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Comparative Analysis and Anatomic Distribution of *Ras* p21, IL-2R, and MEL-14 in Malignant and Hyperplastic Murine Thymus

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The distribution and localization of thymocytes positive for p21 ras, the lymphocyte homing receptor antigen MEL-14, and IL-2 receptors were studied by immunohistology and flow cytometry. Comparisons were made between age-matched normal mice, carcinogen-treated mice at early (stage II) and late (stage III) stages of disease, and cortisone-treated mice. In normal thymus, the majority of cortical and medullary thymocytes are p21 ras positive. MEL-14^{bi}- and IL-2R-positive cells are located in the cortex and comprise less than 5% of the thymus population. Stage II carcinogen-treated animals consistently show increased numbers of MEL-14^{bi} cells in the thymus, with fewer animals having increased numbers of IL-2R positive cells. These populations appear to be different from one another. All stage III animals have MEL-14^{bi}-positive tumor cells, which in 70% of the cases also express IL-2R. Cortisone treatment was used to study nonmalignant proliferation. After cortisone treatment there is a marked increase of p21 ras staining in both the cortex and medulla during the first 72-hour interval. Within 24 hours, 50% of the thymocytes are IL-2R positive, but MEL-14^{bi} cells are not detected. By 48 hours, 90% of the thymus population expresses IL-2R and 50% of the cells are MEL-14^{bi} positive, and this results in a substantial population of cells positive for both IL-2R positive:MEL-14^{bi} markers. This population rapidly disappears by 72 hours, leaving 90% of the cells MEL-14^{bi} positive and less than 10% IL-2R positive. The staining of p21 ras at 72 hours is unusual, showing a speckled, cytoplasmic pattern. In light of our findings, we propose that the first step in thymic lymphomagenesis in carcinogen-treated C57BL/6 mice involves the rare cortical MEL-14^{bi} subpopulation and is thymic dependent. A late stage involves expression of IL-2 receptors by a subset of MEL-14^{bi} cells, thus conferring the potential for autonomous growth and malignancy. (Am J Pathol 1990, 136: 307–317)

The microenvironment of the thymus appears essential for certain stages in the proliferation and differentiation of thymocytes. The thymus consists of thymocytes and stromal cells organized into two major compartments, the medulla and the cortex. The medulla is composed of mature T cells ready for export to the periphery. The cortex is the primary site where stem cells from the bone marrow proliferate and differentiate. The interaction of bone marrow cells with specific lymphoid epithelial structures known as thymic nurse cell complexes[1] located in the outer cortex is required for early events in the T-cell differentiation pathway.[2] Changes in the number of thymic nurse cells occur within the cortex after whole-body irradiation and after treatment with the chemical carcinogen N-methylnitrosourea (NMU).[3,4]

In previous studies we have shown that treatment of low leukemic strains of mice with the chemical carcinogen NMU or γ irradiation induced thymic lymphomas in more than 90% of the animals within 3 to 6 months.[5] The *ras* oncogene is activated frequently by point mutation in these tumors.[6] Although the cellular events occurring during the development of radiation and carcinogen-induced T-cell lymphomas are not fully characterized, abnormal proliferation of cells within the bone marrow and thymus appears to be an obligatory step.[7–10] Recently we have reported that one of the earliest changes that can be detected in the thymus of treated animals is the presence of

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increased numbers of cells bearing receptors for interleukin-2 and/or the lymphocyte-homing antigen.[11]

The relationship between gene expression, localization of T cells within the thymus microenvironment, and proliferative capacity has not been previously determined. In this immunohistologic study the expression of three differentiation antigens were analyzed: the T-cell growth-factor receptor IL-2R,[12] the receptor molecule MEL-14 involved in lymphocyte homing to the peripheral lymphoid tissues,[13] and the p21 protein encoded by the *ras* oncogene.[14] All three antigens were analyzed in thymus tissue from normal age-matched controls and in NMU-treated and γ-irradiated animals at two stages of tumor development. Cortisone-treated mice served as a control group for hyperplastic proliferation not linked to malignant transformation.

In this report we describe the changes in the expression of p21 protein, the interleukin-2 receptor, and the lymphocyte-homing receptor in defined cellular locations within the normal and cortisone-treated thymus and compare it with changes occurring in thymocyte populations from γ-irradiated and NMU-treated mice at early and advanced stages of lymphoma development.

Materials and Methods

Cortisone Treatment

Cortisone acetate (Merck Sharp & Dohme, West Point, PA) was diluted in saline to yield 10 mg/ml. One-month-old female C57BL/6J mice were injected i.p. with 100 mg/g of cortisone acetate. Thymus tissue was removed on days 1 to 5 after treatment. All mice were purchased from the Jackson Laboratories (Bar Harbor, ME).

