Cell Lines Cultured at High Density Are Resistant to Lysis by Tumor Necrosis Factor and Natural Cytotoxic Cells (42854)

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Abstract. **It has been suggested that natural cytotoxic (NC) cell activity and tumor necrosis factor (TNF), the molecular mediator of NC activity, are capable of protecting individuals against the progression of incipient tumors or could be useful in cancer therapy regimens. Much of this speculation arises as a result of *in vitro* studies, on a variety of tumor cells, demonstrating the cytolytic and cytostatic properties of NC and TNF activities.**

Here, evidence is presented showing that certain mouse fibroblast cell lines, generally considered sensitive to NC and TNF lysis, are quite resistant to these lytic activities when cultured at high cell density. Although a soluble factor that renders these same target cells resistant to NC and TNF lysis has been described, no such factor is involved in this high density-induced resistance. Rather, it appears that cell to cell contact of the targets is critical. Moreover, the induced resistance to NC and TNF lysis does not result from loss of either NC recognition determinants or TNF receptors by the target cells, but is the consequence of increased expression of a protein synthesis-dependent resistance mechanism.

These observations raise the issue of the *in vivo* phenotype of cells characterized *in vitro* as sensitive to NC and TNF lysis. It is entirely possible that certain cells which are considered sensitive to NC and TNF activities are, in fact, resistant to these cytolytic activities when growing as tumors (i.e., at high cell density). Should this be the so, NC and TNF cytolytic activities may not function *in vivo* or may function only via some indirect means. [P.S.E.B.M. 1989, Vol 190]

Natural cytotoxic (NC) activity is a cytolytic activity mediated by a multilineage family of cells (1) that utilize tumor necrosis factor (TNF) as their lytic effector molecule (2, 3). There is much speculation and some evidence that NC (4, 5) and TNF (6, 7) activities function *in vivo* for the prevention of tumorigenesis. It is conceivable that NC and TNF activities function by direct lysis of tumor cells or indirectly by affecting other cytolytic or cytostatic activities. Indeed, it has been found that TNF mediates numerous activities, on a wide variety of cell types, and that many of these activities could influence tumor progression. These activities include (i) induction of myeloid cell differentiation (8), (ii) fibroblast growth stimulation (9), (iii) activation of polymorphonuclear cell function via induction of granulocyte-monocyte colony-stimulating factor (10, 11), (iv) cachexia as a result of neoplastic disease (12, 13), (v) interferon-like activity (14, 15), and others.

Evidence presented here demonstrates that certain cell lines that are generally considered sensitive to NC/TNF lysis become quite resistant to NC/TNF lytic activity when maintained in culture at high cell density. Moreover, this high cell density-induced NC/TNF resistance is not mediated by a freely diffusible factor and is not the result of target cells losing NC recognition determinants or TNF receptors. Rather, the high cell density resistance is due to increased expression of a protein synthesis-dependent NC/TNF resistance mechanism. These findings raise important issues regarding both the *in vivo* phenotypes of cells that are known to be NC/TNF-sensitive *in vitro* and the mechanism of NC and TNF activities in the prevention or inhibition of tumorigenesis.

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Materials and Methods

Cell Lines. The cell line B/C-N is a cloned, continuously growing fibroblast cell line derived from a

BALB/c mouse embryo; it is contact inhibited, anchorage dependent, and does not form tumors in syngeneic normal mice or in immunodeficient mice (e.g., athymic nude, ATXBM) (16). 10ME is a cloned cell line derived from B/C-N cells treated with a methylcholanthrene epoxide; it is anchorage independent, contact inhibited, and forms tumors in immunodeficient mice but not in syngeneic normal mice (i.e., 10ME is susceptible to a protective mechanism operative in normal mice but impaired in immunodeficient mice) (16). L929 is a fibroblast cell line derived from an adult C3H/An mouse (cell line CCL 1.2; American Type Culture Collection); it is the L-M derivative of NCTC clone 929. All cell lines were maintained at low cell density in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Unless noted, high cell density cultures were plated at approximately 50% confluency, then utilized 3 days later as targets in the *in vitro* assays (doubling time of all cell lines is 24 hr or less; thus, high density cultures were confluent for approximately 2 days). These cultures are referred to as high density cultures. Maintenance cultures were never allowed to reach confluence.

