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Induction of Reversible Changes in Cell-Surface Glycoconjugates and Lung Colonization Potential by 13-cis Retinoic Acid

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Murine squamous carcinoma cells (KLN205) grown in a medium supplemented with the retinoid, 13-cis retinoic acid (RA), had dose-dependent, selective increases in the expression of certain lectin receptors, which correlated with a dramatic decrease in the ability to form pulmonary colonies ($P = .0003$) (Couch MJ, Pauli BU, Weinstein RS, Coon JS: JNCI, 78:971–977, 1987). These findings suggest a possible relationship between the RA-induced glycoconjugate alterations and the decreased experimental metastatic behavior. We further define the mechanism of RA's action. The finding that RA treatment (5×10^{-6} M, 5×10^{-7} M) did not perturb the cell cycle of KLN205 cells provides further proof that the decreased metastatic behavior is not attributable to any inhibition in the rate of growth or to alterations in the cell cycle. Furthermore, since stable subpopulations with variable lectin binding could not be detected, the mechanism of RA's action does not appear to be due to selection of variant tumor-cell subpopulations. Finally, in a series of experiments designed to determine the reversibility of the RA treatment, the RA-induced decrease in metastatic behavior reverted back to a more metastatic state in the same time frame (3 days) as the reversion of the RA-induced changes in cell-surface glycoconjugate expression. This reversion provides further evidence for a close relationship between the RA-induced modulation of tumor cell-surface glycoconjugate expression and the decreased metastatic behavior; it suggests that transient, reversible modulation of the tumor cell surface may play a role in determining metastatic behavior.

Key words: retinoid, lectin receptors, selection, flow cytometry, lung colony formation, reversible glycoconjugate modulation

We have previously shown that murine squamous carcinoma cells (KLN205) grown in a medium supplemented with the retinoid 13-cis retinoic acid (RA) exhibited a selective, dose-dependent, quantitative increase in the mean level of expression of peanut, soybean, wheat-germ, *Griffonia simplifolia* I, and Concanavalin A lectin receptors, indicating an alteration of glycoconjugate expression in the population. This alteration

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of the cell surface correlated with a drastic decrease in metastatic behavior, as measured by the lung colony assay. The RA-induced decrease in colonization potential could not be attributed to growth inhibition or to decreased cell viability resulting from the RA treatment. Thus, it seemed that the RA-induced alteration of the cell-surface glycoconjugate expression might be related to the decreased metastatic behavior [1].

Because the putative differentiation agent RA is being actively investigated as a potential chemotherapeutic agent [2], we wanted to further define how RA treatment may influence tumor cell metastatic behavior. Specifically, we were interested in whether RA treatment selected for variant tumor cell subpopulations or exerted a modulatory effect on the tumor cell population without eliminating metastatic subpopulations. This has important implications for RA treatment in vivo because selection by RA could induce the generation of new subpopulations, some of which may be resistant to RA treatment and metastatic.

It was also of interest to determine if the RA-induced change of the population's glycoconjugate expression and the decreased metastatic behavior were reversible. If the reversion of the glycoconjugate expression back to control levels coincided temporally with the reversion back to a more metastatic state, this correlation would not only provide further support for a causal relationship between them, but would also argue against selection of subpopulations by the RA treatment. If RA treatment did induce reversible changes in the glycoconjugate expression, then this would provide evidence that transient alterations of tumor cell-surface glycoconjugates may play an important role in determining metastatic behavior.

Using a method that allows the direct effect of RA upon the tumor cells to be studied in a controlled in vitro system, we demonstrated that there was no apparent selection by RA of stable, preexisting variant subpopulations. In addition, the reversible, transient alteration of the cell surface glycoconjugate expression by RA did correlate with reversible changes in the metastatic behavior.

