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Gene Expression and Tumor Cell Escape From Host Effector Mechanisms in Murine Large Cell Lymphoma

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Using in vivo selection methods, we obtained metastatic sublines of the murine RAW117 large cell lymphoma that form multiple liver metastases. The highly metastatic subline RAW117-H10 has a low number of gp70 molecules expressed at the cell surface and low cytostatic sensitivity to activated syngeneic macrophages. This subline was infected with endogenous RNA tumor virus isolated from a high virus-expressing RAW117-P subline of low metastatic potential. After superinfection the H10 subline gradually increased its expression of cell surface gp70 and showed enhanced sensitivity to macrophage-mediated cytostasis, suggesting that gp70 might be involved in host macrophage-mediated surveillance. Culture of RAW117-P and H10 cells in media conditioned by activated macrophages indicated that parental cells are severely growth inhibited in a dose dependent fashion while H10 cells showed almost no effect. Examination of differentially expressed genes in the highly metastatic RAW117-H10 cells by analysis of RNA blots indicated that a mitochondrial gene was expressed at a level that was ~10 times higher in H10 cells than in parental cells. This gene was identified as ND5, which codes for a subunit of NADH dehydrogenase (complex I of the mitochondrial electron transport chain); this complex is the target for an activated macrophage-released cytostatic factor. Among other possibilities, the results are consistent with the suggestion that highly metastatic RAW117 cells may escape macrophage surveillance by decreasing the synthesis of specific cell-surface receptors for cytostatic molecules and increasing the synthesis of specific cellular targets for such molecules.

Key words: tumor metastasis, viral antigens, macrophage cytostasis, differential gene expression, mitochondrial genes

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The interactions of a neoplasm with its host during tumor dissemination are complex and poorly understood [1–3]. Such interactions, for the most part, appear to be mediated at the level of the cell surface. Highly malignant cells express unique cell-surface properties that, in combination with host environment and responses, are important determinants in metastasis formation [1,2, 4–6]. Established tumor models have been used to study differences between nonmetastatic and metastatic neoplastic cells, as well as the role of host tumor surveillance mechanisms in metastasis [2, 3, 6]. Such research indicates that in many metastatic tumors the ability of highly malignant cells to escape host macrophage-mediated cytolysis and cytostasis is important in the metastatic process [3,7–13]. This is reinforced by studies showing that activated macrophages produce soluble respiratory-inhibiting factors (RIF) which inhibit the activity of complexes I and II of the tumor cell mitochondrial electron transport chain and thus cellular respiration [14,29,41,42].

One tumor model that has been useful in examining the role of host response in metastasis is the murine RAW117 large cell lymphoma [15,16]. This tumor system was established by Abelson murine leukemia virus (AbMuLV) transformation to yield parental RAW117-P cells of low malignant potential in syngeneic BALB/c mice. After ten sequential selections for liver colonization, a variant subline (RAW117-H10) was obtained that forms greater than 200 times as many gross liver tumors than does the parental cell line after intravenous or subcutaneous injection [15–17]. Highly metastatic RAW117-H10 cells possess differences in gene expression [18], exposures of cell surface proteins [19] and glycoproteins [17,20], lectin-binding sites [20,21], viral antigens [17,22], two-phase partitioning behavior [23], liver adhesion properties [24], and are less sensitive to macrophage-mediated cytolysis and cytostasis [8,13]. Here we report further studies of the role of macrophage-mediated cytostasis in the metastatic properties of RAW117 cells.

MATERIALS AND METHODS

Cell Lines and Metastasis Assays

RAW117 cells were cultured in petri dishes in Dulbecco-Modified Eagle's medium (DME) supplemented with high glucose (4.5 g/L), 10% fetal bovine serum (FBS), and 25 mM HEPES buffer except for the cells used for RNA isolation and growth curves with activated macrophage conditioned media which were cultured in a 50:50 mixture of DME and Ham's F12, 10% FBS. Cell cultures were used within ten passages (4–6 weeks) from frozen stocks to eliminate possible drift in metastatic properties [16]. All lines were negative for *Mycoplasmas* and other infections [18]. Metastatic potential was assessed by intravenous injection of 5×10^3 viable tumor cells (0.2 ml inoculum) in phosphate-buffered saline (PBS) into groups of 6–8-week-old BALB/c mice [15–18]. Twelve days after the injection mice were killed, and visible tumor nodules were counted and confirmed by histologic analysis [21].

