DIFFERENTIATION ANTIGENS ON SPONTANEOUS
AND TRANSPANTED AKR LEUKEMIAS*

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INTRODUCTION

Cell populations at various stages of T cell maturation are characterized by their expression of specific combinations and levels of surface antigens. In the thymus, for example, the cortical (immature) and medullary (mature) cells are distinguished by expression of several of the Lyt (mouse) or Leu (human) antigens. Similarly, the cytotoxic/suppressor and the helper/inducer subpopulations of mature T cells show distinct antigenic phenotypes in both species. In most cases, these antigens are membrane glycoproteins whose changing pattern of expression during T cell maturation reflects control of specific genes or gene products. Many of these antigens were originally detected with conventional antisera, but are now easily identified using monoclonal antibodies.

We recently used sensitive immunofluorescence techniques with fluorescein-conjugated monoclonal antibodies and a fluorescence activated cell sorter (FACS) to quantitate the

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antigenic expression by T cell populations at various stages of differentiation (11,12). This characterization of normal T cells provides the foundation for quantitative assays of differentiation antigens on T cell leukemias. The potential diagnostic and predictive value of categorizing leukemias into groups based upon surface antigens is largely unknown. An underlying assumption of this approach is that leukemias are arrested at discrete stages of maturation, and therefore, reflect the antigenic phenotype of the cell from which the leukemia was derived. In general, leukemias do express "appropriate" antigens, although there are examples of apparently abnormal patterns of antigen expression by leukemias (9,20). In addition, some leukemias retain a potential to differentiate, resulting in changes in their antigenic phenotype (4,5,7,8).

The high incidence of spontaneous thymomas in AKR mice provides a model system for examining the relationship between leukemia development and surface phenotype. In this report we use monoclonal antibodies to the Lyt-1, Lyt-2, and Thy-1 antigens to examine changes in the thymus at the earliest stages of leukemia development. In addition, the spontaneous thymomas were transplanted in vivo and examined in successive passage generations. Our results suggest that the AKR thymomas arise in an immature precursor cell that is committed to differentiate through the "helper/inducer" T cell lineage.

MATERIALS AND METHODS

Monoclonal Antibodies

The production and characterization of monoclonal rat anti-mouse antibody producing hybridoma cell lines has been described (10). The antibodies used here were derived from hybrid lines 53-2.1 (anti-Thy-1.2), 53-6.7 (anti-Lyt-2), and 53-7.3 (anti-Lyt-1). Anti-Thy-1.1 was derived from hybrid line 19E12. Serum from mice bearing this hybridoma was kindly provided by Dr. R. C. Nowinski.

Immunofluorescence of Cell Suspensions

Direct immunofluorescence of mouse lymphoid cells was performed using fluorescein-conjugated monoclonal antibodies. Monoclonal antibodies were purified and conjugated with fluorescein as previously described (10,11). For staining normal or leukemic cells, reagents were centrifuged for 10 minutes at 100,000 g before use, and 10^6 target cells per well in microtiter plates were reacted with saturation levels of antibody. Cells were stained as previously described (6). Fluorescence measurements were made on a modified FACS II fitted with a logarithmic amplifier.
Thymomas

AKR/J mice, obtained from Jackson Labs, and AKR/Cu mice, obtained from Cumberland Farms, were bred at Stanford. Spontaneous thymomas arose in animals between 6-12 month of age. Spontaneous thymomas of AKR/J mice (Thy-1.1) were transplanted by intravenous inoculation of 5x10^6 cells in young (6-8 week) AKR/Cu mice (Thy-1.2) and spontaneous thymomas of AKR/Cu mice were similarly transplanted in young AKR/J mice.

RESULTS

Leukemia Cells Are Distinguishable From Normal Lymphocytes by Forward Angle Light Scatter

Figure 1 shows a comparison of the light scatter properties of normal thymocytes and spleen cells to cells derived from the thymus and spleen of an AKR/J animal with a spontaneous thymoma. Forward angle light scatter is related to cell size and viability (14). The thymoma cells are distinguishable from most normal cells by this criterion. The large difference in cell size between normal and leukemia cells was consistently observed for the thymomas studied here and provides one of the criteria for distinguishing leukemic and normal cells in animals bearing transplanted thymomas (see below).

