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High-resolution Analyses of Two Different Classes of Tumor Cells *In Situ* Tagged with Alternative Histochemical Marker Genes

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To evaluate interactions of two different tumor cell classes during the establishment of micrometastases at the single-cell level, two different BALB/c 3T3 tumor cell derivatives were established that harbor different histochemical marker genes: bacterial* lacZ *in a EJ-Harvey* ras *transformant (abbreviated LZEJ cells) and human placental alkaline phosphatase (ALP) gene in a human* c-sis *transformant (APSI cells). Several different histochemical staining methods were evaluated, using the distinctiveness of* lacZ *and ALP gene activities, for identification of these cell classes singly or together in the lung after their intravenous injection into nude mice. LZEJ and APSI cells could readily be distinguished from each other after co-injection by using specific and sequential staining protocols of whole organs or sections; staining of host organ cells was minimized. Co-injection of the two tumor cell classes resulted in similar numbers of homogenous microfoci in lungs of LZEJ or APSI cells within minutes after injection that persisted for several hours before clearance of most of them. Furthermore, a significant percentage of foci could be identified containing both classes of tumor cells on whole-organ or section evaluations; these cohabiting foci resisted clearance from lungs. Therefore, use of two different histochemical marker genes to tag different classes of tumor cells provides a powerful approach for determining their* in situ *colocalization, cooperation, or interference with the establishment and development of micrometastases, as well as an opportunity to evaluate gene regulation* in situ *at the single-cell level. (Am J Pathol 1992, 141: 1331–1342)

Although progress has been made in understanding some aspects of the cell and molecular biology of the metastatic process, as well as limited success in controlling metastasis in select model tumor systems,[1–6] studies on formation and development of micrometastases have been hampered because of a lack of suitably sensitive markers to qualitatively and quantitatively discriminate small numbers of tumor cells from the immense background of host cells in target organs. Several methods have been used previously to 'tag' metastatic tumor cells: radioisotopes for evaluating organ distribution[7–9]; specific chromosome markers[10,11] or transfected foreign DNA sequences[12,13] for studying the clonal origin of tumor cells; monoclonal or polyclonal antibodies against tumor-cell–specific antigens for *in situ* studies[14,15]; drug-resistant mutants for clonal interactions and dominance studies[16,17]; melanin-producing melanoma cells for ease of identification of black pigments[18,19]; chemical or fluorescent dyes intercalated into cells[20,21]; and the polymerase chain reaction technique.[22] Unfortunately, these methods cannot be used for both short-term and long-term studies as phenotypic and genotypic markers and in many cases are limited to one (or a few) tumor systems.

To provide a more general marker for tracking tumor cells during progression, we used an ultrasensitive marker gene (*Escherichia coli lacZ*) to genetically and histochemically 'tag' tumor cells.[23] *LacZ* gene codes for a β-galactosidase, the activity of which is detected with a chromogenic substrate (X-gal: 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside), generating an intense blue product that is trapped as a precipitate in the cytoplasm of cells; *lacZ* activity can be detected optimally without interference of host cell galactosidase activity during either whole-organ or methacrylate-embedded section staining.[23] Single tumor cells also can be de-

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tected at sites of invasion of virtually any organ system.[23,24] *LacZ* becomes stably incorporated into the genome of the tumor cells after transfection, facilitating location of tumor cells at various organs after their injection into animals even minutes and hours after injection, thereby allowing high-resolution analyses of the earliest steps in micrometastasis formation.[24,25] Therefore, the *lacZ* gene is a stable and ultrasensitive histochemical marker for metastasis studies, as well as a molecular biologic marker for clonal analyses.[23–26]

To expand this marker gene tagging system for studying potential interactions of multiple tumor cell types as suggested from a growing body of evidence (reviewed in reference 6), we described the design and utility of plasmid vectors containing both an antibiotic resistance gene and one of three histochemical marker genes.[26] Alternative histochemical marker genes on such plasmids would be beneficial for tagging different classes of tumor or nontumor cells, for selecting successfully transfected cells, and for differential analyses of the distributions and locations of two or more cell classes *in vitro* or *in situ* in shared environments.[25,26] Three marker genes were chosen for these analyses for their ultrasensitivity in histochemical staining reactions and for discriminating the activity of the transfected gene, while minimizing background staining from similar gene activities in host target tissues. These were bacterial *lacZ* as described above; *Drosophila* alcohol dehydrogenase generating black-staining cells; and human placental alkaline phosphatase generating red-staining cells.[26] These genes, when transfected into cultured 3T3 cells either singly or collectively, were shown to yield colored products that could readily distinguish single transfected cells from uncolored nontransfected cells and could distinguish different cells expressing the different marker genes.[26] It remained to be shown that these transfected histochemical marker genes could be used for *in situ* analyses of two different cell types.

We now present initial findings of tagging two different classes of tumor cells with different histochemical marker genes and then following the fates of these two tumor cells after their co-injection into athymic nude mice to evaluate possible complementation (or, alternatively, interference) with tumor progression.[6] Human EJ-Harvey *ras*-transfected BALB/c 3T3 cells were tagged with bacterial *lacZ* to establish the LZEJ tumor cell whose micrometastasis and tumor-progressing properties have been described previously.[23,25] In addition, human activated c-*sis* oncogene-transfected BALB/c 3T3 cells have been established in this study carrying the human placental alkaline phosphatase marker gene. Because the *ras* oncogene provides limited metastatic competence for BALB/c 3T3 derivatives in clonally dominant patterns,[25,27] it was of interest to determine if a platelet-derived growth factor (PDGF)-producing tumorigenic cell could complement the *ras*-bearing cell during evolution of micrometastases into overt metastases, particularly in light of the presence of PDGF receptors on 3T3 derivatives. The analyses reported here describe alternative staining protocols for each of these marker genes in cultured tumor cells, in whole organs of recipient nude mice, and in sections of target organs. They also describe the localization and, in some cases, co-localization of the two tumor cell classes during the earliest stages of experimental micrometastasis and suggest mechanisms for the interaction of these two tumor cell classes *in situ*.