Carcinogen Treatment

One-month-old C57BL/6J female mice (80 mice per treatment protocol) received weekly treatments of γ irradiation or NMU, as previously described.[5] Groups of mice were killed and thymus tissue was removed for analysis beginning 4 weeks after NMU treatment up to 18 weeks and beginning 12 weeks after radiation treatment for up to 27 weeks. Staging of the disease was assessed by thymic weight and serologic criteria, as previously described.[11]

Preparation of Tissues

Immediately after the animals were killed, thymus tissue was dissected, weighed, and prepared for flow cytometric and histologic analyses. For frozen sections, thymus tissue was placed on cork discs in OCT (Tissue-Tek, Miles Laboratories, Elkhart, IL) and snap frozen in isopentane precooled in dry ice. Samples were stored at −70 C until sectioned. Frozen sections of C57BL/6J mice were used for all immunophenotype analyses. A portion of one lobe from each thymus was fixed in formalin and embedded in paraffin. Paraffin sections were used for assessment of morphology and p21 *ras* immunoreactivity. The remaining thymus was processed for flow cytometry as a single-cell suspension.

Reagents

Rat monoclonal antibody (MAb) 7D4 (IgM) to murine interleukin-2 receptors (IL-2R)[15] and rat monoclonal antibody MEL-14 (IgG_{2a}) to a lymphocyte-homing receptor[16] were obtained as hybridoma culture supernatants and used undiluted. Rat monoclonal antibody Y13-259 (IgG_1), which recognizes the *ras* gene product p21,[14] was provided by Dr. M. Furth as a purified immunoglobulin preparation and used at 20 to 40 μg/ml. A rat monoclonal antibody (IgG_{2b}) to Thy 1.2 was purchased from Becton Dickinson, Rutherford, NJ.

Immunohistology

Indirect Immunofluorescence Analysis

Cryostat-cut serial tissue sections (4 to 8 μ) were used unfixed or fixed for 10 minutes with either 1% formalin in PBS or cold acetone. Tissue sections were washed several times in PBS, rinsed in 2% bovine serum albumin in PBS (BSA-PBS), and then incubated in a wet chamber with MAb for 1 hour at room temperature, with a predetermined optimal dilution. Sections were washed with PBS and incubated for 45 minutes with fluorescein-conjugated rabbit anti-rat immunoglobulins previously titrated for optimal dilution (1:40 in BSA-PBS) (Cappel Laboratories, Cochranville, PA).[17] Tissue sections were washed extensively in PBS, wet mounted in 90% glycerol in PBS, and examined with a fluorescence microscope equipped with epifluorescence.

Immunoperoxidase Analysis

The avidin-biotin peroxidase complex was the method used on fresh frozen tissue sections and paraffin-embedded tissue sections.[18] Primary antibodies were incubated for 1 hour, and the other steps were similar to those described below for paraffin-embedded tissue sections. Formalin-fixed and paraffin-embedded tissue sections were deparaffinized with xylol and ethanol before use. Sections

Table 1. Gamma-Radiated Mice: Immunotypes of Normal and Tumor Cells.

	THY-1	MEL-14	7D4	RAS
Control	●	○	◒	▨
Control	●	◒	◒	▨
PL-1	●	▨	◒	▨
PL-2	●	◒	○	●
LYM-1	●	◒	●	▨
LYM-2	●	○	◒	▨
LYM-3	●	◒	◒	▨
LYM-4	●	◒	◒	●

●=Homogeneously and Strongly Immunoreactive;
▨=Homogeneously and Weak Immunoreactive;
◒=Heterogeneously Immunoreactive;
○=Undetectable Immunoreactivity.
PL=Early Stage of Disease;
LYM=Lymphoma.

were treated for 30 minutes in 1% hydrogen peroxide in PBS to remove endogenous peroxidase activity. Tissue sections were washed in PBS and then incubated for 20 minutes with 10% normal goat serum to block nonspecific binding to the tissue section. The goat serum was drained and sections were incubated with primary MAb overnight at 4 C. Sections were incubated for 1 hour with secondary antibodies (goat anti-rat biotinylated immunoglobulin at 1:100 in PBS; Vector Laboratories, Burlingame, CA), washed in PBS, and rinsed with 0.05M Tris, 0.1 M NaCl, pH 8. The peroxidase reaction was performed by incubating tissue sections for 6 to 12 minutes with 5 mg of diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) in 100 ml of Tris buffer containing 100 μl of 0.3% hydrogen peroxide. Sections were washed with distilled water, counterstained with hematoxylin, and mounted.

Negative controls included substitution of the primary MAb by another MAb of the same species and subtype, or with PBS alone. Positive and negative controls for *ras* p21 staining consisted of cell lines of known p21 *ras* expression as described previously.[18]

Flow Cytometry Analysis

Thymus tissue was dispersed in Dulbecco's modified Eagle's medium and washed once. Cells were resuspended in PBS-BSA and 0.1% NaN_3 to give 10^7 cell/ml. 10^6 cells were resuspended in 50 μl of antibody (predetermined optimal saturation dilution) and incubated on ice for 30 minutes. Cells were washed twice with wash buffer (PBS-BSA-0.1% NaN_3) and resuspended in a 1:50 dilution of

Table 2. NMU-Treated Mice: Phenotypes of Normal and Tumor Cells.

