ABSTRACT

Blast cells in acute leukemia and lymphoma appear to be "frozen" at various stages of lymphoid cell differentiation. The enzymatic and antigenic phenotypes expressed by these cells often correspond to the gene products of their normal precursors. We have used various immunochemical and enzymatic techniques to identify membrane, nuclear, and cytoplasmic markers associated with the prolactin-dependent Nb2 lymphoma cell line.

The Nb2 cells, whether stationary or in log-phase growth, did not express any surface immunoglobulin. However, 100% of the Nb2 cells bound both a monoclonal antibody raised to rat thymocyte W3/25-HLK, which specifically binds an antigenic determinant on rat T-helper cells, and second monoclonal antibody OX8-HL, which identifies rat nonhelper T-cells.

Transmission electron microscopy showed no evidence of phagocytic vacuoles, and activity of the lysosomal enzyme muramidase was also absent. There was no evidence of the DNA polymerase enzyme terminal deoxynucleotidyl transferase. α-Naphthyl acetate esterase activity was indicated in about 50% of the Nb2 cells by a faint particulate cytoplasmic staining similar to that found in thymocytes. Rosette formation with guinea pig erythrocytes, a property of mature rat thymocytes, was not observed with Nb2 cells.

The data suggest that the Nb2 tumor may have arisen from a thymocyte at an intermediate stage of differentiation. The presence of Thy-like α-naphthyl acetate esterase pattern and the binding of both W3/25-HLK and OX8-HL support the thymic origin and relative immaturity of these lymphoid cells. It is becoming increasingly apparent that a significant proportion of lymphomas and leukemias also originate in undifferentiated thymic cells.

INTRODUCTION

The Nb2 cell line was originally derived from the lymph node of an estrogenized male Noble rat (5). Further studies demonstrated that the growth of this tumor is dependent on the presence of the pituitary hormone prolactin. Currently, Nb2 cells are utilized as a sensitive bioassay for lactogenic hormones (13).

Blast cells in both acute leukemia and lymphoma appear to be "frozen" at various stages of lymphoid cell differentiation (3). The enzymatic and antigenic phenotypes expressed by these blast cells often correspond to the normal gene products of their precursor cells (10). We have used various immunochemical and enzymatic techniques in order to identify membrane, nuclear, and cytoplasmic markers associated with the Nb2 lymphoma. The extreme sensitivity of Nb2 cells to lactogenic hormones (10 pg/ml to 1 ng/ml) makes it an excellent model for studying the biochemistry of cellular proliferation in a hormone-dependent cell line. Determination of both the origin and the degree of differentiation of this tumor provides a rational framework for studying the mechanism of hormonal dependence. Current studies suggest that a large proportion of human lymphoid neoplasms are likely to be of thymic origin.

MATERIALS AND METHODS

Cell Culture. The Nb2 lymphoma cell line was provided courtesy of Dr. Robert Noble (University of British Columbia). These cells were grown in Fisher's medium for leukemic cells of mice supplemented with 10% horse serum, 10% fetal bovine serum, 10^-4 M 2-mercaptoethanol, penicillin (50 μg/ml), and streptomycin (50 μg/ml). All sera and antibiotics were purchased from Grand Island Biological Co., Canada. Cultures were maintained in 1-liter spinner flasks (Belco Glass, Inc.) in a 5% CO2-95% air atmosphere at 37°. Routinely, a concentration of 1 x 10^6 cells/ml was used for all assays.

Detection of Surface Immunoglobulin by Indirect Immunofluorescence. Rabbit anti-rat IgG, goat anti-rat IgA, sheep anti-rat IgE, and rabbit anti-rat IgM were purchased from Miles Laboratories, Inc. (Elkhart, Ind.). Fluorescein-conjugated second antibodies were obtained from Cappel Laboratories (Cochranville, Pa.) and Cedar Lane Laboratories (Hornby, Ontario).

Log-phase Nb2 cells were gently centrifuged and prepared as described previously (8). Briefly, the cells were rinsed 3 times in Hanks' salts and then incubated with the primary antibody for 30 min. Following thorough rinsing, the fluorescein-conjugated second antibody was added, and the cells were incubated again for 30 min. Following a final rinse in Hanks' balanced salts, the cell suspension was examined under a fluorescence microscope. As a positive control for all surface immunoglobulins, a cell suspension was prepared from normal rat spleen.

