

‡  $^{51}\text{Cr}$ -labeled targets at  $10^4$  cells/200-ml well.

§ B:T ratios of 4:1, 2:1, 1:1.

|| Percent specific lysis and percent inhibition of lysis of  $^{51}\text{Cr}$ -labeled targets were calculated as described in Materials and Methods.

labeled targets. CTL activity toward the labeled cells will be inhibited and released  $^{51}\text{Cr}$  will be proportionally decreased. When this assay was performed, inhibition of lysis was observed for all clones and for the parent (Table II). This inhibition was titratable, and similar among all test groups. Two different combinations of targets and DLN yielded the same results (anti-CI 8 DLN on  $^{51}\text{Cr}$ -labeled CI 8 targets, and anti-CI 9 DLN on  $^{51}\text{Cr}$ -labeled CI 9 targets), with all clones and the parent used as blockers (data are shown for anti-CI 8 DLN on  $^{51}\text{Cr}$ -labeled CI 8 targets). Thus, in our hands, tumorigenic and nontumorigenic clones seem to express similar antigens, in similar quantities, and are equally sensitive to lysis by CTL, at least as detected by assays that measure in vivo antigenic recognition or in vitro cytotoxic activity.

*Regressor Cells Influence the Tumorigenicity Phenotype of Progressor Clones.* The parent tumor, RD-1024, used in these studies is a regressor tumor, and does not grow in normal secondary transplant hosts. Nevertheless, both progressor and regressor subpopulations were isolable from the tumor, and were presumably present in the tumor before excision. In consideration of the data that suggest that the growth of cells in a semisolid medium correlates with tumorigenicity, we concede that the cloning procedure may have enhanced the isolation of clones of the progressor phenotype, and that statistically, the relative numbers of regressor and progressor clones obtained by this method may not reflect the actual composition of the tumor. Still, the question remains: why is RD-1024 a regressor? If tumorigenicity implies a lack or a decrease in growth control, why haven't the progressor clones become dominant in such a tumor mass? At some level of regulation, some mechanism must effectively prevent the expression of the progressor phenotype. The results of the cross-reactivity experiments suggest an immunological basis of control. Immunoregulation might be a consequence of the absolute numbers of progressor and/or regressor cells, or possibly, of the relative ratios of progressor to regressor cells, or possibly of the sequential appearance of the two phenotypes during tumor progression. By manipulation of these parameters, one might be able to influence the ultimate tumorigenicity phenotype of a tumor implant.

In an attempt to discover whether the progressor phenotype would be able to predominate at some ratio of regressor to progressor, we constructed composite "tumors," consisting of known proportions of regressor and progressor cells. A known tumorigenic dose ( $10^6$ ) of progressor cells was mixed with varying doses of regressor cells. Groups of mice were inoculated with these mixtures and tumor growth was monitored. Singly challenged controls were injected with the same doses of either progressor or regressor cells. The results of this experiment are shown in Table III. Mice injected only with regressor cells rejected the tumor; CI 8 is nontumorigenic, therefore, at all doses tested. Mice injected with progressor cells alone showed progressive growth of the tumor. All groups of test animals that were injected with both regressor and progressor cells rejected the challenge doses. Even low numbers of regressor cells, i.e., a regressor:progressor ratio of 1:10, resulted in the regressor (parental) phenotype.

To address the question of the nature of the influence, whether direct or indirect, that the regressor exerted over the progressor, we initiated a second experiment in which regressor and progressor cells were injected simultaneously but separately into two different sites on the bellies of the test mice. The outcome was essentially the same as was observed in the mixed challenge, with the progressor control group being

TABLE III  
*Mixed Challenge with Regressor and Progressor Cells*

Group	Challenge*		Incidence	Tumor size‡
	Cl 8	Cl 4		
A Normal mice	10 <sup>6</sup>	10 <sup>6</sup>	0/5	
B Normal mice	5 × 10 <sup>5</sup>	10 <sup>6</sup>	0/5	
C Normal mice	10 <sup>6</sup>	10 <sup>6</sup>	0/5	
D Normal mice	5 × 10 <sup>6</sup>	10 <sup>6</sup>	1/5	13.5
E Normal mice		10 <sup>6</sup>	4/5	139.3
F Normal mice	10 <sup>6</sup>		0/5	
G Normal mice	5 × 10 <sup>5</sup>		0/5	
H Normal mice	10 <sup>6</sup>		0/5	
I Normal mice	5 × 10 <sup>6</sup>		1/5	1.8

\* Tumor cells were administered subcutaneously on the belly in 0.1 ml of alpha MEM without serum.