Low cell density cultures of 10ME contain 250 cells/mm^2, a culture that has been confluent for 1 day contains 2400 cell/mm^2, and cultures that have been confluent for 2 days (typical of those used for these studies) contain about 2800 cells/mm^2.

Assays For NC- , TNF- , and Cytotoxic T Lymphocyte-Mediated Lytic Activities. The assays for NC and TNF lytic activities were essentially identical. Varying numbers of spleen cells from immunologically naive BALB/c mice or titrated doses of recombinant human TNF (kindly provided by Dr. L. Lin, Cetus Corp.) were mixed with 10^4 ^{51}Cr-labeled targets in microtiter wells containing a final volume of either 100 μl or 150 μl of RPMI 1640 medium supplement as described (17). Thus, whether cells are cultured at high or low density, they are assayed at the same effector to target ratios and at the same cell density. The amount of target lysis is proportional to the amount of ^{51}Cr released during the 18-hr assay and was calculated by the formula: percentage specific ^{51}Cr released = 100 × (sample cpm − spontaneous cpm)/(total cpm − spontaneous cpm).

Labeled targets were prepared by incubating cells for 1 hr in a balanced salt solution containing ^{51}Cr (100 μCi/ml). Targets were removed from the plates with 0.02% EDTA and washed three times in medium containing 10% fetal bovine serum. When cycloheximide was added to the assay (5×10^{-3} M), it was added 2 hr after spleen cells and targets were mixed; this has been shown to result in maximum lysis of 10ME cells (16). When nonlabeled targets (i.e., competitors) were used, they were mixed with the ^{51}Cr-labeled targets before addition of spleen cells. Representative experiments are depicted in Table I and Figures 1–5, and all data presented are the arithmetic mean of triplicate assays which have a range of less than 10%.

Cytotoxic T lymphocyte (CTL) activity was measured using spleen cells from C57BL/6 mice boosted 5 days *in vitro* with x-irradiated (1000 rads) stimulator cells from a BALB/c spleen. Varying numbers of effectors were assayed for lytic activity in a 5-hr ^{51}Cr release assay (no NC-mediated lysis can be detected in this short assay period (18)). Percentage of specific ^{51}Cr release was calculated as described above.

Results

Culturing Cells at High Density Reduces Their NC/TNF Sensitivity. The NC lytic mechanism is mediated by TNF (2, 3), consequently, cell lines that are NC sensitive are usually TNF sensitive and vice versa. However, it was observed that when certain NC/TNF-sensitive cell lines were maintained in culture at high cell density for 2 days or more, they manifested a marked reduction in NC/TNF sensitivity. Representative experiments demonstrating this phenomenon are presented in Figure 1. As shown, both the NC and TNF sensitivities of 10ME cells were reduced by culturing the cells at high density (Fig. 1A). Similarly, sensitivity of L929 cells to TNF- or NC-mediated lysis was also reduced when these cells were maintained in culture at high density (Fig. 1B). Cell lines such as B/C-N, which are relatively resistant to NC and TNF lysis, exhibited little or no reduction in NC/TNF sensitivity following culture at high density (Fig. 1C). Although cells cultured at high density manifested resistance to both NC- and TNF-mediated lysis, they expressed no decrease in their sensitivity to CTL lytic activity (Table I).