MATERIALS AND METHODS

Cells and Materials

The murine squamous carcinoma cell line KLN205 was obtained frozen from the American Type Tissue Culture Collection (ATCC CRL 1453), Rockville, MD. The RA was purchased from Eastman Kodak, Rochester, NY. Fetal bovine serum (FBS) (Hyclone, Logan, UT; Lot #110460) was biochemically defined and had low vitamin A activity (carotene, <0.1 ng/ml; retinoic acid, <5 ng/ml; retinyl palmitate, 15 ng/ml; retinyl acetate, <10 ng/ml; retinol, 65 ng/ml). All cell growth medium (minimum essential medium) and supplements, such as L-glutamine, nonessential amino acids, and vitamins, were purchased from GIBCO, Grand Island, NY. Trypsin (used at 0.15% concentration) was purchased from DIFCO (Detroit, MI). Corning (Corning, NY) 75-cm^2 flasks were used in all experiments; ^{3}H-thymidine [(^{3}H)dThd] was obtained from Amersham Corp., Arlington Heights, IL; and Aquasol was purchased from Dupont/NEN Products, Boston, MA. Propidium iodide (PI), trypsin inhibitor, ribonuclease A, and spermine were all purchased from Sigma Chemical Co. (St. Louis, MO). Fluoresceinated lectins (FITC-conjugated *Ulex europaeus* I [*Ulex*], peanut [PNL], wheat germ [WGL], *Griffonia simplicifolia* I [GSL I], Concanavalin A [Con A], and soybean [SBL]) were purchased from Vector Laboratories, Burlingame, CA.

Growth Conditions and Preparation of RA-Supplemented Medium

KLN205 cells were grown in medium containing 10% FBS supplemented with either 5×10^{-6} M, 5×10^{-7} M, or no RA (control). The RA-supplemented medium was made immediately before use from aliquots of a 10^{-2} M RA stock solution as previously described [1]. The cells were grown in a fully humidified 37°C atmosphere containing 5% CO_2. The medium was changed every 48 hr. All experiments were done under subdued lighting.

Cell Cycle Analysis

KLN205 cells were grown in RA-supplemented medium for 15 days. The staining of the tumor cell's DNA with PI was done by following the detergent-trypsin protocol for the preparation of nuclei for flow cytometric DNA analysis, as described by Vindelov [3]. This procedure allowed for good resolution of the DNA peaks and for the absence of nuclei clumping. The nuclei were analyzed on a Coulter Epics V flow cytometer. Ten thousand nuclei were collected for each group of cells analyzed. The data were analyzed with the Coulter Easy PARAI (parametric analysis I) statistical program.

Staining of Tumor Cells with Fluorescent Lectins

KLN205 cells were grown in medium supplemented with 5×10^{-6}, 5×10^{-7}, or no RA (control media) for various intervals, depending on the experiment, and then harvested for staining using a two-stage procedure. Twenty-four hours prior to staining, the exponentially-growing tumor cells were harvested from the tissue culture flask with the use of 0.15% trypsin and then reseeded onto new flasks so that the cells were approximately 60% to 80% confluent. These monolayers were then harvested for staining: the cells were incubated with 5 ml of 10 mM EDTA for 3 min, and then the flasks were struck sharply with the side of the palm of the hand to dislodge the cells from the surface of the flask into a single-cell suspension. Cells harvested in this way had flow-cytometric profiles identical to those of cells harvested with the two-stage trypsin procedure described in the section on lung colony assay.

The cell suspension was then washed and incubated with one of six FITC-conjugated lectins as described previously [1]. The lectins used were *Ulex*, PNL, WGL, GSL, Con A, and SBL. Table I lists the preferential binding specificities of the lectins.

Flow Cytometric Analysis (FCM)

FCM analysis of stained KLN205 tumor cells was performed as previously described [1] with either a Coulter Epics V or a Coulter Epics C flow cytometer.

TABLE I. Lectins and Their Carbohydrate Specificities [24]

Lectin	Sugar
Ulex europaeus I (Ulex)	L-fucose
Peanut lectin (PNL)	D-Gal-(1-3)-GalNAc
Wheat germ lectin (WGL)	(β(1-4)-D-GlcNAc), sialic acid
Griffonia simplicifolia I (GSL I)	D-Gal, D-GalNAc
Concanavalin A (Con A)	D-mannonse, D-glucose
Soybean lectin (SBL)	D-GalNAc