Superinfection of RAW117 Cells

Some RAW117-P cells release infective AbMuLV and Moloney murine leukemia virus (MoMuLV) into culture medium [22]. Such released viruses can be isolated, purified, and used to reinfect susceptible cells such as NIH/3T3 by the procedure of Wong and Gallick [25]. With this method NIH/3T3 cells were completely transformed

in vitro, indicating the presence of infectious AbMuLV, and MoMuLV structural proteins were found expressed in the transformed NIH/3T3 cells [22].

RAW117-H10 cells expressing low amounts of MoMuLV components [17] were superinfected with an equal volume of filtered culture supernatant from RAW117-P cells by suspending 10^6 cells in 2 ml of DME plus 56 μg/ml DEAE Dextran. After a 1-hr incubation at 37°C with mild shaking, the cells were resuspended in 30 ml of DME containing 10% FBS and allowed to grow for a few passages. Mock infections omitted the RAW117-P supernatants. Some of the cultures were further cloned [22].

Viral Glycoproteins

RAW117 cells were labeled metabolically with [^{3}H]-leucine, lysed in a buffered detergent solution, and immunoprecipitated with gp70 antiserum as described previously [22, 26]. Immune complexes were precipitated with formalin-treated *Staphylococcus aureus* and prepared for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 8% polyacrylamide gels, followed by autoradiography [22]. For lectin staining of cellular glycoproteins separated by SDS-PAGE, the procedures of Irimura et al. [20] were used. Cells were lysed with 0.5% NP-40, 0.25 M sucrose, 50 μM $CaCl_2$, 0.4 mM phenylmethylsulfonyl fluoride, 10 mM Tris-HCl (pH 6.7), for 30 min. After centrifugation at 1,000g, the supernatant was collected, mixed with SDS sample buffer, heated at 100°C for 5 min, and subjected to SDS-PAGE using 7.5% polyacrylamide gels [20, 22]. After electrophoresis, the glycoproteins were transferred to nitrocellulose membranes [27], and detected by ^{125}I-concanavalin A (^{125}I-Con A) or peroxidase-Con A (Sigma Chemical, St. Louis, MO) staining [20].

Cytostasis in Macrophage Coculture

Syngeneic peritoneal exudate cells were obtained by intraperitoneal injection of 3 ml of thioglycollate (Difco Laboratories, Detroit, MI) into BALB/c mice [8]. Three days later the peritoneal cavities were flushed with PBS containing 100 μg/ml gentamicin, and the cell suspension was centrifuged at 400g for 5 min at 4°C. After resuspending the cells in DME, aliquots (0.1 ml) of the suspension were placed into wells of a 96-well plate (Costar, Cambridge, MA) at varying cell densities. After a 4-hr incubation at 37°C, the plates were washed twice to remove unattached cells, and 40 μg/ml of poly I:C (Sigma, St. Louis, MO) was added to each well. The DME was replaced after 24 hr with fresh medium containing poly I:C, and RAW117 cells were added. Cytostasis was determined in a 24-hr incubation period using the inhibition of [^{3}H]thymidine incorporation method of Germain et al. [28]. The results were expressed as follows: percent cytostasis $= 1 - (E-T_1)/(C-T_2) \times 100$, where E and C are the [^{3}H]thymidine incorporation of target cells in the presence or absence of activated macrophages, respectively, and T_1 and T_2 are the background incorporation in the presence or absence of macrophages, respectively. Statistical significance was determined by one-way analysis of variance and the significance of differences between sublines by the Mann-Whitney U-test.