NC/TNF Resistance of Cells Cultured at High Density is not Mediated by a Freely Diffusible Factor. It is conceivable that the cells cultured at high density secrete a factor that makes them refractory to NC/TNF lysis; such a factor might be a protease that degrades TNF or, perhaps, something similar to the previously described spleen cell-elaborated factor that reduces the sensitivity to NC/TNF lysis of certain target cells (19). To test whether cells maintained at high density secrete any such factor, 10ME cells were grown at low density in medium taken from high density cultures of 10ME cells. However, no NC resistance was confirmed on the cells cultured in this conditioned medium (data not shown). This suggested that the NC resistance was not the result of a secreted factor but an intrinsic property of the target cells. Alternatively, it was possible that a diffusible factor was produced, but was short-lived and thus unable to function in this type of experiment. To test this alternative, 10ME cells were plated at low density in 35-mm dishes. These dishes were subsequently placed in 100-mm dishes containing either medium alone or high density cultures of 10ME (1-day confluent). In this way, cells at low density and high density shared culture medium but had no physical contact. After 48 hr together, both the low density and

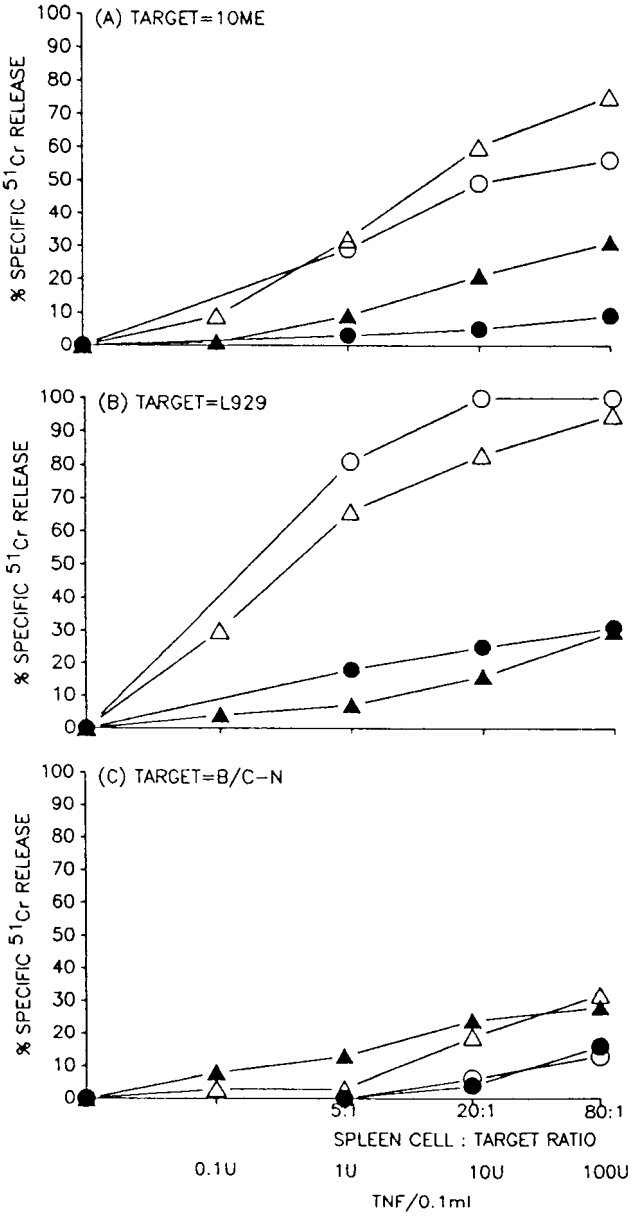

Figure 1. When cultured at low density the cell lines 10ME and L929 are sensitive to lysis by both NC cells and TNF, but when cultured at high density, both cell lines are resistant to NC and TNF lysis. The target cell lines were 10ME (A), L929 (B), and B/C-N (C). △, low density culture conditions and TNF-mediated lysis; ○, low density culture conditions and NC-mediated lysis; ▲, high density culture conditions and TNF-mediated lysis; ●, high density culture conditions and NC-mediated lysis.

high density cells were assayed for their sensitivity to NC and TNF lysis. As shown in Figure 2, the cells maintained at low density were sensitive to NC and TNF lysis regardless of whether or not they shared medium with cells maintained at high density. Since the cells maintained at high density were fully NC and TNF resistant (Fig. 2), these results indicate that the NC/TNF resistance which results from high density culturing is associated with cell to cell contact, not with a secreted product.

