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*J Immunol* 1980; 125:2284-2288; 
http://www.jimmunol.org/content/125/5/2284
THE GLYCOLIPID ASIALO GM1 AS A NEW DIFFERENTIATION ANTIGEN OF FETAL THYMOCYTES

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Antibody directed to the neutral glycolipid "asialo GM1" was found to react with the majority of thymus lymphoid cells lacking characteristic T cell markers in mice at an early embryonic stage (13 days of gestation). The proportion of these asialo GM1-positive cells (asialo GM1 cells) decreased strikingly thereafter, contrasting with an increased fraction of lymphocytes possessing T cell surface markers such as Thy-1, Lyt-1, and Lyt-2. After about 18 gestational days, only a few asialo GM1 cells were detected in the thymus as well as in other lymphoid organs. Double-staining analysis of the embryonic thymocytes (13 days of gestation) with anti-asialo GM1 and anti-Thy-1 demonstrated that thymocytes stained with anti-asialo GM1 did not react with anti-Thy-1, and vice versa. Morphologic examination by immunoelectronmicroscopy demonstrated that these asialo GM1 cells were mainly composed of immature, large lymphoid cells having large nucleoli and relatively abundant cytoplasm with many polysomes. These results suggest that asialo GM1 is present on very early thymocytes and is lost as the mature murine T cell protein antigens Thy-1, Lyt-1, and Lyt-2 develop on these cells. The antibody to this glycolipid is a useful tool for studying embryonic thymic differentiation.

There is substantial evidence that hematopoietic stem cells migrate from bone marrow in adults and from yolk sac and liver in embryos into the thymus, where the migrating stem cells proliferate and differentiate into thymic lymphocytes (1-6). This differentiation pathway in mice is marked by the beginning of expression of T cell-characteristic alloantigens, such as Thy-1, Lyt-1, Lyt-2, 3, and TL (7-11). Although the migrant stem cells are morphologically identified as large lymphoid cells (8, 10, 12-15), they lack known T cell alloantigens and any surface markers that are selectively expressed on them. Therefore, it is still controversial whether Thy-1+ cells are directly derived from multipotential cells or from progenitor cells committed for the T cell lineage.

We have previously reported that cytotoxic treatment with antiserum against a neutral glycolipid, asialo GM1, can eliminate natural killer (NK) cells but not T or B cells (16). Young et al. (17) also observed asialo GM1 on the BCG-induced NK cells. Although uncertain of the lymphoid cell lineage of NK cells, we explored whether this newly discovered marker could be detected on ontogenically immature thymus lymphocytes on which no distinct T cell markers are known. The study reported here proved that antisera against asialo GM1 is able to distinguish thymus lymphocytes lacking any characteristic markers from thymocytes possessing known T cell markers.

MATERIALS AND METHODS

Mice. Pregnant and nonpregnant CBA/N and BALB/c mice were obtained from the Central Institute for Experimental Animals, Kanagawa, Japan; pregnant and nonpregnant BALB/cNH mice raised in the Herzenbergs' laboratory, Stanford, CA, were also used.

Antiserum. Rabbit antiserum against asialo GM1 was prepared by the method described in a previous manuscript (16). Briefly, asialo GM1, previously dissolved by sonic dispersion in saline containing methylated bovine serum albumin and emulsified with an equal volume of complete Freund's adjuvant, was injected into each footpad of each rabbit. The rabbits were boosted 2 wk after the initial injection and bled out 2 wk later. Anti-asialo GM1 was prepared by the same procedure, with purified asialo GM1 as antigen. The specificity of each antiserum was checked by the immunoflocculation test utilizing lecithin cholesterol micelles composed of various glycolipids (16). Fluorescein-conjugated monoclonal anti-Thy-1, anti-Lyt-1, and anti-Lyt-2, produced by hybridomas derived from fusion of the mouse myeloma line NS-1 with immunized rat spleen cells, were all generously supplied by Dr. J. Ledbetter of Stanford. These monoclonal antibodies are designated 30-H12 (Thy-1.2), 53-7 (Lyt-1), and 53-6 (Lyt-2) (18). Fluorescein- and rhodamine-conjugated goat anti-rabbit immunoglobulin (anti-Rg) prepared in our laboratories (Tohkai and Tokyo) was used in the indirect staining for rabbit anti-asialo GM1.

Cell preparation and fluorescence study. Thymuses from fetuses in various gestational stages were removed with cataract knives under a stereomicroscope and pressed between 2 glass slides to make a single-cell suspension. Indirect staining was

Received for publication May 12, 1980
Accepted for publication July 16, 1980

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by a Grant-in-Aid from the Ministry of Education, Culture and Science, Japan, and NIH Grant GM 17367.
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3 Abbreviations used in this paper: NK cell, natural killer cells; Rg, rabbit immunoglobulin; FACS-11, fluorescence-activated cell sorter; HRPO, horseradish peroxidase; DAB, 3,3-diaminobenzidine; BAT, brain-associated T cell antigen; NRS, normal rabbit serum.

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performed for anti-asialo GM₁, and direct staining was used for anti-Thy-1, anti-Lyt-1, and anti-Lyt-2. After washing in medium RPMI 1640 containing 10% fetal calf serum and 0.2% sodium azide, the cell suspensions were incubated with the 1/20 diluted anti-asialo GM₁ (2 × 10⁶/0.1 ml) at 4°C for 30 min. After 3 washings, the cells were stained with fluoresceinated goat anti-RiG at 4°C for 20 min. Stained cells were examined under a fluorescence microscope (Standare IV, Carl Zeiss, West Germany), while a quantitative fluorescence analysis was conducted with a modified fluorescence-activated cell sorter (FACS-II, Becton, Dickinson and Co., Mountain View, CA) fitted with a logarithm: fluorescence signal amplifier as described elsewhere (18-20).