Materials and Methods

Tumor Cell Isolation, Culture, and Injection into Animals

The generation of the LZEJ tumor cell clone has been described previously.[23] Briefly, LZEJ is a BALB/c 3T3 (clone A31) derivative transfected with the human EJ-Harvey-*ras* oncogene, the *neomycin*^{R} gene, and the bacterial *lacZ* gene. This clone stably expresses all three genes during one round of tumor progression.[23] All LZEJ-cultured or tumor-derived cells stain intensely blue on incubation with the β-galactosidase substrate (X-gal)[28] during tumor progression in athymic nude mice, including micrometastasis development (but with limited overt metastasis development) in many secondary organs after subcutaneous, intravenous, or footpad injection.[23,25]

A second tumor cell class was generated from the clone A31 BALB/c 3T3 cells using calcium phosphate transfection[29] of a different oncogene and a different histochemical marker gene. The human activated c-*sis* oncogene, coding for the B-chain of PDGF, was used to evaluate whether a growth-factor–producing tumor cell could complement tumor progression of LZEJ cells during their co-injection, because BALB/c 3T3 cells can be transformed with this oncogene[30] and because all BALB/c 3T3 derivatives possess PDGF receptors.[31] For histochemical detection of this second tumor cell class, the human placental alkaline phosphatase (abbreviated ALP) gene was transfected into these cells, the enzyme product of which yields a different colored product in cultured cells on histochemical staining[26] and whose activity is relatively heat resistant.[32,33] The construction or expression of histochemical marker gene plasmids has been described in detail previously.[26]

Briefly, pRSVPAP harboring the cDNA gene expressing human placental ALP was obtained by cloning a 5.0-

Kb *Hind* III-Xba I ALP gene fragment from the pSV2Apap plasmid into the *Hind* III-*Xba* I sites of the pRc/RSV plasmid.[26] The ALP gene is expressed under regulation of the Rous sarcoma virus long-terminal-repeat promoter (RSV LTR), and its mRNA polyadenylated under regulation of the bovine growth hormone signal.[26] On the same plasmid construct, the *neoR* gene is expressed with the SV40 virus early promoter and the SV40 polyadenylation signal.[26] pRSVPAP was introduced into BALB/c 3T3 cells by calcium phosphate precipitation.[29] Resistant colonies were isolated from tissue culture dishes after 2 weeks of G418 selection (400 μg/ml). One colony with uniform intense reddish-black staining for alkaline phosphatase (see below) was selected for second-round transfection with the pREPIsis plasmid that harbors the human lymphoma c-*sis* oncogene[34] and the *hygromycin*^{R} gene. Successful transfectants were selected in medium containing 200 μg/ml hygromycin B during 2 weeks of growth. Resistant colonies were isolated and screened for ALP activity, as well as for levels of human c-*sis* mRNA. Among many ALP-staining colonies, one particular colony (APSI—abbreviated for Alkaline Phosphatase, SIs expressions) expressed high levels of c-*sis* and ALP mRNAs (W-c Lin and LA Culp, manuscript in preparation). A second colony (abbreviated APB), used for some experiments, expressed high levels of ALP mRNA but undetectable levels of c-*sis* mRNA on Northern blots. The complete tumor-progression properties of clones APSI and APB are described elsewhere (W-c Lin and LA Culp, manuscript in preparation). All transformed or tumor cells were free of *Mycoplasma* and were grown in Dulbecco's modified Eagle's medium supplemented with 10% neonatal calf serum and antibiotics. For tumor analyses, athymic nude mice (strain HSD*nu/nu*) were obtained from the breeding colony and housed in the Athymic Animal Facility of the Cancer Center of Case Western Reserve University; animals were injected with 1×10^5 cells of each tumor cell class into the tail vein intravenously or subcutaneously as indicated. After killing the animals, organs were excised immediately and histochemically stained as described below.

β-*Galactosidase Staining*

Tissue-cultured cells were rinsed with phosphate-buffered saline (PBS) three times, fixed for 5 minutes at 4°C with 2% (vol/vol) formaldehyde/0.2% (vol/vol) glutaraldehyde in PBS, and stained for bacterial *lacZ*-expressing enzyme as described previously.[23,28] Briefly, fixed cells were rinsed with PBS three times and then incubated at 37°C overnight in the staining solution: 1 mg/ml X-gal (or Red-gal where indicated), 20 mmol/l potassium ferricyanide, 20 mmol/l potassium ferrocyanide, and 2 mmol/l $MgCl_2$ in PBS.

For staining of intact organs,[23] selected organs were removed from animals immediately after killing and rinsed thoroughly with PBS. After fixation in 2% (vol/vol) formaldehyde/0.2% (vol/vol) glutaraldehyde in PBS for 60 minutes at 4°C, organs were rinsed with PBS three times and incubated in the X-gal (or Red-gal) staining solution; in addition, Nonidet-P40 and sodium deoxycholate were added to the staining solution to final concentrations of 0.02% (vol/vol) and 0.01%, respectively. After incubation at 4°C overnight, tissues were rinsed with 3% (vol/vol) dimethyl sulfoxide in PBS and then with PBS.[23,28] Samples were stored at 4°C in 0.02% (wt/vol) sodium azide in PBS before microscopic evaluation and photography.

Alkaline Phosphatase Staining

Tissue-cultured cells were rinsed with PBS three times, fixed for 5 minutes at 4°C with 2% (vol/vol) formaldehyde/0.2% (vol/vol) glutaraldehyde in PBS, rinsed with PBS three times, and incubated at room temperature for 30 to 60 minutes with 1 mg/ml X-phosphate in 0.1 mol/l TRIS buffer, pH 10.0. In some cases, the staining solution also contained 1 mg/ml nitroblue tetrazolium (NBT) or iodonitrotetrazolium (INT) to yield differing color products (see Results). Cells were rinsed with PBS and stored at 4°C in 0.02% sodium azide in PBS.