NC/TNF Resistance Caused by Culturing Cells at High Density is Transient. Experiments were conducted to determine whether the change from the NC/TNF-sensitive phenotype to the NC/TNF-resistant phenotype, as a result of culturing cells at high cell density, is permanent and, if not, how long does the NC/TNF resistance last when the cells are reestablished in a low density culture. To this end, 10ME cells were plated such that they were confluent 5 days prior to the NC or TNF assay; at various times after the cells reached confluence they were replated at low cell density. As shown in Figure 3, cells replated at low density 8 hr prior to the cytotoxic assays are as resistant to NC- and TNF-mediated lysis as cells maintained at high density for the entire 5-day period; when cells were replated from high density to low density 20 hr before the cytotoxic assay they began to reexpress sensitivity to NC and TNF lysis. Cells maintained for 2 days at high cell density and then at 72 hr at low density are 80–90% as sensitive to NC and TNF lytic activity as cells continuously cultured at low density. Thus, it appears that it takes 3 days or more for 10ME cells to recover their NC/TNF sensitivity following high density culturing.

Culturing of 10ME Cells at High Density Causes the Increased Expression of a Protein Synthesis-Dependent NC/TNF Resistance Mechanism. NC lysis of target cells involves a series of steps including target recognition by NC effectors, TNF binding to receptors, and activation of a lytic mechanism within the target which eventually results in lysis. Resistance to NC activity could be manifest by blocking any one of these steps or by utilization of a counter-lytic mechanism which "repairs" cellular damage.

Since the cells cultured at high density are resistant to TNF lysis, it seems likely that high density culture conditions affect either TNF binding or subsequent steps in the lytic process and do not affect expression of NC recognition determinants (since such determinants are not required for TNF-mediated lysis). This

Table I. 10ME Cells Cultured at High Density Are *Not* Resistant to Lysis by Cytotoxic T Cells[a]

Target	Percentage specific ^{51}Cr released		
	Spontaneous release (%)	Spleen cell:target cell	
		5:1	20:1
10ME	13	25	61
10ME(HD)	11	32	66

[a] Spleen cells from C57BL/6 mice were immunized *in vitro* against BALB/c spleen cells and subsequently used to determine the sensitivity to CTL lysis of 10ME cells cultured at either low density (10ME) or high density (10ME(HD)).