Detection of T-Cell Helper and Nonhelper Surface Markers. Fluorescein-conjugated swine anti-rabbit IgG and rabbit anti-mouse IgG were purchased from Cedar Lane Laboratories. The mouse anti-rat monoclonal antibodies W3/25-HLK and OX8-HL were obtained from Cappel Laboratories. Cells were prepared as for surface immunoglobulin, although incubation with the additional third antibody was required. Cultures of Nb2 cells were rinsed with Hanks' salts and then incubated with either W3/25-HLK (T-helper marker) or OX8-HL (T-nonhelper marker). Following a thorough rinsing, Nb2 cells were incubated in the same manner with rabbit anti-mouse IgG and swine anti-rabbit IgG. Cell suspensions were examined using a fluorescence microscope.

Intracellular Enzymes. Pararosaniline hydrochloride, α-naphthyl acetate, CaCl2, formaldehyde, NaOH, sodium nitrite, Na2HPO4, KH2PO4, and acetone were purchased from Sigma Chemical Co. (St. Louis, Mo.). Rabbit anti-human muramidase was obtained from Cedar Lane Laboratories and a terminal transferase immunofluorescence assay kit was purchased from Bethesda Research Laboratories (Bethesda, Md.).

Cells were examined for α-naphthyl acetate esterase activity by a method described previously (9). Cell preparations were counterstained in 2% aqueous light green. Nonspecific esterase activity was determined utilizing α-naphthyl acetate in ethylene glycol as a substrate. Isolated rat spleen cells were used as a control.

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Muramidase activity was examined in Nb2 cells following fixation in acetone (8). The Nb2 cells were incubated with rabbit anti-human muramidase labeled with fluorescein and then examined under fluorescent light. TdT activity was determined in a similar manner using reagents from a Bethesda Research Laboratories TdT immunofluorescent assay kit. This antibody has been shown previously to cross-react with rat TdT (2).

**TEM.** Cultured cells were gently centrifuged, stained with osmic acid, and then prepared according to the method of Spurr (11). Sections were examined and photographed using a Phillips Model 301 electron microscope.

**Guinea Pig Erythrocyte Rosette-forming Technique.** Trunk blood was collected in heparinized tubes from normal decapitated guinea pigs. Guinea pig erythrocytes were incubated with Nb2 cells under the conditions described by Takeichi and Boone (12). Normal thymic and spleen cells also were examined. More than 3 erythrocytes binding to a lymphoid cell was considered a positive test.

**RESULTS**

**Surface Markers.** Cultures of Nb2 cells, whether stationary or in log-phase growth, did not express surface immunoglobulin (Table 1). The "capping" phenomenon associated with aggregates of antibody-bound immunoglobulin was absent from all cells examined. In addition, cytoplasmic immunoglobulin was undetectable using a peroxidase-staining technique (data not shown).

A monoclonal antibody raised to rat thymocytes, W3/25-HLK, has been shown to specifically bind to an antigenic determinant on T-helper cells (15). Virtually 100% of the Nb2 cells bound this antibody (Fig. 1). A second monoclonal antibody OX8-HL, which identifies rat nonhelper T-cells (4), bound similarly to 100% of the Nb2 cells. Both antibodies produced patterns of capping which were indistinguishable from each other. The intensity of the binding of both antibodies to Nb2 cells was less than that observed in control cells from a normal rat spleen.

**Intracellular Enzymes.** The lysosomal enzyme muramidase was not detectable in the Nb2 lymphoma (Table 2). Nonspecific esterase activity was absent as was activity of the DNA polymerase enzyme TdT.

The presence of α-naphthyl acetate esterase activity in normal peripheral T-lymphocytes is indicated by a red solitary cytoplasmic nodule or several punctate granules. Although Nb2 cells failed to exhibit this reaction product, about one-third to one-half of the cells examined had a faint localized staining of variable intensity consistent with that seen in normal thymocytes. (Thy-like, Ref. 9). The localized staining was found throughout the cytoplasm of these cells (Fig. 2). We observed a similar pattern in 65% of thymocytes from normal Sprague-Dawley rats.

**Rosette Formation with Guinea Pig Erythrocytes.** Guinea pig erythrocytes have been shown to spontaneously form rosettes with a subpopulation of thymus cells in the rat (12). The proportion of rosette-forming cells in the rat thymus increases with age from fetus to adult. The Nb2 cells do not form rosettes with guinea pig erythrocytes. About 25% of the thymic cells isolated from normal Sprague-Dawley rats demonstrated rosette formation. No rosette-forming cells were detected among spleen cells isolated from these animals. (More than 3 erythrocytes binding to a lymphoid cell was considered a positive test.)