‡ Tumor size is expressed as the mean of the products of two perpendicular diameters of tumor growth.

TABLE IV  
*Double Challenge with Regressor and Progressor Cells*

Group	Challenge*		Incidence		Tumor size‡	
	Cl 8	Cl 4	Cl 8	Cl 4	Cl 8	Cl 4
Experiment 1						
A Normal C3H	10 <sup>6</sup>	10 <sup>6</sup>	0/5	1/5		15.0
B Normal C3H	5 × 10 <sup>5</sup>	10 <sup>6</sup>	0/5	0/5		
C Normal C3H	10 <sup>6</sup>	10 <sup>6</sup>	0/5	0/5		
D Normal C3H	5 × 10 <sup>6</sup>	10 <sup>6</sup>	0/5	0/5		
E Normal C3H		10 <sup>6</sup>		4/5		67.7
F Normal C3H	10 <sup>6</sup>		0/5			
G Normal C3H	5 × 10 <sup>6</sup>		0/5			
H Normal C3H	10 <sup>6</sup>		0/5			
I Normal C3H	5 × 10 <sup>6</sup>		0/5			
Experiment 2						
A 500-rad treated§	10 <sup>6</sup>	10 <sup>6</sup>	4/5	5/5	77.7	185.5
B Normal	10 <sup>6</sup>	10 <sup>6</sup>	1/5	0/5	3.2	
C Normal		10 <sup>6</sup>		4/5		172.1
D Normal	10 <sup>6</sup>		1/5		5.0	

\* Tumor cells were administered subcutaneously on the belly in 0.1 ml of alpha MEM without serum; Cl 8 on the left, Cl 4 on the right.

‡ Tumor size is expressed as the mean of the products of two perpendicular diameters of tumor growth.

§ Mice were treated with 500 rad of gamma irradiation 24 h before challenge with tumor.

the only group of animals growing the tumor (Table IV, experiment 1). The fact that challenge in two sites brings about the same result, as does challenge with a mixed inoculum, suggests that these effects are indirect and that they are mediated by some cell or substance other than the tumor itself. When 500-rad-irradiated mice are doubly challenged with both regressor and progressor cells, tumor masses develop at both sites of injection, whereas normal, untreated mice reject both inocula (Table IV, experiment 2). Because treatment of mice with 500 rad of gamma irradiation is known to abrogate the ability of mice to mount a primary anti-tumor response, leaving the

secondary response intact, these results further reinforce the idea of an immunological mechanism of tumor rejection.

### Discussion

A neoplasm, like a parasite, must possess a means of overcoming host defense mechanisms in order to lose its sensitivity to normal tissue regulatory constraints. If immunosurveillance is a valid concept, then expansion of a neoplastic cell population depends either upon the breakdown of the surveillance system itself or upon the emergence within the tumor of some mechanism that permits the neoplastic cells to proliferate in spite of host regulation. In parasites, these "games" of evasion (21) have developed over evolutionary time. A primary tumor, however, has only one opportunity to express the neoplastic phenotype, so its persistence indicates a more rapid means of escape from host control, one that can be developed over the course of a limited number of cell doubling times. In fact, the concept of tumor progression is centered around the evolutionary capabilities of a neoplasm, in particular, or around the emergence of increasingly tumorigenic variants that possess a growth advantage over existing subpopulations within a tumor or, alternatively, possess means to better evade host defense mechanisms, immune or otherwise (22).