Cell Sorting of KLN205 Cells Using the Flow Cytometer

In two separate experiments, KLN205 cells were sorted into subpopulations on the basis of lectin (PNL) binding ability and cell size. For sorting of KLN205 cells expressing varying amounts of PNL receptor, the cells were plated and harvested with the standard two-stage harvesting procedure (described previously), washed, and then counted. The cells were then stained with lectin (sterile FITC:PNL, 2.5 μg/ml/10^6 viable cells) and incubated on ice for 45 min. After incubation, the cells were washed twice, resuspended at 1×10^6 cells/ml in medium containing propidium iodide (0.15 mg/ml). The cells were then analyzed by FCM to collect a fluorescence histogram; debris and dead cells were gated out as previously described. Sort windows were then set to collect the dimmest-staining 15% and the brightest-staining 15% of the population. After 2 hr of sorting into 2 ml of FBS in 15-ml conical glass collection tubes, the cells were centrifuged and resuspended in 2 ml of medium supplemented with 10% FBS plus antibiotics. An aliquot was reanalyzed by FCM to assess the purity of the sort. The sorted cells were grown in 25-cm^2 flasks and, when 60%–80% confluent, reanalyzed using the two-stage harvesting procedure.

For the sorting of KLN205 tumor cells on the basis of size, a brief (1 min) trypsinization harvest procedure was used. The cells were then analyzed by FCM to collect a forward-angle light scatter profile (an estimate of cell size) and to gate out debris and dead cells. Sort windows were set to collect the smallest 20% and the largest 35% of the population. After 2 hr of sorting, the sorted subpopulations were immediately reanalyzed to assess the purity of the sort. The sorted subpopulations were grown in 25-cm^2 flasks and reanalyzed when they had reached 60%–80% confluency.

Lung Colony Assay

Tumor cells were grown in the presence or absence of RA for various intervals, depending on the experiment. On the day prior to the experiment, KLN205 cells grown in media supplemented with 5×10^{-6} M, 5×10^{-7} M, or no extra RA (control cells) were trypsinized briefly (1 min). The cells were reseeded onto new flasks so that the cells were 60%–80% confluent the next day. This procedure allowed the cells to be harvested for injection with the use of a brief (30 sec) trypsinization. Viable cells, 5×10^4, in 0.2 ml phosphate-buffered saline, were injected into the tail vein of 5-week-old male DBA/2J mice. Viability was determined from the proportion of cells excluding trypan blue (0.1%). The mice were sacrificed 21 days later and the number of lung colonies formed was determined with the use of a dissecting microscope as described by Fidler [4]. Extrapulmonary tumor formation was checked in each group. The Kruskal-Wallis one-way analysis-of-variance test was used to determine if the experimental groups differed from the control group.

RESULTS

Previous studies [1] showed that RA at concentrations of 5×10^{-6} or 5×10^{-7} M in growth medium did not have any effect on the rate of (^{3}H)dThd incorporation into the KLN205 cells. However, it still seemed possible that the cell-cycle distribution of the RA-treated cells might be perturbed, especially because such perturbation had been reported in other systems [5]. Therefore, we wished to determine whether RA treatment caused any differences in the proportion of cells in each cell-cycle compartment. Cell-

cycle analysis of tumor cells grown in RA-supplemented medium (5×10^{-6} M, 5×10^{-7} M RA) for 15 days showed that the DNA histogram profiles were virtually superimposable upon each other. Parametric statistical analysis revealed that RA treatment did not affect a significant alteration in the percentage of cells in the various cell-cycle compartments. For instance, the percentage of control cells in the S phase was 43, whereas cells grown in medium supplemented with 5×10^{-7} M and 5×10^{-6} M RA had 44% and 46%, respectively, of their cells in S phase. Thus, RA at these concentrations induced no alterations in the proportion of cells in each cell-cycle compartment. Because there were no signs of cytotoxicity and no differences in cellular morphology when RA-treated KLN205 cells, which grew as an adherent monolayer, were examined under a phase-contrast microscope, these two noncytotoxic and noncytostatic concentrations of RA were used in all experiments.

When KLN205 cells were grown in RA-supplemented medium for 15 days, dose-dependent quantitative increases in the ability to bind PNL, SBL, Con A, GSL I, and WGL were seen ($P < .001$ when mean log fluorescence channels were compared with control levels). Figure 1a shows the increased binding of PNL by RA-treated tumor cells. Similar profiles were generated when the KLN205 cells were stained with the other lectins, with the exception of *Ulex*. The RA-treated tumor cells stained with *Ulex* did not exhibit the same large difference in lectin-binding ability, compared with control cells, even though the difference was still statistically significant ($P = .02$) (Fig. 1b). Thus, the modulation of the tumor cell-surface glycoconjugate expression by the in vitro RA exposure was dose-dependent and selective.