Lyt-1, Lyt-2, and Thy-1 Phenotypes of Normal Cells

All thymocytes and splenic (Thy-1 bearing) T cells express Lyt-1 antigen; however, there is a clear bimodal distribution of antigen densities that correlates with maturation (Fig. 3). In thymus, approximately 15-20% of the cells stain brightly for Lyt-1. These form a shoulder on the brighter side of the main population. The splenic or lymph node Lyt-1+ cells are about four times brighter than the main thymocyte population and correspond in brightness to the smaller number of bright thymus cells (Fig. 4). Because this latter is the cortisone-resistant thymocyte population (16), these data indicate that levels of Lyt-1 antigen increase during T cell maturation. The mature T cells that express the highest levels of Lyt-1 tend to be Lyt-2 negative (11) and are derived from the "helper/inducer" T cell lineage (2,19).

Lyt-2 bearing cells comprise between 80 and 90% of thymocytes (Fig. 3). The variation in frequency of Lyt-2+ cells in thymus that is seen between individual mice is not a result of estimation errors, which are at most 1 or 2%. The frequency of Lyt-2+ cells in spleen and lymph node of normal mice is usually only 27-40% of the Thy-1+ cells. Again, the variation is between individual mice and not caused by estimation problems. These data indicate that
FIG. 1. Comparison of forward angle light scatter profiles of normal lymphocytes to spontaneous AKR/J leukemia cells using a FACS II. Leukemia cells in either the thymus (c) or spleen (d) are easily distinguishable from normal thymocytes (a) or spleen cells (b).

Lyt-2 is lost from the majority of T cells during maturation, and that Lyt 1+, 2- cells already exist in the thymus. The mature T cells that retain Lyt-2 and low levels of Lyt-1 (11) are derived from the cytotoxic/suppressor T cell lineage (2,19).
FIG. 2. Distinction of AKR/J leukemia cells from normal thymocytes after passage of the leukemia cells in an AKR/Cu recipient. Cells from a spontaneous AKR/J leukemia were transplanted by intravenous injection into an AKR/Cu. Ten days after passage the enlarged thymus was removed from the AKR/Cu recipient and cells were stained with anti-Thy-1.1 (left panel) and anti-Thy-1.2 (right panel). Each dot on the figure represents a single cell. Leukemia (large) cells are positive for Thy-1.1 whereas normal (intermediate sized) thymocytes of the AKR/Cu are positive for Thy-1.2.
For Thy-1.2, we found the immunofluorescence of thymocytes and peripheral T cells closely agreed with previous reports using conventional reagents (3). Thy-1.2 stained thymocytes give a very bright peak with a duller shoulder. This latter corresponds with the hydrocortisone resistant subpopulation. In spleen and lymph node the brighter Thy-1.2 bearing cells were equivalent in brightness to the dullest cells in the thymus (11).

In summary, the results of the quantitative staining analyses for Thy-1.2, Lyt-1, and Lyt-2 antigens in thymus, lymph node, and spleen imply the following changes during T cell maturation: (a) Lyt-1 increases substantially; (b) Thy-1 decreases substantially; and (c) Lyt-2 is lost from most cells. Thus, by comparing thymus, spleen, and lymph node cells on a population basis, Lyt-1 and Thy-1 antigen densities appear to change in opposite directions during T cell development but frequencies remain essentially the same. In contrast, Lyt-2 changes in frequencies of positive T cells, but only slightly in density on positive cells.

**Lyt-1 and Lyt-2 Phenotypes of Spontaneous Thymomas**

There was some variability in Lyt-1 and Lyt-2 phenotypes among spontaneous thymomas. In general, however, the thymomas could be divided into two categories (Table 1). One category of thymomas showed a uniform phenotype that was Lyt-1 bright and Lyt-2 negative. This phenotype is characteristic of mature T cells (medullary thymocytes or peripheral T) of the helper subpopulation (see above). Of the 16 AKR/J and AKR/Cu spontaneous thymomas examined here, six showed the Lyt-1 bright, Lyt-2 negative phenotype.

Leukemias were transplanted in vivo by intravenous injections of 5x10^5 cells into the congenic AKR strain. Thus, AKR/J leukemias (Thy-1.1^+^) were transplanted into AKR/Cu recipients and AKR/Cu leukemias (Thy-1.2^+^) were transplanted into AKR/J recipients. When leukemias arose in the recipients, usually by 8-14 days after passage, the Thy-1 phenotype was assayed and used to unequivocally identify the leukemia cells and distinguish them from normal cells of the recipient (see Fig. 2).

Two of the leukemias described above were transplanted and assayed after in vivo passage (Table 1). The Lyt-1 and Lyt-2 phenotypes remained constant.