Cytostasis in Macrophage Conditioned Media

Activated macrophages release soluble factors that inhibit tumor cell growth by blocking mitochondrial electron transport and cellular respiration [14]. Media conditioned by activated macrophages was obtained as previously described [14]. Briefly,

mice were immunized i.p. with 1×10^7 colony-forming units of BCG (*Bacillus calmette guerin*, strain *M. bovis*, Connaught Labs, Toronto, Ontario, Canada), 25 and 4 days before harvesting cells. The resulting plastic nonadherent cells were activated with 50 ng/ml endotoxin and the media conditioned by such cells was stored at −20°C for further use. Macrophage-conditioned media (MCM) was concentrated tenfold by filtration over a YM10 ultrafiltration membrane (Amicon Corp., Lexington, MO) and added to cultures to produce the indicated effective concentration (e.g., 100% concentration was made by adding 0.1 vol of 10× concentrate). These were then added to RAW117 cell cultures, and the effects on cell viability and growth determined [14].

Differentially Expressed RAW117 Genes

Total RNA was prepared from RAW117 cells [18] and the polyadenylated mRNA was separated by oligo(dT)-cellulose (type 30; Collaborative Research, Waltham, MA) chromatography [30]. RNA aliquots were heated at 60°C for 10 min, electrophoresed in a 2% agarose gel containing 6 M urea and 25 mM sodium citrate and visualized by staining with 1 μg/ml ethidium bromide for 30 min [31]. RNA was electrotransferred to freshly prepared DBM paper in 50 mM sodium acetate, pH 4.5 [32], and hybridized to plasmids containing specific gene sequences, labeled with ^{32}P-dCTP by the random primer method [33–35]. RNAs on DMB were probed using cloned mitochondrial DNA. RNA blots were prehybridized overnight in 50% formamide, 1% glycine, 0.2% SDS, 5× SSPE (150 mM NaCl, 10 mM $NaH_2PO_4.H_2O$, 1 mM EDTA, pH 7.4), 100 μg/ml tRNA, and 2× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin). Hybridization was performed in the same solution as prehybridization except that $2–4 \times 10^7$ cpm of radioactive probe was added. After a 24-hr incubation, the blots were washed 5 times (250 ml each) in 2× standard saline citrate (SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.2% SDS; and five times in 0.1× SSC, 0.1% SDS. The blots were then dried and exposed to Kodak XAR-5 film for 24-48 hr at −80°C.

Recombinant mitochondrial DNA clones were established from covalent closed circular DNA isolated from cultured mouse LA9 cells. The purified DNA was cleaved with either *Pst* I or *Hinc* II and recombinants were constructed using the vector pACYC177 [36] and transfected into *Escherichia coli* strain HB101. One of these clones was used in construction of a mitochondrial DNA vector [37]. The original clones were characterized only by their ethidium bromide-stained patterns in agarose gel electrophoresis. The clones have subsequently been confirmed by blot hybridization with nick-translated mouse mitochondrial DNA (D.L. Robberson and H. Eberspracher, unpublished results) and are designated according to an acronym of the restriction fragment they contain as follows: pMt1-Pst-1, pMt1-Pst-2, pMt1-Hnc-1 through pMt1-Hnc-5 in descending order of insert fragment size. We note that these designations are changed from those originally assigned [37]. These probes were synthesized using the random primer method from mouse mitochondrial genome fragments [33]. To make the ND5 gene probe, mitochondrial clone pMt1-Hnc-1 was restricted using *Hind* III and *Xho* I, and the resulting fragments were separated by electrophoresis on a 2.0% agarose gel in TBE (90 mM Tris base, 22 mM boric acid, 1 mM EDTA, pH 8.0) buffer. The band corresponding to gene ND5 was excised and electroeluted using Isco Model 1740 sample concentrators (ISCO, Lincoln, NB) in an

IBI model QSH gel electrophoresis apparatus (IBI Inc., New Haven, CT) as described [38] and the resulting DNA was labeled by the random primer method.