Although a monoclonal origin has been assumed for the great majority of tumors and has been convincingly shown for several tumors (monoclonal spike in myelomas and single G-6-PD isoenzyme in tumors arising in heterozygotes), most tumors are heterogeneous in a number of phenotypic characteristics. Morphology, histology, and karyotype often show evidence of heterogeneity within individual tumors (1-3). Hormone sensitivity, drug resistance, metastatic potential, and growth kinetics have all been demonstrated to be heterogeneous among tissue culture lines derived from tumors by artificial selection pressures (6-8). Immunological non-cross-reactivity between tumor fragments excised from different areas of the same fibrosarcoma can be added to this compendium of phenotypic variant types (23). Elegant statistical analysis of parent and clonal lines has suggested the probable preexistence of this heterogeneity *in vivo*, at least in regard to the formation of lung foci in a popular metastasis model (8, 24).

It is possible that some of these variants simply represent components of the myriad life support systems available to the tumor as it grows, and are not actually included in the neoplastic cell population. Alternatively, minor subpopulations may represent neoplastic variants that lead a cryptic existence within the tumor, but are totally irrelevant to the growth or non-growth of the incipient tumor. Finally, phenotypic variants may play an obligate role in tumor progression, and it is to this end that heterogeneity within tumors is generated, that is, to enhance the progressive growth of neoplastic cells within primary tumors.

Tumors induced in mice by treatment with UV radiation are unique in that the majority of these tumors will not grow when they are transplanted into normal syngeneic hosts. These UV regressor tumors progress, however, when they are transplanted into recipients previously treated with subcarcinogenic doses of UV light, a known immunosuppressive agent. Using adoptive transfer protocols, it has been shown that UV-treated animals possess suppressor T cells ( $T_s$ ) with functional specificity for the common antigens (TAA) on UV tumors. This  $T_s$  population must have arisen in conjunction with and consequent to UV irradiation, because it is

present in UV-treated animals that have never undergone tumor challenge. By extension to the primary host, it has been postulated that the preexistence of this  $T_s$  response generated by UV light is responsible in part for the emergence of skin tumors in chronically irradiated animals (25). Thus, it appears that UV light possesses both immunosuppressive and carcinogenic activity in an irradiated host, and that the former precedes the latter. There are theories that deal with the actual mechanisms giving rise to suppressive activity in UV-treated animals (26). For our purposes, we shall simply assume that such suppression is present and functioning in these animals.

Our interest in this phenomenon extended beyond  $T_s$  cells, however, and beyond the implications that the function of this subset of regulatory cells may have for the host's perception of tumor. We felt it might be useful to scrutinize the tumor itself for insight into this dichotomous situation where a UV tumor grows in the primary host but not in normal syngeneic transplant hosts. Other investigators (9, 10) using other systems have been able to generate tumor variants with differing grades of tumorigenicity, apparent upon transplantation into appropriate hosts. These model systems all include mutagenesis or chemical or viral carcinogenesis to facilitate detection and isolation of cell lines differing in tumorigenicity phenotype. In light of the heterogeneity that has been observed in tumors, we considered the possibility that these variants might reside *in vivo* within virgin UV tumors, and that they could be isolated by standard cloning techniques.

The cloning of one of these UV regressor tumors in soft agarose has resulted in the isolation of stable clones that exhibit heterogeneity of tumorigenicity phenotype. Cl 8 does not grow in normal syngeneic mice at any tested tumor dose. Cl 4 and Cl 9 both grew when they were inoculated at a dose of  $10^6$  cells/mouse or greater. We have shown that these clonal subpopulations are immunologically cross-reactive, and that a rejection response elicited toward a regressor clone can be responsible for the secondary or concomitant rejection of a progressor clone. Preliminary findings also suggest that regressor cells are capable of initiating an activity that results in the rejection of an 8-d implant of progressor tumor (data not shown). In this case, positive effector activity elicited by the regressor was capable of overriding any suppressive activity established by implantation of progressor tumor 8 d earlier. Experiments are underway to explore the limits of this regressor-induced activity, with respect to the interval between progressor and regressor challenge. It is possible that the positive effector activity induced by the regressor is not absolute. At more extended intervals, or possibly at shorter intervals, between progressor and regressor challenge, one may find the regressor cells incapable of prevailing over existing suppressive mechanisms active in a progressor tumor-bearing animal, resulting in continued growth of the progressor tumor. A second possibility would be progressive growth of both regressor and progressor implants, implying an upset in the balance between positive and negative immunoregulatory activities.