**Light Microscopy.** Giemsa-Wright staining of cytopsin preparations of Nb2 cells from suspension culture and paraffin sections of Nb2 tumor grown s.c. in Sprague-Dawley rats revealed undifferentiated lymphoblasts. The cytoplasm of both cultured cells and cells from the solid tumor was scanty, and many cells contained a horseshoe-shaped nucleus. A prominent nucleolus was visible in most cells. Numerous mitotic figures were present. Following a s.c. injection of the cultured Nb2 cells, a solid tumor grew which appeared homogeneous with the few host fibroblasts present.

**TEM.** The Nb2 lymphoma cells averaged between 7.5 μm in diameter under stationary conditions and 9.0 μm during log-phase growth. The cytoplasm of these cells contained numerous polyribosomes and many mitochondria (Fig. 3). Cytoplasmic inclusions resembling internalized plasma membrane commonly were observed. Rough endoplasmic reticulum, cytoplasmic vacuoles, and Golgi bodies were very sparse or absent in these cells.

The large nuclei of these cells usually contained one promi-
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ment nucleolus. Heterochromatin was distributed along the nuclear membrane and around the nucleolus. Nuclear inclusions were not observed in these cells. These features are consistent with a previous report on the ultrastructure of the normal rat thymus (7).

DISCUSSION

The absence of surface immunoglobulin indicates that the Nb2 lymphoma is not likely to be of B-cell origin. In addition, the absence of both cytoplasmic vacuoles (TEM) and the lysosomal enzyme muramidase suggests that this tumor is not of the monocyte series. The solitary large red nodule which demonstrates the presence of a-naphthyl acetate esterase was not present in the Nb2 lymphoma, indicating that this tumor is not of T-cell origin.

The mouse monoclonal antibody W3/25-HLK recognizes a subset of peripheral T-lymphocytes which mediate helper graft versus host and mixed lymphocytic reactions (14). The mouse monoclonal antibody OX8-HL will bind with all T-lymphocytes that are W3/25-HLK negative and which are responsible for functions such as the suppression of induction of antibody-forming cells (4). Together, both antibodies will bind 90% of peripheral T-lymphocytes in the normal rat. No subset of peripheral T-cells has been identified which binds both antibodies.

All Nb2 cells examined, from both stationary and log-phase growth cultures, bound both W3/25-HLK and OX8-HL. The intensity of fluorescence of both antibodies was greater in the stationary cultures. This is consistent with the observation that the volume of growing Nb2 cells is twice that of stationary Nb2 cells. An increase in cell surface area without a corresponding increase in surface marker number would result in a decrease in fluorescence.

Thymocyte differentiation is often accompanied by the loss of membrane marker proteins. For example, murine thymocytes express the Thy 1.1 antigen, while mature peripheral T-cells do not (1). The selective expression of either W3/25-HLK or OX8-HL antigens in peripheral T-cells of the rat may be linked to the specialized functions of these 2 cell types. The observation that W3/25-HLK and OX8-HL produce a more intense fluorescent pattern on their respective target cells in the normal rat thymus and the Nb2 tumor corresponds to the initial expression of membrane marker proteins. For example, murine thymocytes like pattern confirm the thymic origin and relative immaturity of these lymphoid cells. The phenomenon of prolactin-regulated growth in Nb2 cells raises several questions regarding the nature of this hormonal dependence. If indeed these tumor cells are “frozen” at a particular stage of thymocyte development, the presence of functional prolactin receptors suggests the possibility that this hormone may be involved in normal thymocyte maturation. Alternatively, the prolactin dependence observed in these cells may be the result of malignant transformation and/or subsequent selection processes. Further investigation of the role of prolactin in thymocyte maturation and in the growth of leukemias and lymphomas of thymic origin may reveal a subset of lymphoid tumors in which growth is prolactin dependent.

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REFERENCES

Fig. 2. α-Naphthyl acetate esterase activity in Nb2 cells. Cells with "Thy-like" pattern are indicated by arrows. × 200.

Fig. 3. TEM of log-phase growth Nb2 cells. Rough endoplasmic reticulum, cytoplasmic vacuoles, and Golgi are sparse or absent. Heterochromatin is seen distributed along the nuclear membrane. × 8300.
Thymic Origin of the Prolactin-dependent Nb2 Lymphoma Cell Line


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