RESULTS

Superinfection of RAW117-H10 Cells

The biological properties of uninfected RAW117 sublines were similar to those previously described [15–18]. Intravenous injection of 5×10^3 RAW117-P cells produced few liver (median = 0) or lung (median = 0) tumor colonies, while RAW117-H10 cells produced large numbers (median > 200) of liver tumor colonies but few (median = 1) lung tumor nodules (Table I). Parallel studies showed that RAW117-H10 cells have a low level of expression of gp70 compared to RAW117-P cells [16,17,22].

To increase the expression of gp70 in low gp70-expressing RAW117-H10 cells we superinfected such cells with endogeneous RNA tumor virus obtained from RAW117-P cells. Soon after superinfection, increases in gp70 were not found, but by 9–13 culture passages some superinfected RAW117-H10/P cultures showed increases in gp70 expression (Fig. 1) (the designation "/P" is used to indicate cultures superinfected by virus from the parental cells). In contrast, mock-infected H10 cells did not show increased expression of gp70 (Fig. 1). Superinfected H10/P cells heterogeneously expressed gp70, and cell clones derived from superinfected H10/P cells expressed from relatively low to high levels of gp70 (data not shown). Many cell clones showed substantially increased levels of gp70 with respect to mock-infected H10 cells (Fig. 2). Soon after infection the superinfected H10/P cells with a low expression of gp70 had metastatic properties similar to uninfected H10 cells (median liver tumor nodules > 200) (Table I), while several culture passages later some of the superinfected H10/P cells (e.g., cell line 4) had higher levels of gp70 expression (Fig. 2) and were less metastatic (median liver tumor nodules ~50), while mock-infected H10 cells were highly metastatic, similar to uninfected H10 cells (Table I).

Since H10 cells were found previously to be much less sensitive to activated macrophage-mediated cytolysis and cytotasis [8], we examined the abilities of poly I:C-activated macrophages to inhibit the growth of uninfected, mock-infected, and superinfected H10/P cells. At effector:target ratios > 10 there was little difference in activated macrophage-mediated cytostasis, but at lower effector:target ratios (3 to 7), the superinfected H10/P line 4 cells were significantly ($P < 0.001$) more sensitive to

TABLE I. Properties of Uninfected, Mock-Infected, and RNA Tumor Virus-Superinfected RAW117 Cells

RAW117 subline	Passage No.	Infection	Median liver tumors (range)[a]	Survival[a]
P	10	None	0 (0,5)	10/10
H10	10	None	>200 (>200)	0/10
H10/P(M)[b]	26	Mock infection	>200 (>200)	0/10
H10/P(4)[c]	12	Superinfection	>200 (80, >200)	3/10
H10/P(4)[c]	28	Superinfection	51 (18,>200)	8/10

[a]BALB/c mice (8 week old) were injected intravenously with 5×10^3 viable tumor cells and experimental metastases and survival determined 12 days later.
[b]Mock-infected RAW117-H10 cells from Figure 1.
[c]Line 4 superinfected RAW117-H10 cells from Figure 1.