	THY-1	MEL-14	7D4	RAS
Control	●	○	◒	▨
Control	●	◒	◒	▨
PL-1	●	◒	◒	●
PL-2	●	◒	▨	▨
L -1	●	◒	◒	●
L -2	●	▨	◒	●

●=Homogeneously and Strongly Immunoreactive;
▨=Homogeneously and Weak Immunoreactive;
◒=Heterogeneously Immunoreactive;
◑=Isolated Cells Immunoreactive.
○=Undetectable Immunoreactivity.

fluoresceinated goat-anti-mouse immunoglobulin (Cappel) or fluoresceinated mouse-anti-rat immunoglobulin (Jackson Immunoresearch Laboratories) for 30 minutes on ice. After two washes, samples were resuspended in 1 ml of wash buffer and 20,000 cells were analyzed in an Ortho 50 H cytofluorograph (Becton-Dickinson). The forward light-scatter profile of normal thymocytes stained with MEL-14 antibody was used to set the appropriate computer gating of fluorescence signals to score only for the MEL-14^{hi} cells. MEL-14^{hi} cells comprise 3% of the normal thymus population.[13] The percentage of specific marker-positive cells is determined by subtraction of the percentage of cells that stain with another MAb of the same species and subtype.

Results

Tables 1 and 2 summarize the immunoreactivities of MEL-14, 7D4 (IL-2R), and p21 *ras* MAbs on sections of thymus tissue removed at different intervals following γ irradiation

Table 3. *Expression of Thymocyte Differentiation Antigens in Carcinogen-Treated C57BL/6 Mice at Different Disease Stages*

Disease stage*	N†	Thymus (mg ± SEM)	% Animals positive‡ MEL-14^{hi}	% Animals positive‡ IL-$2R^+$
Control	21	61 ± 3	0	0
II	32	59 ± 3	88	38
III	17	226 ± 27	100	70

* Thymus tissue from untreated control animals, matched for sex and age, was analyzed in parallel with thymus tissue from carcinogen-treated animals. Treated animals were analyzed at weekly intervals beginning 4 to 18 weeks after NMU-treatment or 12 to 27 weeks after γ-irradiation treatment.

† N, number of animals analyzed.

‡ Thymus tissue was analyzed for surface markers by flow cytometry. The number of animals (%) that differed from age-matched controls by one standard deviation in percentage of marker positive cells over control values is given. Control value for MEL-14^{hi} or IL-2R <4%.

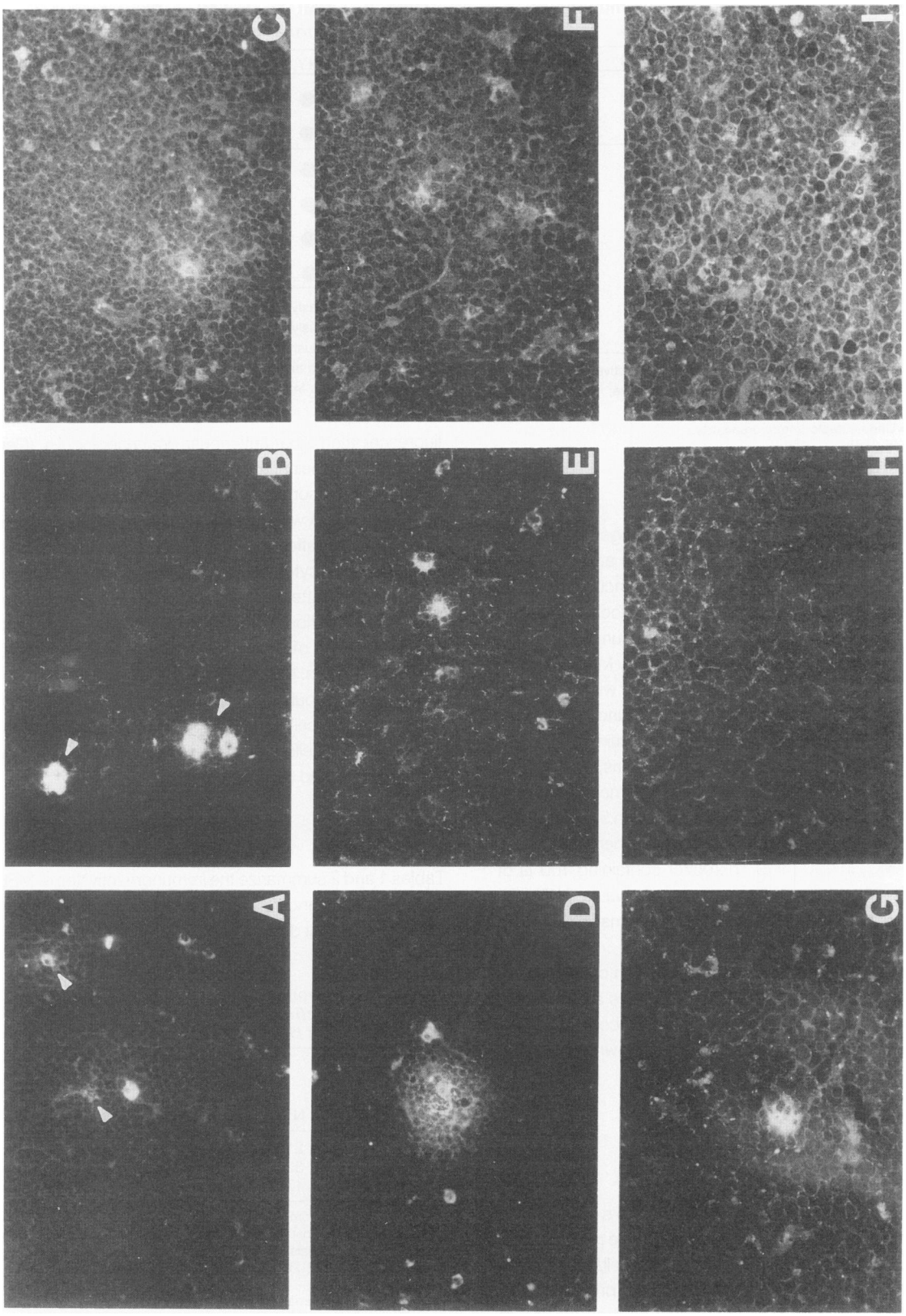
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Figure 1. *Localization of MEL-14, 7D4 (IL-2R) and p21* ras *antigens in normal thymus* (A–C), *early stage of disease* (D–F), *and frank lymphoma* (G–I) *of carcinogen-treated mice.* A: *MEL-14 positive dendritic thymic cells denoted by arrows (×200);* B: *7D4 positive isolated subcapsular thymic cells denoted by arrows (×100);* C: *weak and diffuse p21* ras *protein staining (×200);* D: *isolated groups of immunoreactive cells with MEL-14 MAb (×200);* E: *diffuse and weak immunostaining with 7D4 MAb (×200);* F: *homogeneous expression of p21* ras *by Y13-259 MAb (×200);* G: *homogeneous and weak expression of MEL-14 antigen by lymphoma cells (×400);* H: *diffuse localization of 7D4 antigen by lymphoma cells (note speckled immunostaining pattern of the membrane) (×400);* I: *strong and homogeneous immunoreactivity of Y13-259 MAb in lymphoma cells (×200).*