The second category of spontaneous thymomas was characterized by the presence of more than one population of large "blast-like" cells. The multiple populations were distinguished by their differential expression of Lyt antigens (Table 1). For example, one spontaneous thymoma (Fig. 3) showed two populations of cells that differed in both Lyt-1 and Lyt-2 phenotypes. The Lyt-1 phenotypes were
FIG. 3. Expression of Lyt-1 and Lyt-2 antigens on normal thymocytes and on spontaneous thymoma cells derived from an 8 month old AKR/Cu. Panels (a) and (b) show Lyt-1 and Lyt-2 antigens on normal thymocytes compared to background (autofluorescence). Panels (c) and (d) show Lyt-1 and Lyt-2 antigens on the leukemia population compared to background (autofluorescence). Antigens were detected by staining with fluorescein-conjugated monoclonal antibodies and analyzed using a FACS II fitted with a logarithmic amplifier.
# TABLE 1 Phenotypes of Spontaneous AKR/J and AKR/Cu Thymomas

**Category I:** Thymomas with a uniform phenotype similar to mature T cells of the helper/inducer lineage.

<table>
<thead>
<tr>
<th>Thymoma</th>
<th>Passage Healthcare</th>
<th>Surface Phenotype Healthcare</th>
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<tbody>
<tr>
<td></td>
<td>Generation Healthcare</td>
<td>Lyt-1 Healthcare</td>
</tr>
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<td>&lt;5 &lt;95</td>
</tr>
<tr>
<td>AKR/J #2</td>
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<td>&lt;5 95 &lt;5 &gt;95 &lt;100</td>
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<tr>
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<td>AKR/Cu #1</td>
<td>1</td>
<td>&lt;5 &gt;95 &lt;5 &gt;95 &lt;100</td>
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**Category II:** Thymomas with at least two populations corresponding to both "immature" and "mature" T cell phenotypes.

<table>
<thead>
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<th>Surface Phenotype Healthcare</th>
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</thead>
<tbody>
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</tr>
<tr>
<td>AKR/Cu #10</td>
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1AKR/J leukemias were passaged in AKR/Cu recipients and AKR/Cu leukemias were passaged in AKR/J recipients. Passage generation 0 refers to the spontaneous leukemia, 1 refers to the first passage in the congenic strain and 2 refers to the second passage in the congenic strain. In each passage, the thymus was removed and assayed for the presence of leukemia cells:

Surface-antigens were analyzed by immunofluorescence with fluorescein-conjugated monoclonal antibodies and a FACS II.
Lyt-1 was always present on 100% of thymoma cells; however, two distinct Lyt-1 antigen densities were seen and are referred to as dull and bright.

Lyt-2 antigen was either present or absent.

FIG. 4. Expression of (a) Lyt-1 and (b) Lyt-2 on thymocytes and spleen cells from a normal young AKR/Cu compared to (c) Lyt-1 and (d) Lyt-2 on leukemia cells from thymus and spleen of a 10 month old AKR/Cu. Antigens were detected by staining with fluorescein-conjugated monoclonal antibodies and analyzed using a FACS II fitted with a logarithmic amplifier.
Lyt-1 "dull" and Lyt-1 "bright", whereas the Lyt-2 phenotypes were Lyt-2 positive and Lyt-2 negative. For both of these antigens, their quantitative expression on the two populations seen in this thymoma correlate with their expression on normal immature (cortical) and mature (medullary) thymocytes. Thus, the two populations seen in this thymoma appear to reflect cells at different stages of differentiation.

When the spleen from the same animal bearing the spontaneous thymoma was examined (Fig. 4), the large cells were not identical in Lyt phenotype to those seen in the thymus. The predominant phenotype was Lyt-1 "bright", Lyt-2 negative, although a small number of Lyt-1 "dull" and Lyt-2 negative cells were seen.

The second category of spontaneous AKR thymomas, those with at least two distinct populations of large cells, is typified by the thymoma described above. Ten of the 16 AKR/J and AKR/Cu thymomas examined were of this category (Table 1). Several of these thymomas were transplanted in vivo and examined in successive passage generations. Results of this analysis (Table 2) showed that both populations of cells grew in the recipient animals and thus qualified as leukemia cells. However, the relative proportions of the two populations showed variability, with the Lyt-1 "bright", Lyt-2 negative (mature phenotype) population expanding at the expense of the Lyt-1 "dull", Lyt-2 positive (immature phenotype) population (Table 1 and Fig. 5).

After the second transplant generation, the mature phenotype population was the predominant population remaining and these leukemias appear identical to the leukemias in the first category. Thus, it is tempting to speculate that the Category I leukemias are similar to the Category II leukemias at a later stage of development.