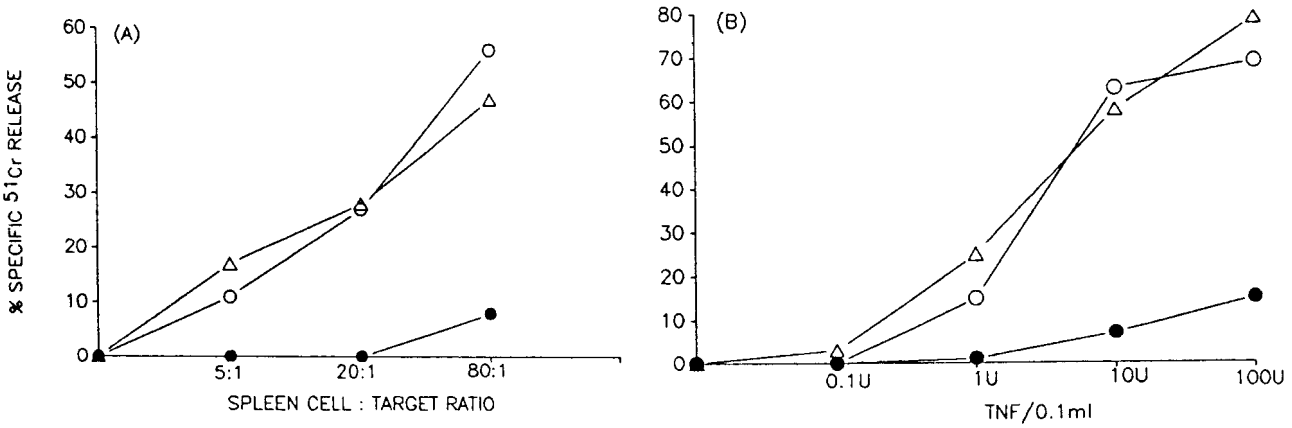

Figure 2. Low density-cultured 10ME cells remain sensitive to NC- and TNF-mediated lysis even if they share medium with cells cultured at high density. This indicates the NC resistance that results form high density culture conditions is not mediated by a secreted product. (A) spleen cell- and (B) TNF-mediated lytic activities. ○, 10ME cells from low density cultures; △, 10ME cells from low density cultures that shared medium with 10ME cell maintained at high density; ●, 10ME cells maintained at high density that shared medium with low density cultures of 10ME.

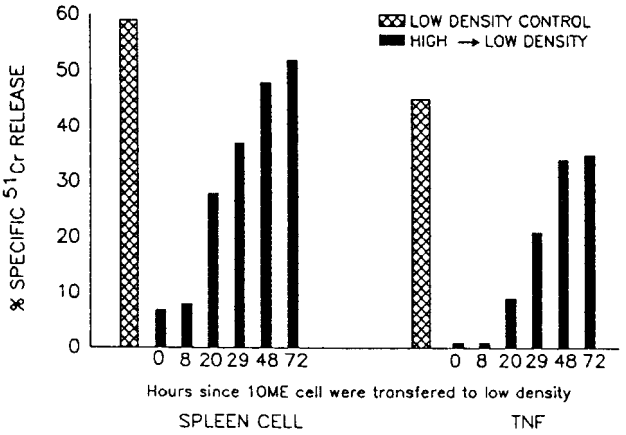

Figure 3. 10ME cells maintained at high density require more than 2–3 days to reexpress the level of NC and TNF sensitivity of control (low density) 10ME cells. The control cells were continuously cultured at low density. High density cultures of 10ME were transferred to low density at various intervals, as indicated, prior to the cytolytic assays. Spleen cell to target ratio of 20:1; 1 unit of TNF in 0.1 ml.

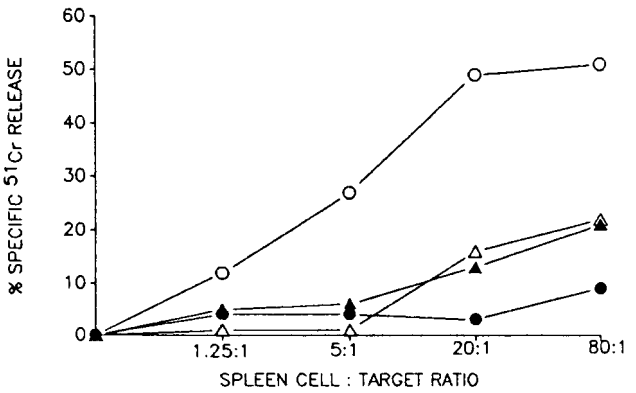

Figure 4. 10ME cells cultured at high or low density are equally effective as competitors against the NC-mediated lysis of ^{51}Cr-labeled 10ME target cells. The competitor to target ratio was 7:1. ○, 10ME target cells cultured at low density, no competitors; △, 10ME target cells cultured at low density, 10ME competitors cultured at low density; ▲, 10ME target cells cultured at low density, 10ME competitors cultured at high density; ●, 10ME target cells cultured at high density, no competitors.

was confirmed utilizing competition experiments. As shown in Figure 4, nonlabeled 10ME cells cultured at high or low density are equally effective at inhibiting NC lysis of labeled 10ME targets. This indicates that both cell types express equivalent levels of NC recognition determinants, despite the fact that one cell type is NC sensitive while the other is quite NC resistant.