For whole-organ staining, removal of organs from mice was followed with three PBS rinses, fixation for 60 minutes at 4°C with 2% (vol/vol) formaldehyde/0.2% (vol/vol) glutaraldehyde in PBS, heat treatment at 65°C for 30 minutes in PBS, rinsing with PBS three times, and incubation at 4°C overnight with 1 mg/ml X-phosphate (and in select cases 1 mg/ml NBT or INT to yield differing color products—see Results), 0.02% NP-40, and 0.01% sodium deoxycholate in 0.1 mol/l TRIS buffer, pH 10.0. After staining, tissues were rinsed with 3% (vol/vol) dimethyl sulfoxide in PBS and PBS alone for storage at 4°C in 0.02% sodium azide in PBS. It should be noted that the heat treatment is essential for eliminating host tissue alkaline phosphatase activity while leaving the transfected plasmid-expressed enzyme activity unaffected.

Embedding and Sectioning

These protocols were essentially as described previously,[23,25] with some exceptions to preserve both β-galactosidase and alkaline phosphatase activities of marker genes. Briefly, tissues were fixed and stained for marker enzymes first (essential to stain for β-galactosidase first

because of its heat sensitivity and then for alkaline phosphatase after the heat treatment to eliminate host organ activity). Then, in preparation for sectioning, stained tissues were fixed again with 4% (vol/vol) paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4, for 3 hours at 4°C; rinsed with phosphate buffer; incubated for 3 hours each with 7% and then 15% (wt/vol) sucrose in phosphate buffer; and dehydrated under vacuum for 10 minutes each in 50% and then 95% cold acetone; finally, for 20 minutes in 100% cold acetone. Samples were infiltrated with JB4-A methacrylate monomer overnight at −20°C under vacuum and transferred to embedding molds for filling with embedding medium (20 ml JB-4A, 0.5 ml JB-4B, and 0.09 g catalyst). After 24 to 48 hours' incubation at −20°C under vacuum to allow polymerization, sections (4 μ thick) were cut with a glass knife on a Lipshaw 45 Rotary microtome. In some cases, sections were counterstained with neutral red or methyl green solutions and finally mounted with Permount (Fisher Scientific, Fairlawn, NJ).

Photomicrography

Photomicrographs of cultured cells were obtained with a Nikon Diaphot-TMD microscope equipped with a Microflex AFX. Photomicrographs of whole organs were generated on a Nikon SMZU dissecting microscope equipped with a Microflex UFX. Fujichrome 64T film was used to produce photomicrographs, although it has been shown that other films and their processing are useful for amplifying some color products.[26]

Materials

The following materials were obtained for these studies: Permount, acetone, and microscope slides from Fisher Scientific Co., Fairlawn, New Jersey; Dulbecco's modified Eagle's medium and G418 sulfate from GIBCO, Grand Island, New York; neonatal calf serum from Biologos, Inc., Naperville, Illinois; glutaraldehyde from Eastman Kodak Co., Rochester, New York; HEPES, napthol AS-BI phosphate, fast red TR, neutral red, methyl green, potassium ferricyanide, potassium ferrocyanide, paraformaldehyde and formaldehyde from Sigma Chemical Co., St. Louis, Missouri; hygromycin B from Boehringer-Mannheim Biochemicals, Indianapolis, Indiana; glycol methacrylate embedding kit (JB-4) from Polysciences, Inc., Warrington, Pennsylvania; 5-bromo-4-chloro-3-indolyl-β-D galactopyranoside (X-gal), 6-chloro-3-indolyl-β-D-galactopyranoside (Red-gal), 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate), NBT, and INT from Research Organics, Cleveland, Ohio.

Results

Double-staining protocols are essential for effective resolution of two different tumor cell populations in host animal tissues. The two tumor cell populations can be genetically 'tagged' with different histochemical marker genes, the enzyme products of which yield very different colored products that are easily detected at the single-cell level in tissues of recipient animals.[24] The recent description of three different marker genes, expressed and differentiated by their characteristic colored products in cultured cells,[26] can now be applied to *in situ* analyses of tumor progression. By using different substrates for different histochemical enzymes, we can provide many possibilities for experimental analyses of multiple-cell systems.

For bacterial *lacZ*-tagged cells, the cultured LZEJ cells show intense blue staining with X-gal substrate (Figure 1A) as described previously.[23] An alternative substrate, Red-gal, which generates a red product, also was tested. It produces intense red staining with LZEJ cells (Figure 1B), a color product easily differentiated from the blue X-gal product. For ALP-tagged cells (APSI), the naphthol-ASBI (or naphthal-ASMX) and fast red protocols traditionally used to identify endothelial cells enriched in alkaline phosphatase activity[23] are not suitable for whole-organ staining because of high background staining from fast red, as well as the relatively low sensitivity of this protocol (data not shown). Therefore, another alkaline phosphatase staining protocol was developed for this application. The X-phosphate/NBT protocol is far more sensitive and can be used for whole-organ staining, as well as with heat inactivation to reduce background staining of host tissues.[32,33] This protocol resulted in intense black staining (with a reddish cast seen at higher magnifications) of cultured APSI cells with X-phosphate/NBT (Figure 1C).

The testing of other potential histochemical substrates with APSI cells in culture showed other possibilities. When the alkaline phosphatase staining protocol was modified by different combinations of X-phosphate, NBT, and INT, different color products were generated. For example, the reddish-black color produced by X-phosphate/NBT (Figure 2A) changes to reddish-brown color using X-phosphate/INT combination (Figure 2B). When X-phosphate was used as substrate alone, blue staining was observed (Figure 2C). When mixtures of LZEJ and APSI cells were stained in culture, the blue (X-gal) or red (Red-gal) staining of LZEJ cells was easily distinguished from the reddish-black (X-phosphate/NBT) staining of APSI cells (data not shown).