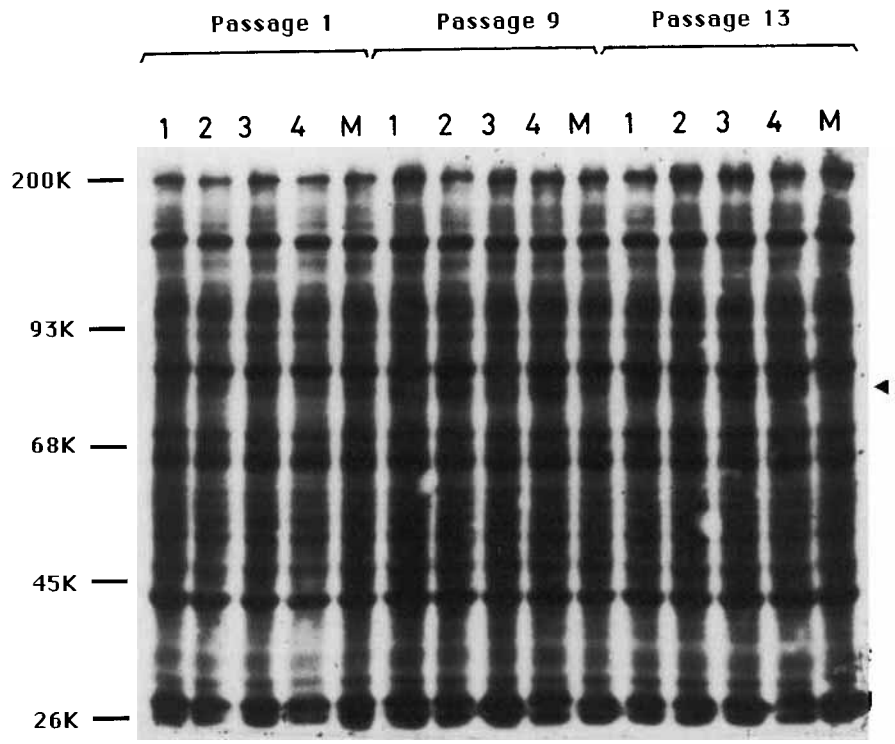

Fig. 1. Cellular levels of viral gp70 in RAW117-H10 cells at various passage numbers with or without endogenous virus superinfection. Superinfection was performed on five cultures of RAW117-H10 cells and electrophoresis, Western blotting, and ^{125}I-Con A staining were performed as described in Materials and Methods. **Lanes 1–4:** Superinfected RAW117-H10 cell lines 1 to 4. **Lane 5 (M):** Mock-infected RAW117-H10 cells. Arrow indicates position of gp70. Molecular weight markers are myosin (M_r ~200,000), phosphorylase b (M_r ~92,500), bovine serum albumin (M_r ~68,000), ovalbumin (M_r ~45,000), and alpha-chymotrypsinogen (M_r ~25,700).

macrophage-mediated growth inhibition (Fig. 3). Mock-infected H10/P cells behaved like uninfected H10 cells in such assays (Fig. 3).

Cytostasis in Macrophage-Conditioned Media

Activated macrophages synthesize and secrete factors that can inhibit tumor cell respiration [14]. By harvesting the culture supernatants of BCG/endotoxin-activated macrophage cultures and adding these to cultures of RAW117 cells, differences in susceptibility to such factors can be demonstrated. Growth rate experiments showed that RAW117-P cells were sensitive in a dose-dependent fashion to growth inhibition by macrophage culture supernatants, but RAW117-H10 cells were relatively resistant (Table II).

Differentially Expressed RAW117 Genes

Previous results indicated differences in gene expression between RAW117-P and -H10 cells [18]. Therefore, we examined some of the differentially expressed RAW117 cell genes to see if they encoded potential target molecules for macrophage cytostatic activities. Since mitochondrial respiration, specifically complexes I and II

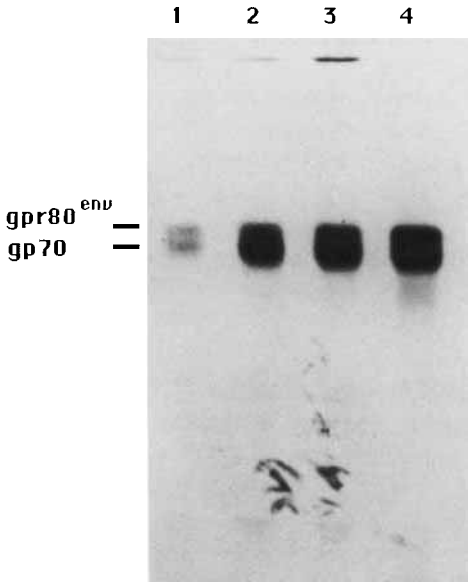

Fig. 2. SDS-PAGE- of RAW117-P, superinfected RAW117-H10, and mock-infected RAW117-H10 cell lysates and anti-gp70 immunoprecipitates. Cells were metabolically labeled with [^{3}H]-leucine and lysed as described in Materials and Methods, and precipitation was performed by adding anti-viral gp70 and protein-A. **Lane 1:** Mock-infected H10 (10 passages). **Lanes 2,3:** H10/P lines 2 and 4 (superinfected with MoMuLV) lines 2 and 4 (10 passages). **Lane 4:** RAW117-P.