It has been shown that certain NC/TNF-resistant cells express a protein synthesis-dependent NC/TNF resistance mechanism (3, 16). When the NC/TNF resistance mechanism is blocked by inhibitors of protein synthesis, these cells become NC/TNF sensitive. To determine whether such a mechanism is operative in cells cultured at high density, cycloheximide, a protein synthesis inhibitor, was added to the NC assay of 10ME cells maintained at high or low density. 10ME cells, which are normally NC sensitive, are even more sensitive when assayed in the presence of cycloheximide (Fig. 5). Moreover, the 10ME cells that are NC resistant

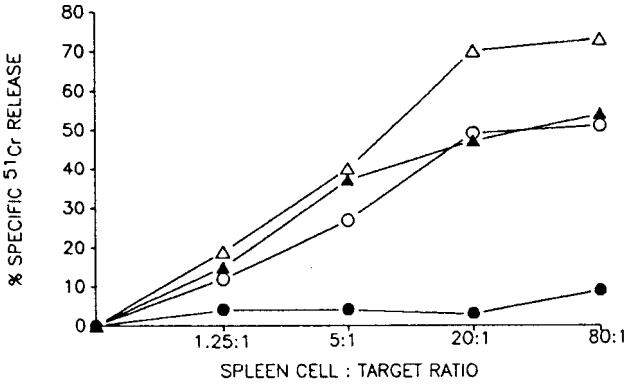

Figure 5. The sensitivity to NC lysis of cells cultured at high density is greatly increased by addition to the NC assay of a protein synthesis inhibitor (cycloheximide). This indicates the NC resistance that results from high density culturing is caused by a *de novo* synthesized protein(s) that is in lower concentration in cells cultured at low density. ○, 10ME cells cultured at low density; ●, 10ME cells cultured at high density; △, 10ME cells cultured at low density and assayed in the presence of cycloheximide; ▲, 10ME cells cultured at high density and assayed in the presence of cycloheximide.

as a consequence of culturing at high density become quite sensitive to NC lysis in the presence of cycloheximide (Fig. 5). Similarly, TNF sensitivity is markedly increased in the presence of cycloheximide (data not shown). These findings suggest that culturing 10ME cells at high cell density induces the increased expression of a protein synthesis-dependent NC/TNF resistance mechanism which is responsible for the NC/TNF resistance of these cells.

It is noteworthy that the *in vitro* NC/TNF lysis of L929 cells in an 18-hr assay, unlike the lysis of 10ME, is strictly dependent on protein synthesis (3). Thus, our experimental procedure that utilizes cycloheximide blocks all lysis of L929 and, therefore, does not permit the determination of whether the NC/TNF resistance of L929 cultured at high density is also protein synthesis dependent. (L929 treated with cycloheximide can be lysed in assays that are prolonged beyond 18 hr.)

Discussion

It has often been proposed that TNF and NC activity are involved in the prevention of tumorigenesis (3–7). Evidence in support of this comes from both *in vivo* and *in vitro* studies of their tumoricidal activities and because the selection of NC-resistant variants from NC-sensitive cell lines is associated with a concurrent selection for increased tumorigenicity. However, despite the fact that both TNF and NC effectors can lyse certain target cells *in vitro*, there is little evidence that it is the NC/TNF cytolytic activity that actually functions *in vivo* by direct lysis of target cells; it is conceivable that these effectors function indirectly. Indeed, TNF, the mediator of NC activity, is known to mediate a variety of activities (8–15), many of which might affect tumor growth, and there is reason to believe that NC activity can function by facilitating a T cell-mediated immune response (20).

In experiments presented here, we showed that certain cells, when maintained in culture at high cell density, manifest a marked reduction in sensitivity to NC and TNF lysis (Fig. 1). Moreover, this induced NC/TNF resistance is not mediated by a freely diffusible factor, but appears to require proximal interactions or cell to cell contact (Fig. 2). Additionally, the NC/TNF resistance does not result from a reduction in the expression of NC recognition determinants (Fig. 4) or TNF receptors (Fig. 5), but is caused by an increased expression of a protein synthesis-dependent resistance mechanism (Fig. 5), and is similar or identical to the NC/TNF resistance mechanism expressed by many NC/TNF-resistant progressor tumor cells. Recall that this protein synthesis-dependent resistance mechanism confers no resistance to CTL lysis (Table I and reference (18)).