We previously used such a double-staining combination with X-gal and naphthol-ASBI to identify LZEJ micrometastatic cells and their topological relationships to

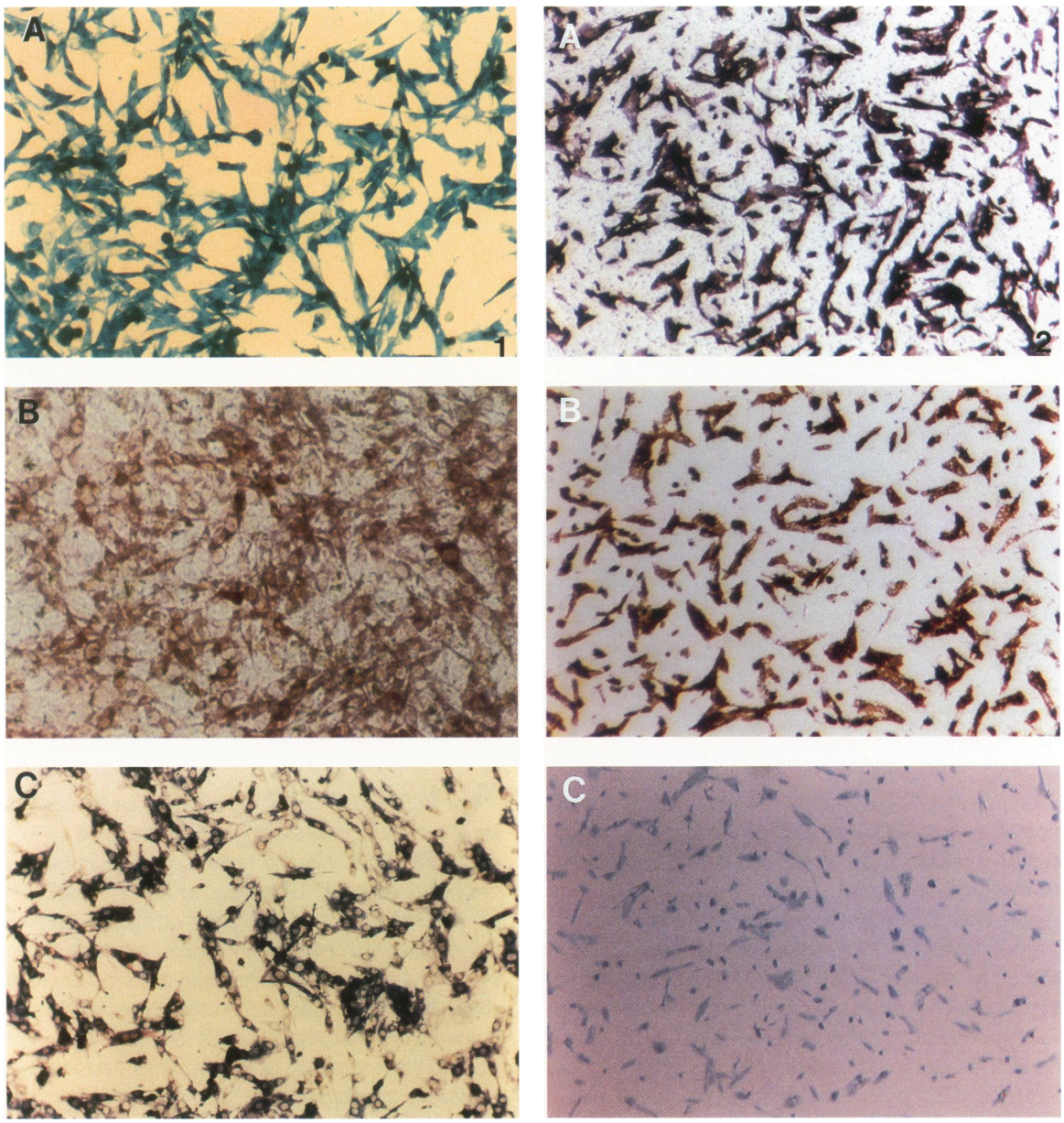

Figure 1. *Alternative histochemical staining of LZEJ cells compared with APSI cells.* **A:** *Blue staining of cultured LZEJ cells with X-gal substrate (×180).* **B:** *Red staining of LZEJ cells with Red-gal substrate (×180).* **C:** *Reddish-black staining of cultured APSI cells with X-phosphate/NBT (×180). It should also be noted that LZEJ cells did not stain at all with X-phosphate/NBT and that APSI cells did not stain at all with X-gal or Red-gal (not shown).*

Figure 2. *Alternative histochemical staining of cultured APSI cells.* **A:** *Reddish-black staining with X-phosphate/NBT (×54).* **B:** *Reddish-brown staining with X-phosphate/INT (×54).* **C:** *Blue staining with X-phosphate only (×54). It should also be noted that LZEJ cells did not stain with any of these protocols (not shown).*

neighboring blood vessels.[23] A similar application of Red-gal and X-phosphate/NBT staining was tested in this study to determine the specificity of these new staining protocols and whether LZEJ tumor cells could be distinguished from blood vessel endothelial cells. This requires that the tissue sections not be heat-treated, to avoid inactivation of alkaline phosphatase in endothelial cells. As shown in Figure 3, the black staining of X-phospate/NBT showed excellent blood vessel endothelial cell staining in the kidney cortex in the absence of any tumor cells (Figure 3A, open arrow) and in the lungs that also harbor LZEJ tumor cells (Figure 3B, open arrow). When this staining approach was used in combination with Red-gal staining to detect LZEJ tumor cells (Figure 3B, black arrows for LZEJ cells and open arrows for blood vessel endothelial cells), one can readily relate the topology of tumor cells to blood vessels. Therefore, this new double-staining approach, using Red-gal to detect *lacZ*-tagged