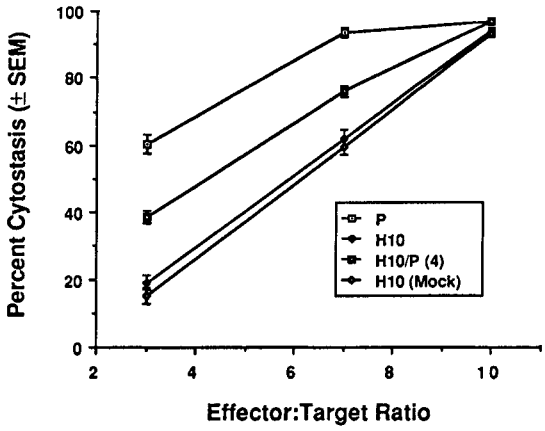

Fig. 3. Sensitivities of RAW117 cells to poly I:C-activated macrophage-mediated cytostasis. Macrophage-mediated cytostasis was performed at various effector:target cell ratios as described in Materials and Methods. At effector:target ratios of 3 and 7, superinfected RAW117-H10/P (line 4) is significantly different ($P < 0.001$) from RAW117-H10 by the Mann-Whitney U-test.

TABLE II. Growth Rates of RAW117-P and H10 Cells in Culture With Media Conditioned by Activated Macrophages

Cells	Effective MCM conc.[a] (%)	Doubling time (hr)
H10	0	9.41 (0.258)[b]
H10	33	11.15 (0.398)
H10	67	14.33 (0.651)
H10	100	15.05 (0.717)
P	0	9.12 (0.268)
P	33	18.81 (1.107)
P	67	30.10 (0.983)
P	100	44.66 (2.390)

[a]Effective concentration of macrophage-conditioned media (MCM) in culture.
[b](SEM).

of the mitochondrial electron transport chain, are the target for a class of macrophage mediators [14,42], we examined mitochondrial gene expression in RAW117-P and -H10 cells using probes derived from the entire mouse mitochondrial genome and portions of the mitochondrial genome. The resulting Northern analyses indicated that a mitochondrial gene, ND5, was expressed at higher levels in H10 than in parental RAW117 cells (Fig. 4). ND5 encodes a subunit of the NADH dehydrogenase of complex I [43,44]. Examination of Northern blots indicates that ND5 is expressed at ~ 10 times higher levels in H10 than parental cells, while the rest of the structural genes in the mitochondria are expressed at nearly equivalent amounts with the hybridization ratio of H10 to P approximately equal to 1.88 (range 1.2–2.8). Deviation of this ratio from unity is due to imprecision in the amount of RNA loaded on the gel.

DISCUSSION

When RAW117 cells are selected in vivo for enhanced metastatic properties, differences can be found in the cellular properties of the highly metastatic cells [8,13,16–24]. The most dramatic change is in the expression of Moloney leukemia virus-encoded gp70. In addition to in vivo selection for metastasis, highly malignant RAW117 cells have been selected in vitro for decreased binding to immobilized lectins [21], increased partitioning in dextran-polyethylene glycol two-phase systems [23], and increased binding to hepatic endothelial cells (R. Tressler and G.L. Nicolson, in preparation). Selected RAW117 cells and unselected cell clones show a good correlation ($r = 0.93$) between loss of gp70 and metastatic potential [16]. Highly metastatic RAW117 cells are also significantly less sensitive to activated macrophage-mediated cytolysis and cytostasis [8,13]. If macrophage-mediated effector systems are suppressed in vivo, the metastatic potentials of the low malignant RAW17 sublines are increased dramatically [13], suggesting a relationship between metastasis, gp70 expression, and macrophage-mediated surveillance mechanisms.