and NMU treatment, respectively. No differences were observed in the reactivities of the MAbs when comparing immunofluorescence and immunoperoxidase stainings (data not shown). Table 3 summarizes the flow cytometric analysis of surface markers of thymocytes from the same mice analyzed by immunohistochemistry.

Tissue from representative early stage and frank thymic lymphomas stained for the same panel of antigens is shown in Figure 1. Figures 2 and 3 illustrate the immunofluorescence staining patterns of the MAbs detecting T-cell homing and growth-factor receptor antigens and p21 *ras* in normal mouse thymus after cortisone treatment.

Immunohistology of Thymocytes from Normal Mice

Four control thymus were analyzed to assess the immunophenotypes and distribution of thymocyte subpopulations of normal C57BL/6J mice.

Thy 1.2

The majority of thymocytes express the Thy-1 antigen but in differing amounts. Staining intensity can be used to distinguish cortical (Thy-1^{hi}) from medullary (Thy-1^{lo}) compartments of the thymus.[19,20] In the normal thymus, the subcapsular area stains intensely with Thy-1 MAb, while only a few immunoblasts under the thymic capsule are negative for Thy-1 antigen. The medulla shows a weaker and more diffuse immunoreactivity with the Thy-1 MAb (data not shown). The Thy 1.2 antibody was used as a reference for the staining pattern of thymocytes within the thymus section.

MEL-14

Recirculating T and B cells express homing receptors for lymph node high endothelial venules,[16] which are recognized by the MAb MEL-14. Only 3% of thymocytes express high levels of the homing receptors, MEL-14^{hi}[21] and they are located within the cortex.[13,20]

In this study, frozen sections incubated with MEL-14 MAb showed that the majority of the cortical and medullary cells were unreactive for this antibody. Positive cells were identified in both the thymic cortex and the medulla (Figure 1A). Foci of positive cells occurred in the thymic cortex with random distribution in small groups and represented 2% to 5% of the total cell population of the normal mouse thymus. Dendritic cells located mainly in the medulla showed intense immunostaining.

IL-2R

In the adult thymus only 2% to 3% of the total thymocyte population express receptors for the T-cell growth factor IL-2.[22] Murine IL-2 receptors are detected by the MAb 7D4.[15] IL-2R-positive thymocytes have been described in the subcapsular area of the cortex and scattered throughout the inner cortex.[22,23]

In this study, thymic sections stained with 7D4 MAb showed negative medullary zones. Isolated positive cells were located in the subcapsular area. Dendritic cells in the medulla were negative for 7D4 immunoreactivity (Figure 1B).

p21 ras

The three genes of the *ras* family (H-, K-, and N-*ras*) are normally expressed in thymocytes of the adult mouse at the RNA level[24] and encode similar proteins (p21 *ras*), all of which are recognized by the MAb Y13-259.[14]

Immunohistochemical analysis of thymic sections stained with Y13-259 MAb showed a homogeneous and diffuse immunostaining of the cortical thymocytes. Medullary cells, although positive, were only weakly reactive. Dendritic cells located in the medulla were more intensely stained than the surrounding cells for the p21 *ras* product (Figure 1C).