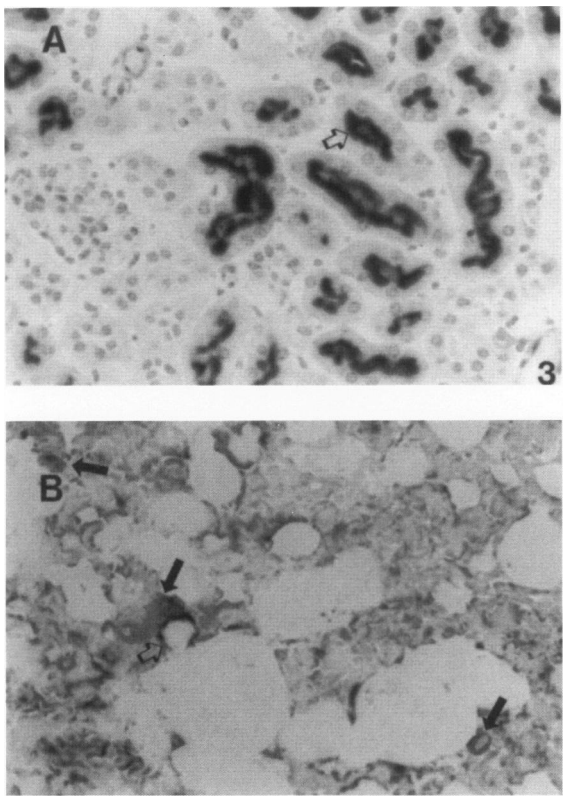

Figure 3. *Alkaline phosphatase staining of kidney and lung sections—costaining of LZEJ tumor cells and blood vessels.* **A**: *Alkaline phosphatase staining of a kidney section was performed with the X-phosphate/NBT protocol, but without heat pretreatment to preserve endothelial cell alkaline phosphatase activity. The black staining of blood vessel endothelial cells at kidney cortex region is indicated with an open arrow for one example (×360).* **B**: *Lung sections were obtained from animals injected IV with 1 × 10^5 LZEJ cells and stained first with Red-gal solution to detect tumor cells (staining red) and then with X-phosphate/NBT substrate without heat treatment to detect blood vessel endothelial cells (staining black). Open arrow indicates one black staining blood vessel and black arrows indicate red-staining LZEJ tumor cells (×360).*

tumor cells and X-phosphate/NBT without heat treatment to detect endothelial cell alkaline phosphatase, provides alternative colors with conservation of cell-type specificity and sensitivity.

Alternative staining protocols then were tested for *in situ* identification of micrometastatic foci from LZEJ or APSI cells after their injection singly into athymic nude mice. Figure 4A illustrates Red-gal staining of microfoci in the lung soon after intravenous injection of LZEJ cells. This Red-gal staining is as sensitive as X-gal staining for identifying LZEJ cells, as shown previously for blue-staining microfoci,[23,24] although the background rose color of lung tissues may provide better contrast with X-gal staining in some cases. Sections of Red-gal–stained lung showed similar sensitivity to that of X-gal–stained lung sections (data not shown).

During staining of lungs from APSI-injected animals, high background staining was observed with X-phosphate combinations if organs were stained as described previously for blood vessel staining.[23] Because the transfected placental form of human alkaline phosphatase is more tolerant to high temperature, organs were incubated at 65°C for 30 minutes to inactivate the enzyme activity of host organ cells.[33] With this heat treatment, background staining in the lung, kidney, and liver were completely eliminated after overnight incubation with X-phosphate/NBT solution at 4°C (data not shown). As shown in Figure 4, use of X-phosphate as the only substrate produced blue-staining APSI foci (Figure 4B) soon after intravenous injection of APSI cells alone; alternatively, X-phosphate/NBT produced reddish-black staining (Figure 4C). Both staining methods produced excellent contrast with host organ tissues, although X-phosphate- or X-phosphate/NBT-stained microfoci generated somewhat fuzzier images than the X-gal– or Red-gal–stained LZEJ microfoci. Whether this observation is related to the localization of the enzymes (alkaline phosphatase is membrane-associated in transfectants; bacterial β-galactosidase is cytosol-localized) or to some other unknown parameter requires further study. The X-phosphate/INT staining for ALP-tagged APSI cells was not suitable for whole-organ staining, because the background color of lung tissues tended to obscure the reddish-brown color of the X-phosphate/INT product (data not shown).

Because a double-staining procedure is essential for histochemical analyses of two different cells tagged with different marker genes, we then examined various combinations of alternative substrates for both β-galactosidase and alkaline phosphatase enzymes when mixtures of both LZEJ and APSI cells were injected into animals. In this case, LZEJ and APSI cells were mixed 1:1 in a tube before injection into the tail vein. (Simultaneous inoculation of the mixture into culture dishes established that the