Even though we used grossly nontoxic concentrations of RA in all experiments, we were concerned that RA treatment could be selecting for subpopulations of tumor cells that bound more lectin molecules or were larger cells. This possible selection of variant subpopulations could explain the quantitative increases in glycoconjugate expression seen on the RA-treated cells. To determine if there were preexisting subpopulations of cells that RA treatment could select for, the original KLN205 cell population was first labeled with FITC:PNL and then sorted, with the flow cytometer, on the basis of lectin-binding ability. We collected the dimmest-staining 15% and the brightest-staining 15% of the cells using the sort windows shown in Figure 2a. The cells were collected for 3 hr, and aliquots were immediately reanalyzed by FCM, which showed an effective separation based upon lectin-binding ability (Fig. 2b,c). The sorted subpopulations were expanded in tissue culture for 8 days, and then the progeny of the dim- and bright-sorted cells were stained with FITC:PNL and reanalyzed. The dimly and the brightly staining subpopulations (Fig. 2d,e) were indistinguishable from each other upon reanalysis. Apparently both had shifted back toward the original population's profile. Similar results were seen in another experiment, where the KLN205 cells were sorted into 'large' and 'small' cell subpopulations on the basis of forward-angle light scatter (a measurement of cell size). The smallest 20% and the largest 35% of the cell population were collected. When these subpopulations were reanalyzed 13 days later, both subpopulations had reverted back to the original population's profile. Thus, the sorting experiments argue against selection of variant large, lectin-binding tumor cell subpopulations because there were no stable, preexisting subpopulations in the original population of KLN205 cells that could be selected for by the RA treatment.

In this system, where stable tumor cell subpopulations with variable lectin binding could not be detected, the reversibility and kinetics of the cell-surface glycoconjugate alteration induced by RA were examined. We sought to relate reversible alterations in

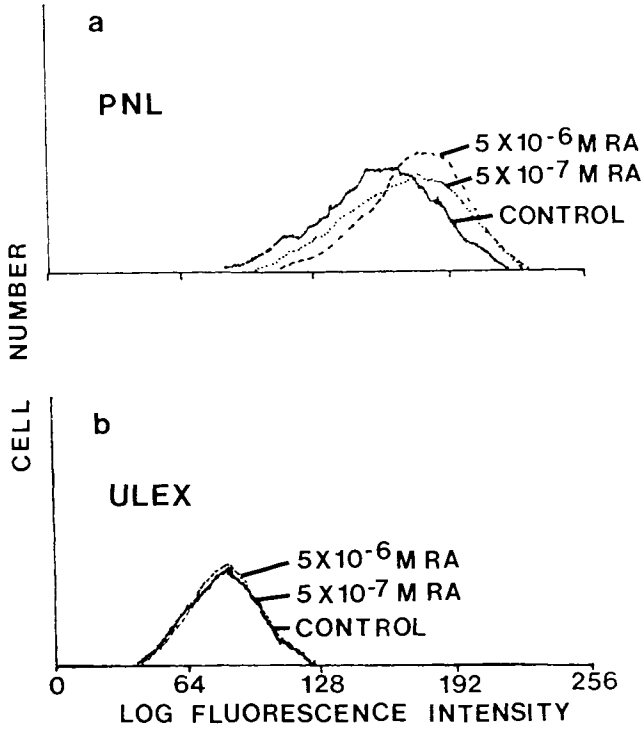

Fig. 1. Selective, dose-dependent quantitative increases in the binding of fluoresceinated lectins by KLN205 cells grown in RA-supplemented medium for 15 days. KLN205 cells were harvested with the two-step EDTA procedure and labeled with FITC:PNL before FCM analysis (**a**). FITC:*Ulex* was used to stain the RA-treated tumor cells (**b**). Each histogram was generated from the analysis of 5,000 viable cells.