Immunohistology of Thymocytes from γ-Irradiated and NMU-Treated Mice at Different Stages of Disease

A large series of mice (80 animals per treatment protocol) were treated either with γ irradiation or NMU and killed at intervals during the latent period of tumor development.[11] In this analysis, six cases of frank thymic lymphomas (stage III of disease) and four early lymphomas (stage II of disease) were studied (Tables 1 and 2). Early lymphomas are characterized by a thymic weight of less than 100 mg and an increase in the numbers of MEL-14^{hi} cells.[11] No major differences were observed between treatment

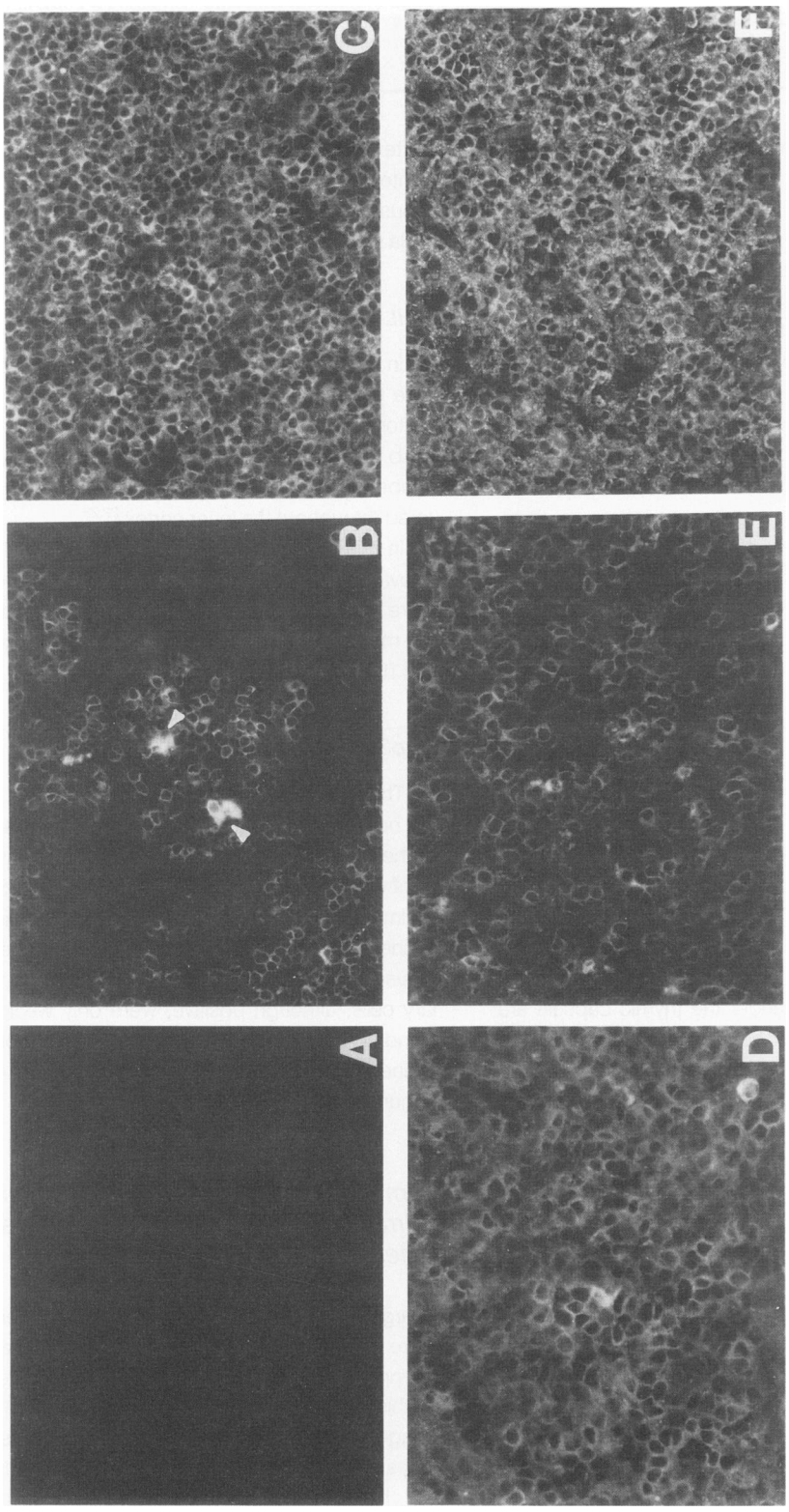

Figure 2. *Localization of MEL-14, 7D4(IL-2R) and p21* ras *antigens in thymus of cortisone-treated mice at day 1* (**A–C**) *and day 3* (**D–F**) *after treatment.* **A**: *MEL-14 antigen was not detectable on day 1 after cortisone treatment;* **B**: *7D4 antigen was expressed by isolated thymic cells surrounding dendritic cells denoted by arrows;* **C**: *homogeneous and diffuse staining with Y13-259 mAb detecting p21* ras; **D**: *heterogeneous and weak expression of MEL-14 antigen at day 3 after cortisone treatment;* **E**: *patchy expression of 7D4 antigen by thymic cells;* **F**: *increased expression of p21* ras *detected by Y13-259 MAb at day 3 after cortisone treatment (note speckled immunostaining pattern of the cytoplasm) (×200).*