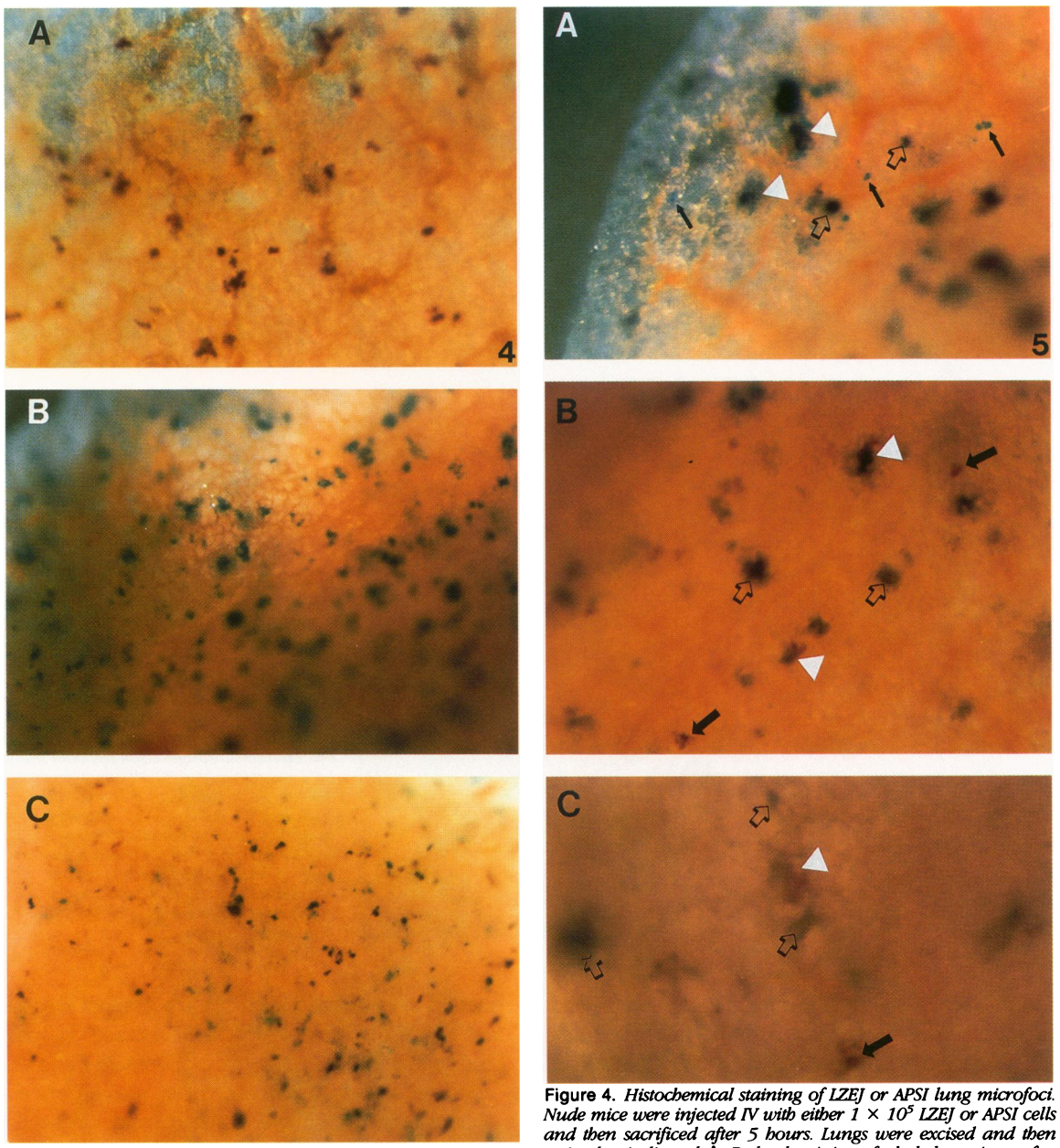

Figure 4. *Histochemical staining of LZEJ or APSI lung microfoci. Nude mice were injected IV with either 1 × 10^5 LZEJ or APSI cells and then sacrificed after 5 hours. Lungs were excised and then stained as indicated.* **A**: *Red-gal staining of whole lung tissue after LZEJ injection (×45). Red-staining microfoci of LZEJ cells are easily localized relative to small blood capillaries.* **B**: *X-phosphate staining of whole lung tissue after injection of APSI cells (×45).* **C**: *X-phosphate/NBT staining of whole lung tissue after injection of APSI cells (×45).*

Figure 5. *Double-staining of lung microfoci after co-injection of LZEJ and APSI cells. Lungs were removed from mice, given an IV injection of a mixture of 1 × 10^5 LZEJ and 1 × 10^5 APSI cells, at 1 hour postinjection. Tissues were fixed, stained for β-galactosidase activity (X-gal in* **A**; *Red-gal in* **B** *and* **C**) *first, and then stained for alkaline phosphatase (X-phosphate/NBT in all panels) after heat treatment.* **A**: *Blue-staining LZEJ microfoci are indicated by small black arrows and reddish-black staining APSI microfoci are indicated with open arrows. Two double-stained foci are indicated by white arrowheads (×100).* **B**: *Some red-staining LZEJ microfoci are indicated by large black arrows and reddish-black staining APSI microfoci are indicated by open arrows. Two double-stained foci, containing both LZEJ and APSI tumor cells, are indicated by white arrowheads (×120).* **C**: *At higher magnification (×320), a typical red-staining LZEJ microfocus is indicated by a large black arrow and reddish-black staining APSI microfoci are indicated by open arrows. Also, one double-stained microfocus is indicated by the white arrowhead.*

vast majority of the population were single cells.) Lungs were removed from animals injected with a mixture of LZEJ and APSI cells at 1 hour after injection, fixed, and histochemically stained. Because of the heat sensitivity of bacterial β-galactosidase, X-gal or Red-gal staining was performed first with fixed organs, followed by 65°C heat treatment and then X-phosphate/NBT staining. As shown in Figure 5A, three classes of microfoci could be distinguished easily soon after injection: well-isolated blue-staining LZEJ microfoci (small black arrows), isolated