cell-surface glycoconjugate expression to the lung-colonization potential of the tumor cells by performing modifications of experiments that we had done earlier [1]. After 1 day in RA-supplemented media, the KLN205 cells showed no differences in their ability to bind any of the lectins. These same cells had no alteration in their lung-colonization capacity, compared with control cells ($P = .39$). After 4 days, large dose-dependent increases were seen in the binding of all the lectins ($P < .0001$) except *Ulex* ($P = .02$). The KLN205 cells grown in RA for 4 days had a decreased ability to form lung colonies (as measured by the median number of colonies formed). However, the range was so large that the difference between the RA-treated and control groups was not statistically significant ($P = .22$). By 15 days of RA treatment, however, the KLN205 cells exhibited dose-dependent, quantitative increases in the binding of PNL, GSL I, WGL, SBL, and Con A, but the increased binding of *Ulex* was of a much smaller magnitude. When injected into mice, the cells exposed to RA for 15 days had a markedly altered ability to form lung colonies ($P = .0003$). Indeed, their ability to form lung colonies was completely abrogated by the RA treatment.

To determine if the RA-induced changes in cell-surface carbohydrate structures were reversible, we grew the KLN205 cells in the RA-supplemented medium for 15 days and then immediately returned them to the control medium for 3 days. Figure 3

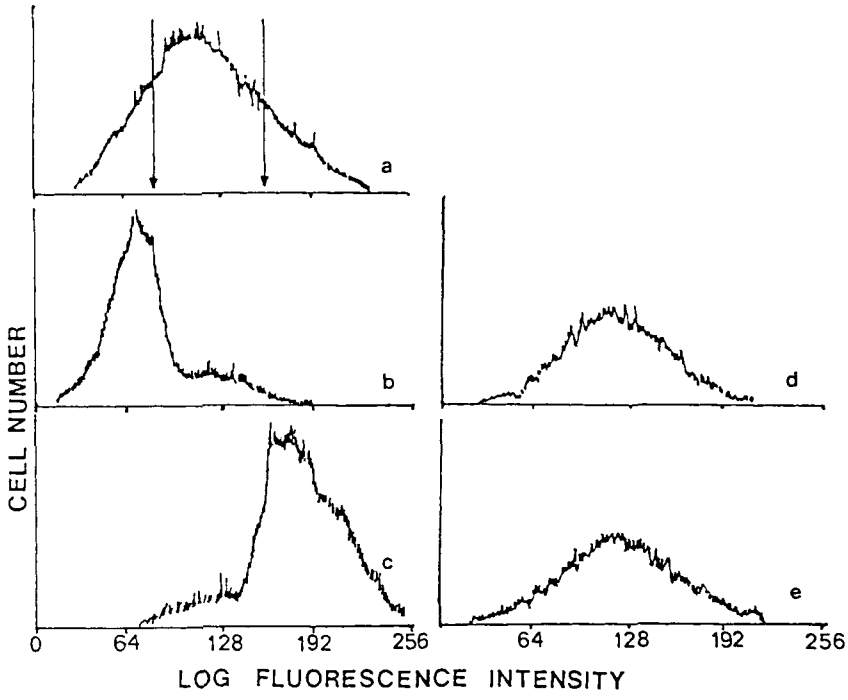

Fig. 2. Cell sorting and reanalysis of KLN205 cells stained with FITC:PNL. KLN205 cells were labeled with FITC:PNL, and sort windows were set as shown by the arrows (**a**). After the sterile sorted cells were collected, an aliquot of the dim and bright cells was reanalyzed by FCM. The PNL binding profile of the brights (**b**) and the dims (**c**) is shown immediately after sorting. After expansion in tissue culture for 8 days, the progeny of the sorted cells were stained with FITC:PNL and again analyzed by FCM: sorted bright (**d**) and sorted dim (**e**).

demonstrates the reversion of the glycoconjugate expression of these cells back to control levels. Although only the reversion of the increased expression of PNL receptors is shown, similar results were obtained when the other lectins (except *Ulex*) were used to stain the tumor cells. Because RA-treated KLN205 cells labeled with *Ulex* never had the same large quantitative increase in glycoconjugate expression exhibited when other lectins were used, the reversion of *Ulex* receptor expression did not seem remarkable.

To examine the reversibility of the RA-induced decrease in lung-colonization potential, we grew KLN205 cells in RA-supplemented medium for 15 days, then transferred the RA-treated tumor cells into the control medium for 3 days immediately before injecting them into mice. Returning the KLN205 tumor cells to control media for 3 days caused them to revert back to a more metastatic state (Fig. 4). Because both the RA-induced alteration of tumor cell-surface glycoconjugate expression and the RA-induced decrease in colonization potential reverted back to control levels in the same time frame, the transient alteration of glycoconjugate expression may be an important determinant of the metastatic phenotype.