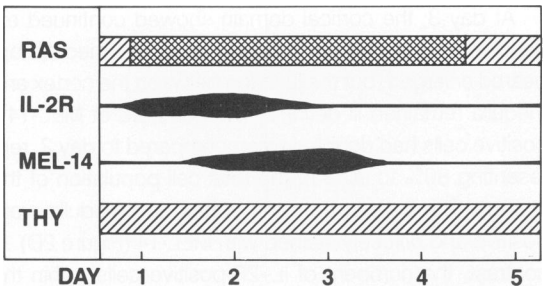

Figure 3. *Schematic representation of the changes in p21* ras, *IL-2R (7D4) and MEL-14 expression in normal thymus from day 0 to day 5 after cortisone treatment. Width of the bars denotes relative frequency of marker positive cells. The area of crossbatched bars indicates increased intensity of p21* ras *staining.*

groups when comparing stage II of the disease. Similarly, the results of stage III tumors were the same regardless of inducing agent. Although some variations in staining patterns were observed between individual cases at each disease stage, the results will be presented as the overall pattern of changes observed at stage II or at stage III of lymphoma development (Tables 1 and 2 and Figure 1). In addition to the data obtained by immunostaining of frozen sections, flow cytometric analysis was performed on the remaining thymus tissue (Table 3).

Early Stage of Disease

More than 70% of animals at early stages of disease (stage II) show morphologic changes in the thymus ranging from typical hyperplasia to lymphoblastic lymphoma.[11]

Thymus sections from stage II animals showed derangement of corticomedullary areas with rich cellularity. Thymic hyperplasia was observed in all treated mice, regardless of carcinogen used. Foci of cells showing atypical hyperplasia were identified in two of two NMU-treated mice and in one of two γ-irradiated animals.

The staining patterns of MEL-14, IL-2R, and p21 in diseased animals differed from those observed in age-matched control mice (Figure 1 and Tables 1 and 2). In general, the numbers of positive cells and the staining intensity was increased. Foci of MEL-14-positive cells were localized around dendritic cells, which are positive themselves for MEL-14 (Figure 1D). These groups of cells were intensely stained, showing a heterogeneous pattern of reactivity. By flow cytometry, 28% of thymocytes were MEL-14^{hi} positive (range, 10% to 65%, ±3 SEM). The immunoreactivity of IL-2R-positive cells was more variable in stage II animals at 27% (range, 10% to 78%, ±7 SEM) In animals with increased numbers of IL-2R-positive cells, the cells were weakly and diffusely stained (Figure 1E). These cells tended to be isolated throughout the cortex. At this earlier stage of disease, two independent thymocyte populations can be distinguished by the differences in MEL-14 and IL-2R staining reactivities and distribution within the thymus. The characteristic pattern of p21 immunostaining for this group of stage II animals was diffuse and weak reactivity (Figure 1F).

Lymphomas

At late stages of disease when animals show frank tumors at autopsy, the thymus shows an expanded cortex with little or no medulla remaining. Tissue sections of lymphomas showed effacement of the typical thymic architecture. The thymus was filled with large, hyperchromatic and undifferentiated tumor cells. Many mitotic cells were observed in both groups of treated mice. The majority of stage III animals showed not only an increased frequency of all three marker-positive cell populations but also an increased immunostaining reactivity in most cases (Figure 1G to I and Tables 1 and 2). MEL-14^{hi}-positive cells have a heterogeneous pattern of immunostaining with weak immunoreactivity (Figure 1G). Groups of positive and negative tumor cells were observed in the expanded cortical areas. Flow cytometric analysis showed 38% (range, 12% to 80%, ±4 SEM) MEL-14^{hi}-positive cells. A different pattern of immunostaining was observed for IL-2R-positive cells showing a diffuse and speckled staining pattern (Figure 1H). Approximately 33% (range, 13% to 78%, ±7 SEM) of cells were IL-2R positive. All tumors were consistent in p21 reactivity showing, in some cases, a homogeneous strong, and in others, a weaker pattern of immunostaining (Figure 1I).

Flow Cytometric Analysis of Thymocytes from γ-Irradiated and NMU-Treated Mice at Different Stages of Disease

Flow cytometric analysis was performed on thymocytes from 21 age-matched control animals, 32 stage II animals with early disease, and 17 stage III animals with frank tumors. Frozen sections of thymus tissue from representative cases (four stage II; six stage III) were used to assess immunohistology (Tables 1 and 2 and Figure 1). The percentage of positive cells obtained by flow cytometry closely agreed with the results obtained by immunostaining for each case analyzed. The results of the analysis for MEL-14- and IL-2R-positive thymocytes in animals at the two stages of disease are shown in Table 3.

The earliest and most consistent change observed in thymocytes of 88% (27 of 32) stage II γ-irradiated and NMU-treated animals was an increased frequency of MEL-14^{hi}-positive cells. At the same time, only 38% (12 of 32) of the same animals showed significant increases in the frequency of IL-2R-positive cells.

All of the animals with advanced lymphoma, regardless of treatment protocol, had increased frequencies of MEL-14^{hi} cells and 70% (12 of 17) showed increased frequency in IL-2R-positive cells. These results are consistent with the increased representation of a rare subpopulation of MEL-14^{hi} thymocytes in the thymus during the latency period, a subset of which also express receptors for IL-2.