Assuming that both regressor and progressor subpopulations existed in the original tumor, and that they are also present in the transplant, one must conclude that the presence of regressor cells in a heterogeneous transplant suppresses in some manner the expression of the progressor phenotype. We have shown that cytotoxic cells can be generated against the regressor population that act *in vitro* to kill related tumor cell clones in a cross-reactive manner. In all probability, it is this effector function that results in the elimination of a tumor implant in a normal animal, despite the presence

within the implant of cells with progressive growth potential. In a UV-irradiated host, the preexisting regulatory  $T_s$  cell population prevents the generation (or the expression) of a positive effector response and a transplant can assume the progressor phenotype. A UV tumor in UV-treated hosts retains its heterogeneity, however, because after excision from a UV tumor-bearing animal and transplantation into a normal animal, the original regressor phenotype is reestablished. The same suppressor mechanism may have been operative in the primary, autochthonous host, in that UV light caused the expansion of a  $T_s$  cell population with specificity for the cross-reactive antigens on UV tumors, such that no effective positive cytotoxic response could be raised against the incipient neoplasm, and it grew progressively as a tumor, though populated with both regressor and progressor cells.

The fact that regressor Cl 8 shows antigenic disparity with the host and that Cl 4 and Cl 9 possess the same disparity, although they are tumorigenic, suggests that antigenicity is perhaps not the ultimate criterion for growth or rejection of a tumor implant, or by extension, for tumorigenicity in a primary host. Our experiments suggest that positive or negative immunoregulatory responses can be elicited by individual clones of cells. We would propose that a balance between these activities exists within a tumor-bearing animal, and that heterogeneity within a tumor contributes significantly to the immune perception of the host to the tumor, and thus also to the final outcome of the host-environment-tumor interaction.

### Summary

We have shown that both regressor and progressor clones can be isolated from a UV regressor tumor, RD-1024. Although the daughter clones are characterized by differences in tumorigenic potential in normal transplant hosts, they nevertheless seem to express the same major tumor rejection antigens, because immunization with either the regressor parent tumor, RD-1024, or with regressor Cl 8 protects against subsequent challenge with progressor Cl 4 or Cl 9. Consistent with the *in vivo*-generated data is the evidence that draining lymph node cells with functional specificity for regressor Cl 8 are capable of cross-reactive cytotoxicity in an *in vitro* chromium release assay.

We have demonstrated an indirect interaction occurring *in vivo* between regressor and progressor cells, in that Cl 8 cells have the ability to influence the outcome of simultaneous or sequential challenge with Cl 4 or Cl 9 cells. Because 500 rad of gamma irradiation has been shown to compromise the ability of mice to respond to a primary challenge with tumor, an immunological mechanism is implicated in the ultimate rejection of progressor tumor in a doubly challenged host.

The importance of these results lies in the knowledge that these interacting subpopulations have been isolated directly from a tumor growing *in vivo* and that no selection pressure has been exerted on the cells greater than the short *in vitro* culture period necessary for the isolation and expansion of individual clones. The apparent immunoregulatory potential in a tumor-bearing animal is thus seen to be modified in accordance with the phenotypic heterogeneity of the cells within that tumor.