10ME, the fibroblast cell line used for most of these studies, was derived from a nontransformed cell line (i.e., B/C-N) via chemical carcinogenesis. The parental cell line, B/C-N, is nontumorigenic whereas 10ME forms tumors only in immunodeficient mice (ATXFL or athymic nude mice), i.e., 10ME is intrinsically tumorigenic but susceptible to host protective mechanisms operative in normal mice (16). By serial passage of 10ME cells in immunodeficient mice, then normal mice, variants were selected that do form tumors in normal mice (16). Consistent with the hypothesis that NC or TNF function in the prevention of tumorigenesis, these variants are NC and TNF resistant (3). Further experiments have shown that *in vivo* or *in vitro* selection for NC resistance yields variants that are capable of forming tumors in normal mice (4). Thus, selection of 10ME cells for the ability to form tumors in normal mice selects for NC and TNF resistance and selection for NC resistance selects for the ability to form tumors in normal mice. It therefore appears likely that, for 10ME, escape from NC or TNF lysis is a necessary step on the pathway to becoming a progressor-type tumor.

Our analysis of the *in vivo* or *in vitro* selected NC/TNF-resistant variants of 10ME has revealed that they all express NC recognition determinants and TNF receptors. In fact, all are NC/TNF resistant via a protein synthesis-dependent NC/TNF resistance mechanism which is similar or identical to that expressed by the 10ME cells cultured at high density. Thus, the phenotype of 10ME cells maintained at high cell density is the same as that expressed by variants of 10ME that have been selected for the progressor tumor phenotype. This raises critical questions as to the *in vivo* phenotype of 10ME cells and other cells categorized as NC/TNF sensitive in *in vitro* assays and whether NC and TNF activities can function *in vivo* by direct lysis of target cells. It is possible that cells growing as tumors in mice are resistant to NC/TNF lysis regardless of their *in vitro* NC/TNF sensitivity and the antitumor activity attributed to NC and TNF activities function indirectly. Indeed, evidence for such an indirect mode of action has been revealed by others (21).

It has been reported that there is a correlation between the expression of gap junctions and resistance to TNF lytic activity (22). These studies showed that CHO cells, which are TNF sensitive when cultured at low density, become resistant to TNF lysis when the culture density reaches 80–90% confluent and at which time there is a concomitant increase in expression of gap junctions. As a control, the cell line L929 was used since it had been reported to be gap junction deficient (23). These investigators (22) found high density cultures of L929 expressed no increase in resistance to TNF lysis; thus, there was support for their hypothesis that cells united by gap junctions are resistant to the cytolytic action of TNF. Although our experimental procedures were quite different from theirs (e.g., they assayed lytic activity on mitomycin C-treated cells whereas we utilized untreated cells; their high density cultures were 80–90% confluent whereas ours were 99%

confluent), we got strikingly different results. We found that L929 cells maintained at high density expressed a marked reduction in both NC and TNF sensitivity (Fig. 1B). Although we have not assayed the expression of gap junctions in L929 cells, if we assume our L929 cells are gap junction deficient, as reported (23), then the TNF resistance of L929 cannot be a function of gap junction expression. Although our experiments are somewhat contradictory to those previously reported (22), they do not negate the conclusion that gap junction expression can be associated with resistance to TNF lysis. Indeed, our experiments indicating that cell to cell contact is required for the induction of NC/TNF resistance are consistent with the assumption that gap junctions are involved in the phenomenon. However, even if one assumes that gap junctions are part of the induced NC/TNF resistance mechanism, this provides, at best, an incomplete explanation of the NC/TNF-resistant phenotype. Moreover, the methods used to coordinately modulate gap junction expression and TNF sensitivity (i.e., culturing at high cell density, treatment with a phorbol ester or 8-bromo-cAMP, or infection with Rous sarcoma virus) are known to have a wide variety of effects apart from their effects on gap junction expression. Thus, the role of gap junction expression, as it relates to NC/TNF resistance, remains quite uncertain.

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