Immunohistology of Thymocytes from Cortisone-Treated Mice

To ascertain whether the immunohistologic changes observed in the thymus of carcinogen-treated animals were due to the proliferation of malignant cells, a group of animals was treated with cortisone to compare changes due to proliferation of normal cells in regenerating thymus tissue. Groups of cortisone-treated mice were sacrificed at 24-hour intervals (three animals per day) from day 1 to day 5 after treatment. The immunohistology of representative animals is shown in Figure 2. Figure 3 summarizes the changes in the expression of p21 *ras*, MEL-14, 7D4 (IL-2R), and Thy-1 antigen during the 5-day interval.

Cortisone treatment rapidly and selectively eliminates the majority of cortical cells within 24 hours of administration, giving rise to a disorganized corticomedullary region, a small undefined cortex, and an expanding hypocellular medulla. The rare population of cortical MEL-14^{hi}-positive cells present in the control mice was no longer observed by day 1 (Figure 2A). Approximately 50% to 60% of the thymocytes in the subcapsular and inner cortical regions were IL-2R-positive compared with less than 3% observed in normal thymus tissues (Figure 2B). The majority of cells in both the cortex and medulla showed stronger homogeneous immunostaining for p21 *ras* than is observed in the control. Dendritic p21-positive cells were not observed in these sections (Figure 2C).

Two days after cortisone treatment the repopulation of the cortex of the thymus was clearly evident. Although the thymic architecture was still disorganized, the cortical zone was expanding. Within the expanding cortical region 40% to 50% of the cells showed a heterogenous staining pattern with MEL-14. In addition, 20% to 30% of the medullary cells also showed a weak and heterogeneous immunoreactivity with MEL-14. More than 90% of the cortical thymocytes were weakly positive for IL-2R, while the medulla was negative. Clearly more than 40% of the cortical IL-2R-positive cells also expressed the MEL-14^{hi} lymphocyte-homing receptor antigen. The p21 *ras* reactivity was very similar to that on day 1, showing strong and homogeneous reactivity for most cortical and medullary cells.

At day 3, the cortical domain showed continued expansion and increased cellularity, and the medulla appeared enlarged, but the junction between the cortex and medulla remained ill defined. The numbers of MEL-14^{hi}-positive cells had doubled when compared to day 2, representing 80% to 90% of the total cell population of the thymus. The cells in both the cortex and medulla were positive and diffusely stained with MEL-14 (Figure 2D). In contrast, the numbers of IL-2R-positive cells within the cortical population decreased 5% to 10% of the population (Figure 2E). The MEL-14^{hi}:IL-2R-positive population, if present at 72 hours, was greatly diminished in number. The pattern of p21 *ras* immunoreactivity remained strongly and homogeneously positive, which was similar to days 1 and 2. A speckled pattern of reactivity was clearly evident in both the membrane and cytoplasm (Figure 2F).

At day 4, the thymus showed decreased cortical hypercellularity and although no well-defined corticomedullary junction was apparent, the thymus structure in the cortex and medulla was reorganized. The staining pattern of MEL-14^{hi}- and IL-2R-positive cells was similar to the control animals. A few scattered groups of cells located in the subcortical region were weakly staining for MEL-14. The remaining cortex and medullary areas were unreactive, including the dendritic cells that stain intensely with MEL-14 in normal thymus tissue. IL-2R-positive cells represented approximately 5% of the total thymus population and were located exclusively in the subcapsular area of the cortex and randomly distributed within the inner cortex. The p21 immunoreactivity was similar to that observed in controls, with one exception. Approximately 30% of the cortical cells showed a patchy and weak immunoreactivity rather than the homogeneous and diffuse immunostaining pattern of thymocytes from untreated animals.

Finally, at day 5 the thymus structure was fully reorganized and showed good corticomedullary definition and normal cellularity in the cortex and medulla. At this time, all staining patterns for MEL-14, IL-2R, and p21 antigens were normal (Figure 1A to C and Figure 3).

Discussion

A comparative analysis of the frequency and distribution of p21 *ras*-, MEL-14^{hi}- and IL-2R-positive thymocytes was made between normal thymus tissue and tissue from carcinogen-treated mice at two stages of lymphoma development. Differences were found for MEL-14 and IL-2R markers during the latent period of tumor growth, suggesting that cells in which these genes are expressed play a role in malignant T-cell proliferation.

Three *ras* genes, H-, K- and N-*ras*, comprise a family of highly conserved genes that encode similar protein (p21 *ras*) products.[18] In tissues of adult mice, these three genes show differential expression in all the tissues examined at the RNA level.[24] For thymus tissue, only N- and K-*ras* genes are expressed.[24] Increased p21 *ras* expression occurs in rapidly proliferating cells, eg, regenerating liver tissue following partial hepatectomy,[25] and is associated as a marker for cells within basal layers of several self-renewing tissues, eg, skin, colon, and mammary lobules.[18] In this study, we examined the patterns of immunostaining for p21 *ras* by its distribution within the thymus as well as the general level of gene expression by intensity of tissue staining to determine if changes in expression were associated with proliferation.