**DISCUSSION**

Previous studies of differentiation antigens on spontaneous AKR leukemias have described a variety of possible Lyt phenotypes (21), rather than a uniform phenotype that might be expected from transformation of a single type of thymus cell. These results have suggested that AKR thymomas can arise from cells at more than one stage of differentiation. However, these studies did not transplant the thymomas to see if the phenotype was stable. Lyt phenotypes of long term cell lines derived from AKR leukemias also have been reported (15), but these studies did not investigate the early events in the development of the thymomas. Our studies benefit from the quantitation obtained by using monoclonal antibodies and a FACS, and by transplantation of spontaneous thymomas in Thy-1 congeneric
animals that allowed unequivocal identification of growing thymoma cells in the recipient animals.

Many of the spontaneous AKR/J and AKR/Cu thymomas that we examined showed at least two populations of large "blast-like" cells distinguishable by surface expression and density of Lyt-1 and Lyt-2 antigens. The multiple distinct populations of large cells seen in these thymomas correlate with both immature and mature T cell phenotypes when compared to Lyt-1 and Lyt-2 expression on normal T cells at various stages of differentiation. Furthermore, both the "immature" and "mature" cells in the thymoma qualify as transformed malignant cells since both are able to grow when transplanted. At successive passages, however, the relative proportions of the "immature" and "mature" populations changed, with the "mature" phenotype thymoma cells tending to predominate by the second or third generation.

There are at least two hypotheses that could account for our results. First, it may be that thymomas arising spontaneously in AKR mice are polyclonal, that is, multiple transforming events may occur simultaneously in the thymus resulting in tumor cells derived from both immature and mature T cells from the cortical and medullary areas of the thymus. In this case, it should be possible to derive stable AKR thymomas of both types. Secondly, the AKR thymomas may result from transformation of a T cell precursor that is committed to differentiate through the "helper/inducer" T cell lineage. In this case, the thymomas population with a "mature" phenotype (bright Lyt-1, Lyt-2 negative) may derive by differentiation of the thymoma population with the "immature" phenotype (dull Lyt-1, Lyt-2 positive). By separating and transplanting them independently, it should be possible to determine if the "immature" population gives rise to the "mature" population.

Our finding that thymoma phenotypes can be different in the thymus versus the spleen of the same animal lends support to the differentiation hypothesis. Since the AKR thymomas arise in the thymus, the thymoma cells that metastasize to other lymphoid organs may differentiate faster in the peripheral tissues. The differentiation hypothesis is further supported by the specific patterns of surface antigen changes during thymoma transplantation, including (a) the Lyt-1 and Lyt-2 antigens each changed in one direction, i.e., Lyt-1 dull→Lyt-1 bright, and Lyt-2 positive→Lyt-2 negative, the same directions of change that normal T cells show during differentiation (11,16); and (b) some thymomas that initially show only the "immature" phenotype, in later transplant generations show changes in first one of the Lyt antigens and then the other (see AKR/Cu #5 thymoma). If differentiation is not occurring, then it becomes necessary to postulate at least three distinct transformed populations, each of which can predominate at different transplant generations.
FIG. 5. Lyt-1 (a) and Lyt-2 (b) antigens on a spontaneous AKR/J thymoma (AKR/J #5; see Table 1) and Lyt-1 (c) and Lyt-2 (d) antigens after passage of the thymoma in an AKR/Cu recipient. Antigens were detected by staining with fluorescein-conjugated monoclonal antibodies and analyzed using a FACS II fitted with a logarithmic amplifier. For detection of the lymphoma cells, large cells were analyzed and >95% of the population after passage was Thy-1.1 positive.
The mechanisms involved in development of spontaneous thymic leukemias in AKR mice are largely unknown. The thymus plays a central role, since thymectomy prevents the development of disease, and AKR streaker mice (congenitally athymic) do not develop leukemia (1,18). Although the leukemogenesis is associated with the replication of endogenous C-type viruses, these viruses are replication competent and lack an identifiable transforming gene such as that found in the replication defective mammalian sarcoma viruses. Furthermore, the latent period of leukemogenesis is much longer than that seen for tumors induced by the sarcoma viruses. Rather than transforming directly by introduction of a transforming gene into a target cell, it has been proposed that leukemogenesis results from chronic immune stimulation by viral antigens (13,17). Our results, showing that AKR thymomas arise from either polyclonal transformation or stimulation of a precursor cell to expand and differentiate, support the idea of chronic immune stimulation.

The usefulness of identifying normal differentiation antigens on leukemia cells is illustrated by this manuscript. In addition to the potential value of subclassifying human leukemias for therapeutic decisions, it is likely that such an approach will produce new insights into fundamental aspects of the disease process.

REFERENCES