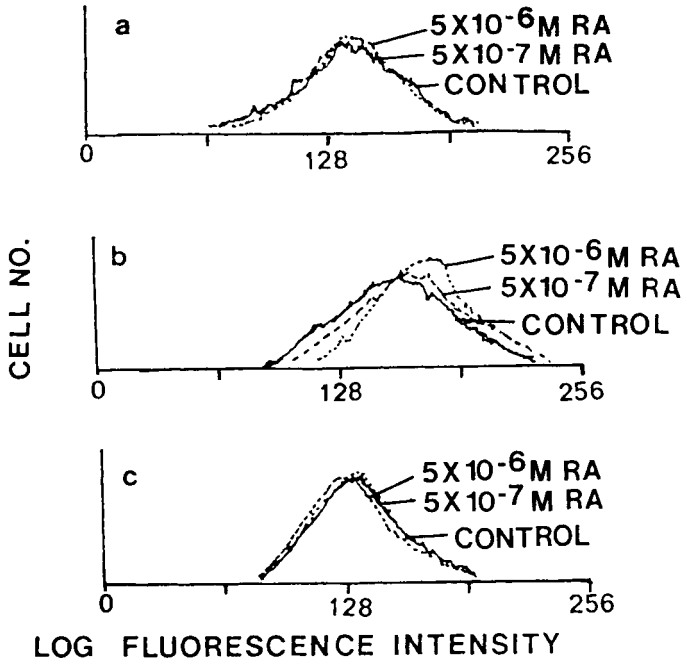

Fig. 3. The reversibility of the RA-induced quantitative increases in the binding of fluoresceinated lectins by KLN205 cells. Tumor cells were grown in RA-supplemented media for 1 day and stained with FITC:PNL for FCM (**a**). After 15 days in RA-supplemented medium, the tumor cells exhibited a dose-dependent increase in PNL receptor expression (**b**). However, when the KLN205 cells were immediately transferred to the control medium for 3 days, the cells no longer had the same quantitative increases in glycoconjugate expression (**c**).

DISCUSSION

Treatment of tumor cells with RA has previously been associated with changes in glycoconjugate expression [6], altered cellular adhesion [7], and growth inhibition [8,9]. Whereas studies have shown that tumor cells grown in retinoid-supplemented medium have a decreased ability to form pulmonary colonies [10–12], this effect may be attributed to the inhibition of proliferation by the retinoid treatment because tumor cell growth was inhibited by the concentrations of RA used in those studies. Since retinoids may act as potent immunoregulatory agents and have diverse other effects in vivo [13], it is difficult to study the direct effect of RA on the tumor cells when retinoids are administered to a tumor-bearing animal.

We have shown, using a system designed to study the direct effect of RA upon metastatic tumor cells, that RA treatment (5×10^{-6} M and 5×10^{-7} M) caused dose- and time-dependent alterations in tumor cell-surface glycoconjugate expression. This modulation was stable for as long as the KLN205 murine squamous carcinoma cells remained in the RA-supplemented growth medium up to 80 days [1]. RA at these concentrations caused no growth inhibition or alterations in the cell cycle of the KLN205 cells. The observation that decreased lung-colonization potential occurred only after glycoconjugate changes were present on the tumor cell's surface suggested an association