The distribution of the p21 *ras* protein in normal thymus tissue and tissue from animals with early and late stages of disease was similar, as assessed by the pattern of immunostaining. The most notable difference in p21 *ras*-positive thymocytes between normal and diseased animals was in their morphology. An exception was the pattern of immunostaining in the thymus of cortisone-treated animals 72 hours after treatment. These thymocytes showed strong staining with a speckled appearance of the cytoplasm (Figure 2F), a pattern not encountered previously in thymus tissue from age-matched control or carcinogen-treated animals. We conclude from these observations that rapidly proliferating cells in regenerating thymus tissue differ from proliferating malignant cells as well as proliferating normal cells within the cortical compartment based on the differences in cytoplasmic staining of the p21 *ras* protein.

There are several reports of increased p21 *ras* protein in human colon, mammary, and bladder carcinomas.[26–28] We compared levels of p21 *ras* expression in thymus tissues from normal, early (stage II), and late (stage III) stages of disease. We also compared the level of p21 *ras* expression with the presence or absence of activated *ras* oncogenes in the same tumors. First, only one half of the lymphomas (stages II and III) showed increased p21 *ras* expression. Therefore, proliferation of malignant thymocytes can occur without increased expression of p21 *ras* protein, similar to the situation observed in normal thymus tissue. Second, in the tumors known to contain activated *ras* genes [N-*ras* (Table 1, LYM-3), K-*ras* (Table 2, L-1 and L-2)], or a novel non-*ras* transforming DNA sequences (Table 1, LYM-1 (11)), there was no clear correlation between elevated levels of p21 *ras* protein and the presence of activated *ras* genes.

All mature lymphocytes normally express lymphoid organ-specific homing receptors that preferentially allow migration to peripheral lymph nodes or to Peyer's patches.[29] MEL-14 MAb recognizes the antigen involved in thymocyte adherence to peripheral node endothelial venules.[16] In the normal adult thymus, less than 5% of the thymocytes express high levels of this antigen.[13,21] However, 72 hours after cortisone treatment, more than 90% of T cells are MEL-14^{hi} positive. These MEL-14^{hi}-positive thymocytes represent a population within the thymic cortex that give rise to mature, functional, peripheral T cells.[13,21,29] It is not surprising, therefore, to find that many thymic lymphomas, whether induced by virus, γ irradiation, or chemical carcinogen, are MEL-14^{hi} positive.[11,16,30,31]

We found that the earliest and most consistent change associated with the onset of disease in the thymus of carcinogen-treated animals was an increased frequency of MEL-14^{hi}-positive thymocytes. By immunohistology, clusters of MEL-14^{hi}-positive cells were observed in the cortex. Most likely they correspond to the hyperplastic foci seen in hematoxylin and eosin stained sections. Careful morphologic studies of thymus during the latent period following virus, irradiation, or carcinogen treatment shows neoplastic changes similar to those we have described here.[32–34] In these reports, separate foci of lymphoblasts, often in only one of the thymic lobes, were present in the cortex, expanding during later stages of the disease to obscure the medulla.

Proliferation of mature thymocytes in peripheral lymphoid tissues is dependent on the presence of T-cell growth factor IL-2 and expression of IL-2 growth-factor receptors after antigenic stimulation.[12] However, within the normal adult thymus, which consists of a large proliferative cortical compartment of immature T cells, less than 5% of the thymocytes express IL-2 receptors.[22] Therefore inappropriate expression of IL-2R may be associated with malignant proliferation. Several cell lines derived from murine T-cell lymphomas are known to express IL-2 receptors as well as secrete IL-2.[35,36]

In this study, an increased frequency of IL-2R-positive cells was observed at both early and late stages of disease. Immunohistologic analysis of serial sections indicated that the IL-2R-positive population detected early in the disease was different from the MEL-14^{hi} cell population. However, by late stages of disease, many MEL-14^{hi}-positive tumor cells were also IL-2R positive as determined quantitatively by flow cytometry. These findings suggest several possibilities for tumor development. First, the MEL-14^{hi}-positive cells are tumor cells, while the IL-2R-positive cells represent a normal subset of thymocytes that increase in frequency due to the disease process itself. Second, both the MEL-14^{hi}- and the IL-2R-positive cells represent distinct populations of tumor cells. Finally, there is one population of MEL-14^{hi}-positive tumor cells that subsequently express IL-2 receptors late in the disease. The evidence from immunohistology, flow cytometry, and analysis of tumor clonality (E.W. Newcomb, W. Bayona and A. Pellicer, manuscript in preparation) support the latter hypothesis.

The sequence of events described here during the development of carcinogen-induced thymic lymphomas would suggest that IL-2R-positive cells appearing early in the disease may play a role in progression to malignancy. Activated T cells, in addition to expressing IL-2R, produce IL-2.[12] Thus, the IL-2R-positive cells in stage II animals may serve as a source of IL-2 to support tumor cell proliferation during the thymic-dependent stage.[37] Constitutive expression of IL-2R on the MEL-14^{hi} subpopulation, which appears later in the disease, may coincide with autonomous cell growth.

Using this animal model system, we will be able to selectively isolate and further characterize IL-2R-positive and MEL-14^{hi}:IL-2R-positive cells present at defined stages of thymic lymphoma development and compare them to phenotypically similar populations found in cortisone-treated thymus. Although these cells may be derived from the same precursors, presence of activated *ras* oncogenes or some other chromosome abnormality may contribute to malignant behavior.[11,38]

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