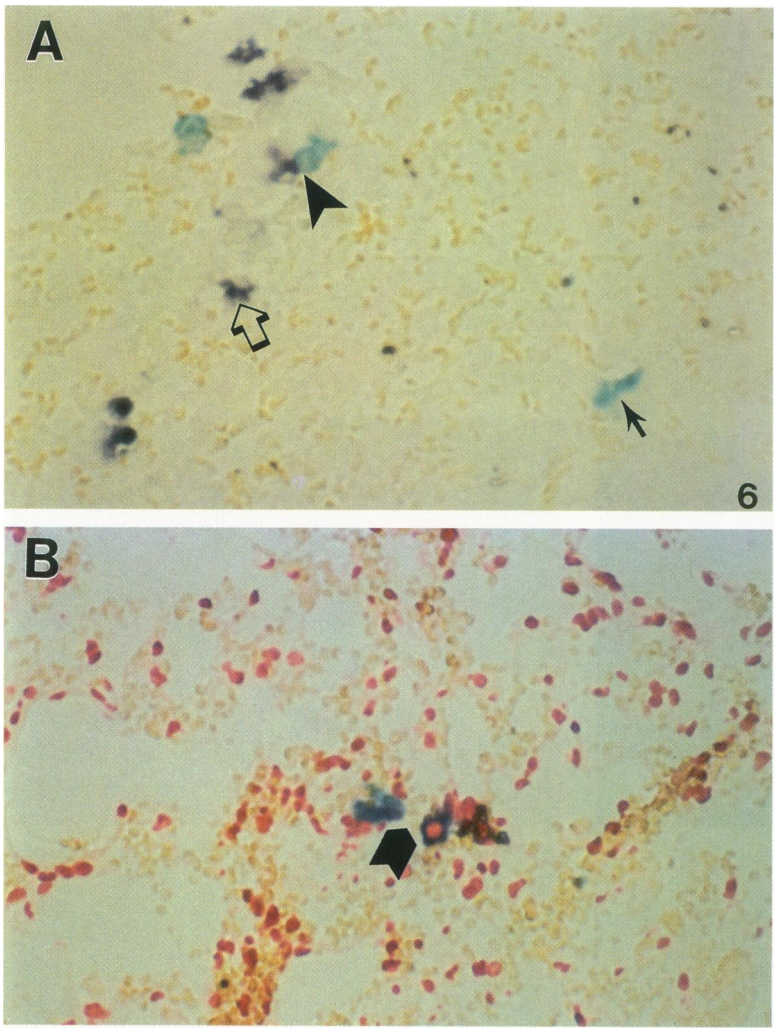

Figure 6. *Detection of double-staining microfoci in lung sections. The experiment described in the legend to Figure 5 was performed, except one animal was sacrificed at 1 hour after injection* (**A**) *and a second animal sacrificed at 24 hours* (**B**). *Lungs were excised, fixed, embedded, and double-stained as described in the Materials and Methods.* **A**: *A section—1 hour after co-injection and without counterstaining—contains blue-staining LZEJ microfoci (e.g., small black arrow) and reddish-black staining APSI microfoci (e.g., open arrow) that are well separated, as well as a focus that harbors both cell types (black arrowhead).* **B**: *Even 24 hours after coinjection, colocalized foci containing both tumor cell classes are evident (solid black arrowhead) in a neutral red counter-stained section. Both panels, ×120.*

reddish-black-staining APSI microfoci (open arrows), and a significant number of microfoci that contained both cell types and were double-stained (white arrowheads). This last result indicates some degree of co-localization of two different tumor cell populations in the same micrometastatic foci.

Other double-staining protocols were tested as well for animals co-injected with both classes of tumor cells. As shown in Figure 5B, Red-gal–staining LZEJ microfoci (large black arrows) and X-phosphate/NBT-staining APSI microfoci (open arrows) could also be easily distinguished, as well as double-staining microfoci (white arrowheads). Also, the combination staining using X-phosphate only and Red-gal provides reasonably good contrast between red-staining LZEJ cells and blue-staining APSI cells (data not shown).

To evaluate co-localization of two tumor cell classes at greater resolution, 4-μ-thick sections of double-stained lung from LZEJ/APSI intravenously co-injected animals were evaluated (Figure 6). Soon after injection, the three classes of tumor cell microfoci could be readily resolved in sections. In the case of Figure 6A, this section was not counterstained. Individual microfoci of LZEJ and APSI cells, well separated from each other, are very apparent (Figure 6A). Furthermore, some microfoci in sections showed close proximity of both tumor cell classes to each other (black arrowhead in Figure 6A). This co-localization persisted at the 24-hour time point (Figure 6B, black arrowhead) when >98% of both tumor cell classes had been cleared out of the lung and the basal level of micrometastatic foci had become established.[24] Counterstaining of sections with neutral red assisted the identification of neighboring host lung cells without adversely affecting the resolution of the two tumor cell types (Figure 6B). The clearance of both tumor cell microfoci was maximized by 24 hours, whereas a significant fraction of microfoci persisted for days or weeks after this time point. Although several thousand foci per lung could be enumerated between 5 minutes and 5 hours after injection, only 200 to 300 foci persisted beyond 24 hours. Of these

'established' microfoci, however, a larger proportion of them contained both tumor cell classes than observed at earlier time points, suggesting that co-localization of the the two cell types provides a more efficient invasion/establishment mechanism in the lungs of co-injected animals. The mechanisms for this more efficient establishment can now be studied more effectively by the approaches described here.

Discussion

In this report, we have demonstrated the versatility of using histochemical marker genes in several different contexts. First, different substrates can be used for each marker gene enzyme that 'light up' tagged cells with different colors, as summarized in Figure 7. Using bacterial *lacZ*, transfected tumor cells will generate intense blue staining with X-gal substrate or red staining with Red-gal substrate. Human placental ALP gene activity in transfected tumor cells can be detected as blue, black, reddish-black, or reddish-brown product, depending on which X-phosphate combination substrate is used. These permutations for two different marker genes provide considerable versatility for maximizing sensitivity of detection of single tumor cells (or small collections of them) in virtually any organ of the recipient animal. Although one combination may be more effective during whole-organ staining, another combination may be more effective during section analyses of lung, brain, or liver.