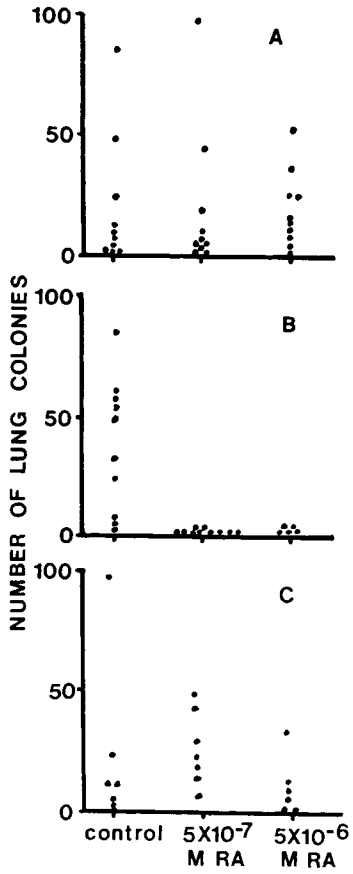

Fig. 4. The reversible RA-induced decrease in colonization potential by KLN205 cells. Tumor cells grown in RA-supplemented medium for 1 day had no decrease in their ability to form lung colonies (**A**). After 15 days of RA treatment, the ability of the KLN205 tumor cells to form pulmonary colonies was drastically decreased (**B**). When these cells were transferred back to control medium for 3 days, there was a reversion to a more metastatic state (**C**).

between the RA-induced decrease in lung-colonization potential and the modulation of glycoconjugate expression.

Abundant evidence supports a mechanistic relationship between cell-surface glycoconjugate expression and the malignant phenotype in other experimental tumor systems [14,15]. For instance, a tumorigenic, nonmetastatic variant cell line had lectin-binding profiles that differed both from the parent cells and from a metastatic variant line [16]. In another system, the expression of Con A receptors on rat hepatocarcinoma metastatic variants was predictive of lung-colonization potential; the mannose residues were thought to be involved in the attachment of tumor cells to endothelial cells [17].

Thus, it is quite plausible that RA-induced alterations in tumor cell-surface carbohydrate expression may influence the metastatic behavior of the KLN205 cells. It is also perhaps possible that RA treatment in vitro rendered the cells highly immunogenic and thereby unable to grow in vivo. However, this seems unlikely because the cells appear to recover from the RA treatment within 3 days. There probably would not be

enough time for immune cells to become sensitized to the recovering tumor cells in vivo and mount an immune reaction against them. Even if animals did become sensitized to the RA-treated cells, any RA-induced antigenic differences would have disappeared by the time that cytotoxic T cells could attack the tumor cells. Altered susceptibility of the tumor cells to natural cytotoxic effector cells is a more likely mechanism, especially because they have been shown to regulate lung colonization. This possible mechanism is being actively investigated in our laboratory.

The following evidence supports the idea that the RA treatment did not select for preexisting tumor cell subpopulations. First, the RA concentrations used were nontoxic and therefore did not exert apparent selection pressure. Second, differences in the ability to bind lectins or cell size (which might affect the total amount of lectin bound) were not stable or heritable, as judged by sorting appropriate subpopulations and expansion in culture. Finally, the alterations in glycoconjugate expression and the metastatic behavior were both rapidly reversible after the RA treatment was removed, which would not occur if selection of subpopulations had occurred. If RA is used as a chemotherapeutic agent, this finding may have important implications because tumor cell subpopulations interact in what has been described as a 'tumor ecosystem' to limit the generation of new variant subpopulations [18]. Thus, a chemotherapeutic agent that does not exert a selection pressure will not be prone to cause clonal instability or increased tumor cell heterogeneity [19]. Furthermore, if RA decreased metastasis by elimination of metastatic subpopulations, the development of resistant metastatic subpopulations with strong growth advantages would be virtually inevitable. However, caution must be exercised in extrapolating the results of this report to any effect that retinoids may have as chemotherapeutic agents, because all the work was done with mice.

The reversibility of both the RA-induced cell-surface glycoconjugate changes and the RA-induced alterations in lung-colonization potential not only were further proof of a close relationship between the two but also emphasized the importance of transient, reversible changes in cell surface glycan expression in determining tumor-cell lung-colonization potential. These studies are paralleled by reduced metastatic capacity of tumor cells treated with tunicamycin, which also induces reversible glycosylation changes [20,21]. It is interesting that Poste [22] has suggested that the microenvironment may act to reversibly modulate the competence of a tumor cell to complete a step in the metastatic process. For instance, alterations in the supply of tumor cell nutrients may induce a transient phenotypic modulation of the tumor cell.

Mice fed a retinoid-supplemented diet for 15 days had abnormal PNL and *Ulex* staining of frozen sections taken from the ear, tail, and gut [23]. This finding is interesting because it suggests a role for retinoids as in-vivo modulators of cell-surface glycoconjugate expression. Thus, it is reasonable to speculate that retinoids may have antineoplastic action in vivo by reversibly modulating tumor cell glycoconjugate expression.

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