We therefore modified RAW117 cell surface gp70 by superinfection with endogenous RNA tumor virus. Superinfection had been used previously to alter the cell

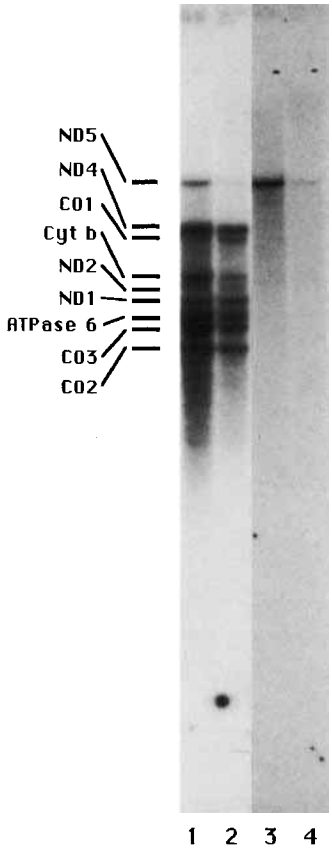

Fig. 4. Blot of RAW117-P and RAW117-H10 poly A+ RNA. **Lane 1:** RAW117-H10 probed with total mitochondrial DNA. **Lane 2:** RAW117-P probed with total mitochondrial DNA. **Lane 3:** RAW117-H10 probed with ND5 specific DNA. **Lane 4:** RAW117-P probed with ND5 specific DNA.

growth, antigenic [39], and malignant [40] properties of rodent cells. By superinfecting highly metastatic low gp70-expressing RAW117-H10 cells with endogeneous RNA tumor virus from some high-expressing low malignant parental cells, we were able to increase gp70 expression and sensitivity to macrophage-mediated cytostasis while lowering metastatic potential in vivo. This suggests that these properties are interrelated and that one of the steps in cytostasis is macrophage recognition of RAW117 cells through a viral-encoded or related structure at the cell surface.

To examine further the mechanism of macrophage-mediated growth inhibition of RAW117 cells, we tested for the ability of activated macrophages to release soluble factors that could differentially affect RAW117 cells [14,41,42]. We found that soluble factors from activated macrophages that are capable of inhibiting the respiration of tumor cells can inhibit significantly the growth of RAW117-P cells, but have little effect on highly metastatic RAW117-H10 cells. These factors are now being purified and characterized, and they have in common the ability to inhibit tumor cell respiration by blocking mitochondrial electron transport systems [14,42].

Previously we found that highly metastatic RAW117-H10 cells express differentially only a few genes compared to low metastatic RAW117-P cells [18]. We have

identified one of these differentially expressed genes as a subunit of NADH dehydrogenase of complex I of the mitochondrial electron transport chain, the target for a macrophage-released cytostatic factor called respiration-inhibiting factor (RIF) [14,29,41,42]. The overexpression of mitochondrial gene ND5 could be related to the ability of H10 cells to escape macrophage-mediated cytostasis, but support for this notion must await further studies on the role of RIF-like molecules in inhibiting the respiration and growth of RAW117 cells.

The limitations in our studies are that the data are correlative and do not definitively prove that gp70 and electron transport components are directly involved in RAW117 cell escape from macrophage surveillance mechanisms. Additional experiments, such as blocking the ability of macrophage cytostatic molecules to bind to RAW117 cells and demonstrating that increased levels of mitochondrial NADH dehydrogenase or other complex I or II components can overcome the cytostatic action of molecules like RIF, will be necessary. Preliminary experiments indicate that there are differences in the steady-state levels of mitochondrial proteins in RAW117-P and -H10 cells, including those thought to be involved in electron transport. Further research should determine whether overproduction of specific respiration components can allow malignant cells to circumvent the action of molecules such as RIF.

Our studies suggest that highly metastatic RAW117 cells may be altered in several characteristics that allow these cells to disseminate and survive at secondary organ sites. In addition to the ability of highly metastatic RAW117 cells to escape host surveillance mechanisms, they also have the characteristics to adhere better to target organ sites [24] and respond differentially to paracrine growth factors released from different organs [45]. Collectively, these properties probably allow highly malignant RAW117-H10 cells to metastasize to liver with high efficiencies.

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