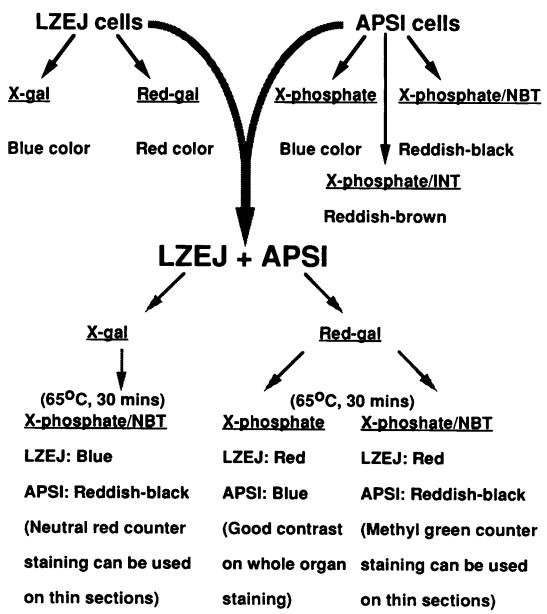

Figure 7. *Double-staining protocols for LZEJ and/or APSI cells in shared environments. The alternative staining protocols developed in these analyses are shown for two situations. First and at the top of the figure, analyses of either LZEJ or APSI cells singly can utilize different histochemical reactions. Second, mixtures of the two tumor cell classes can use these alternative staining protocols to distinguish localities of the two tumor cell classes, as well as host tissue counterstaining for more effective contrast in analyses of sections with neutral red or methyl green.*

Second, these approaches permit detection of two different tumor cell populations in commonly occupied organs. Staining for *lacZ* activity in LZEJ tumor cells was not compromised by staining for ALP activity in APSI cells in the same whole organs or in sections; the converse relationship was also true. Moreover, these experiments demonstrate that these staining reactions must be performed in the prescribed order to 1) conserve optimal staining of both tumor cell classes and 2) minimize host tissue enzyme activities that could potentially reduce specificity for detection of tumor cells. Several factors contribute to the success of these double-staining protocols. The background staining of β-galactosidase and alkaline phosphatase activities can be minimized by taking advantage of unique properties of transfected histochemical marker enzymes: neutral pH for bacterial β-galactosidase activity (*versus* the low pH optimum of host cell galactosidases in lysosomes) and heat stability for human placental alkaline phosphatase (versus lability of blood vessel activity on heat treatment). These double-staining protocols could provide many new possibilities for evaluating tumor cell: tumor cell (or tumor cell: host cell) interactions *in vitro* or *in situ*.

Third, although the *lacZ* marker gene has been successfully used in several tagging systems, new marker systems would be beneficial in studying multiple cell classes. *Drosophila* ADH (alcohol dehydrogenase) and human placental ALP genes provide this versatility.[25,26] Placental alkaline phosphatase provides advantages with its multiple color histochemical protocols that can be used for specific experimental requirements. Another substrate, menadiol diphosphate, has been reported to have excellent sensitivity and more versatility[35] and remains to be tested *in situ*. The human origin of the ALP gene and of the *sis* proto-oncogene makes them suitable genetic markers for Southern blot analysis during clonal studies in a mouse tumor progression model system.[23]

Finally, the establishment of APSI and LZEJ cell systems, harboring different oncogenes and different histochemical marker genes, has permitted examination of possible interactions between two different tumor cell classes *in vitro* and *in situ*. The results of experiments described here permit several comparisons between these two tumor cell systems. First, APSI cells stably establish themselves as microfoci in lungs for several hours after intravenous injection in a pattern that parallels similar processes of LZEJ cells reported previously.[25] Second, the vast majority of APSI microfoci are cleared from the lung by the 24-hour time point, as also shown previously for LZEJ cells[25] and for other tumor cell systems.[36] Therefore, these two results indicate that there is little specificity

for the oncogene that transforms the 3T3 cells and the transient establishment/clearance of most foci from the lung; perhaps any transformant follows the same pattern. In this latter regard, it will be interesting to insert a histochemical marker gene into BALB/c 3T3 cells without transforming them and then evaluating their association with the lung after intravenous injection.

The experiments reported here also demonstrate that a significant number of microfoci, persisting in the lung for several hours after co-injection of equal numbers of LZEJ and APSI cells, contain both classes of tumor cells. With two different tumor cell classes within close proximity of each other, there is a possibility, if not a likelihood, that one tumor cell class provides a 'factor' or environment that permits the second tumor cell class to be more successful in the transformation from micrometastases to overt metastases.[6] Alternatively, one cell class could contain an antagonist that reduces the efficiency of the second cell class in these processes. That double-staining foci persist beyond the 24-hour time point and are more readily apparent in section analyses at later time points suggests that foci containing both tumor cell classes have selective advantage over homogeneous foci during establishment in the lung, ie, they are not cleared as efficiently as homogeneous foci. One possible mechanism for this success in early establishment, remaining to be proven conclusively, is the contribution of PDGF by APSI cells that may permit more successful growth and micrometastasis development by LZEJ cells. Alternatively, LZEJ cells could contribute an environmental 'factor' or 'factors' that stabilize APSI cells in the lung with closest proximity.

Other experimental paradigms suggest growth factor cooperation between two different cell classes in various tumor model systems.[31,37] Platelet-derived growth factor or EGF in the vicinity of micrometastases facilitate their growth in some instances.[38–41] Oncogene expression in some rodent and human tumor cell lines leads to increased expression and secretion of PDGF,[42] which could mediate increased cell division activity of cells in the environment. Alternatively, *ras* oncogene expression in some cells leads to dissociation of PDGF-dependent up-regulation of c-*fos* or c-*myc* proto-oncogenes.[43,44] Factors produced by neighboring tissue fibroblasts play antithetical roles in growth regulation of melanoma cells, dependent on their metastatic competence or incompetence.[45]

The ability to precisely localize two different tumor cell classes at the single-cell level with alternative histochemical marker genes provides opportunity to evaluate gene regulation in these two different cell populations at the earliest stages of micrometastasis development. Thus, the polymerase chain reaction and *in situ* hybridization technologies can be used to precisely identify which tumor cells are expressing which genes *in situ*.[22] Also, "histo-blotting" using molecular biologic probes can be used in combination with histochemical marker enzyme activities to achieve similar goals.[46] In any case, these high-resolution methods offer considerable optimism that important new concepts will emanate on intercellular cooperation during micrometastasis formation, development, or degeneration.

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