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## References

1. Becker, F. F., K. M. Klein, S. R. Wolman, R. Asofsky, and S. Sell. 1973. Characterization of primary hepatocellular carcinomas and initial transplant generations. *Cancer Res.* **33**: 3330.
2. Mitelman, F. 1971. The chromosomes of fifty primary Rous rat sarcomas. *Hereditas.* **69**:155.
3. Henderson, J. S., and P. Rous. 1962. The plating of tumor components on the subcutaneous expanses of young mice. *J. Exp. Med.* **115**:1211.
4. Law, L. 1952. Origin of the resistance of leukemic cells to folic acid antagonists. *Nature (Lond.)*. **169**:628.
5. Schabel, F. M., Jr. 1975. Concepts for systemic treatment of micrometastases. *Cancer Res.* **35**:15.
6. Sluysers, M., and R. Van Nie. 1974. Estrogen receptor content and hormone responsive growth of mouse mammary tumors. *Cancer Res.* **34**:3253.
7. Heppner, G. H., D. L. Dexter, T. DeNucci, F. R. Miller, and P. Calabresi. 1978. Heterogeneity in drug sensitivity among tumor cell subpopulations of a single mammary tumor. *Cancer Res.* **38**:3758.
8. Kripke, M. L., E. Gruys, and I. J. Fidler. 1978. Metastatic heterogeneity of cells from an ultraviolet light induced murine fibrosarcoma of recent origin. *Cancer Res.* **38**:2962.
9. Boon, T., and O. Kellermann. 1977. Rejection by syngeneic mice of cell variants obtained by mutagenesis of a malignant teratocarcinoma cell line. *Proc. Natl. Acad. Sci. U. S. A.* **74**: 272.
10. Patek, P., J. Collins, and M. Cohn. 1978. Transformed cell lines susceptible or resistant to in vivo surveillance against tumorigenesis. *Nature (Lond.)*. **276**:510.
11. Kripke, M. L. 1974. Antigenicity of murine skin tumors induced by ultraviolet light. *J. Natl. Cancer Inst.* **53**:1333.
12. Spellmann, C. W., and R. A. Daynes. 1978. Ultraviolet light induced murine suppressor lymphocytes dictate specificity of anti-ultraviolet tumor immune responses. *Cell. Immunol.* **38**:25.
13. Woodward, J. G., and R. A. Daynes. 1979. Cell mediated responses to syngeneic UV induced tumors. I. The presence of tumor associated macrophages and their possible role in the in vitro generation of cytotoxic lymphocytes. *Cell. Immunol.* **41**:304.
14. Daynes, R. A., P. A. Fernandez, and J. G. Woodward. 1979. Cell mediated immune response to syngeneic UV induced tumors. II. The properties and antigenic specificities of cytotoxic T lymphocytes generated in vitro following removal from syngeneic tumor-immunized mice. *Cell. Immunol.* **45**:395.
15. Daynes, R. A., C. W. Spellmann, J. G. Woodward, and D. A. Stuart. 1977. Studies of the transplantation biology of ultraviolet light induced tumors. *Transplantation (Baltimore)*. **23**: 343.
16. Dexter, D. L., H. M. Kowalski, and B. A. Blazar. 1978. Heterogeneity of tumor cells from a single mouse mammary tumor. *Cancer Res.* **38**:3174.
17. Freedman, V. H., and S. Shin. 1974. Cellular tumorigenicity in nude mice. *Cell.* **3**:355.
18. Old, L. J., E. J. Boyse, and D. A. Clarke. 1962. Antigenic properties of chemically induced tumors. *Ann. N. Y. Acad. Sci.* **101**:80.
19. Coggin, J. H., Jr., and N. G. Anderson. 1974. Cancer differentiation and embryonic antigens. Some central problems. *Adv. Cancer Res.* **19**:105.
20. Parmiani, G., and G. Invernizzi. 1975. Alien histocompatibility determinants on the cell surface of sarcomas induced by methylcholanthrene. I. In vivo studies. *Int. J. Cancer.* **16**: 756.
21. Bloom, B. 1979. Games parasites play: how parasites evade immune surveillance. *Nature (Lond.)*. **279**:21.

22. Foulds, L. 1969, 1975. *Neoplastic Development*. Academic Press, Inc., New York. Vol. 1: 41; vol. 2:17.
23. Prehn, R. 1970. Analysis of antigenic heterogeneity within individual 3-methylcholanthrene induced mouse sarcomas. *J. Natl. Cancer Inst.* **45**:1039.
24. Fidler, I. J., and M. L. Kripke. 1977. Metastasis results from preexisting variant cells within a malignant tumor. *Science (Wash. D. C.)*. **197**:893.
25. Roberts, L. K., and R. A. Daynes. 1980. Modification of the immunogenic properties of chemically induced tumors arising in hosts treated concomitantly with ultraviolet light. *J. Immunol.* **125**:438.
26. Lynch, D. H., M. F. Gurish, and R. A. Daynes. Relationship between epidermal Langerhans cell density, ATPase activity and the induction of contact sensitivity. *J. Immunol.* In press.