**Immunoelectronmicroscopic assay.** The cells incubated with pepsin-digested F(ab)₂ fragments of anti-asialo GM₁ at 4°C for 30 min were reacted with F(ab)₂ fragments of goat anti-RiG coupled with horseradish peroxidase (HRPO) by Nakane's method (21). After washing, the cells were fixed in 2% glutaraldehyde for 20 min on ice and washed in phosphate-buffered saline (pH 7.2) 3 times, then preincubated with 3,3-diaminobenzidine (DAB) (20 mg/ml) as substrate for HRPO in Tris-HCl buffer (pH 7.6) and treated with dimethyl sulfoxide at 1% for 20 min at room temperature. They were further incubated in Karnovsky solution (DAB-H₂O₂ solution) for 5 min. After osmium fixation and gradual alcohol dehydration, thin sections cut with a LKB-8800 microtome were examined under an electronmicroscope JEM-100C (JEOL, Tokyo, Japan). The staining of sections with metal salt was omitted in some sections to discriminate reaction products from nonspecific density.

**RESULTS**

Proportion changes of asialo GM₁-positive cells in the thymus from various gestational stages by fluorescence microscope. Embryonic thymus cells incubated with anti-asialo GM₁ at various gestational stages were examined by fluorescence microscope after indirect staining with fluoresceinated goat anti-RiG. As shown in Figure 1, more than 90% of the cells from the thymus were positive at 12 gestational days, whereas less than 2% of the cells from the same thymus were stained positively with anti-Thy-1 (Fig. 1). From 12 to 19 days of gestation there was a rapid decrease of asialo GM₁+ cells accompanied by a reciprocal increase in proportion of Thy-1 positive cells (Fig. 1). At birth, few asialo GM₁+ cells were detectable in the thymus (Table I). Also, we observed only a small percentage of asialo GM₁+ cells in adult spleen, bone marrow, lymph node, as well as fetal liver (Table I). The specificity of staining with anti-asialo GM₁ was determined by absorbing antisera with mixed micelles containing purified asialo GM₁ or asialo GM₂.

**Table 1**

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>Staining Sera</th>
<th>% Positive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymocytes from 13-day embryos</td>
<td>Anti-asialo GM₁</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Anti-asialo GM₁ absorbed with asialo GM₁</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>Anti-asialo GM₁ absorbed with asialo GM₂</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Anti-asialo GM₁ absorbed with normal rabbit sera</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>Anti-asialo GM₁ absorbed with anti-Thy-1</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Thymocytes from adults (6 wk)</td>
<td>Anti-asialo GM₁</td>
<td>8</td>
</tr>
<tr>
<td>Fetal liver cells from 15-day embryos</td>
<td>Anti-asialo GM₁</td>
<td>11</td>
</tr>
<tr>
<td>Spleen cells from adults (6 wk)</td>
<td>Anti-asialo GM₁</td>
<td>8</td>
</tr>
<tr>
<td>Bone marrow cells from adults (6 wk)</td>
<td>Anti-asialo GM₁</td>
<td>7</td>
</tr>
</tbody>
</table>

*Cells were stained by indirect method by using fluoresceinated goat anti-RiG.

As shown in Table I, anti-asialo GM₁ absorbed with asialo GM₁ did not stain thymocytes from a 13-day embryo, whereas the antisera absorbed with asialo GM₂ stained as many cells as did unabsorbed antisera.

Analysis of cell membrane markers of thymocytes from various gestational stages in FACS. Since it is known that the frequencies not only of Thy-1+ but also of Lyt-1+ and Lyt-2+ cells in the thymus change during gestation (11, and unpublished data by Habu, S., Beverley, P. C. L. and Raff, M. C.), we compared these changes along with changes in frequencies of asialo GM₁+ cells using quantitative fluorescence analysis on the FACS. Log fluorescence profiles of embryonic and adult thymocytes stained with various antisera are depicted in Figure 2. Although not shown for simplicity, unstained cells or control cells treated with normal rabbit serum (NRS) and the second-step reagent, fluorescein-conjugated goat anti-RiG, gave peaks that returned to baseline by log 2 fluorescence intensity units. At day 13, although only a small percentage of cells stained above background with anti-Lyt-1 and anti-Lyt-2, more than 50% of lymphocytes reacted with anti-asialo GM₁. 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Anti-asialo GM$_1$-positive (asialo GM$_1^+$) cells showing reaction products on their surface were found mainly among the large blast cells showing relatively abundant cytoplasm with scanty organelles and little endoplasmic reticulum (Fig. 4). They also had fine heterochromatin attached to the nuclear membrane, and distinct nucleoli (Fig. 4). These features of asialo GM$_1^+$ cells were consistent with immature thymic lymphocytes and proved to be distinct from thymic epithelial cells that might have contaminated cell suspensions. No reaction products are seen on the cells that were incubated with NRS instead of anti-asialo GM$_1$.

DISCUSSION

The results obtained in this study clearly indicate that rabbit antibody directed to the neutral glycolipid, asialo GM$_1$, can be utilized to detect immature lymphocytes in the thymus of early embryonic stages that lack any characteristic T cell markers. Although there is no direct evidence in this study to suggest that asialo GM$_1^+$ cells in the thymus are progenitors of Thy-1$^+$ thymocytes, the clear inverse relationship between the proportion of Thy-1$^-$ cells and asialo GM$_1^+$ cells during the gestational stages tempts one to assume that asialo GM$_1^+$ cells in the thymus may convert to Thy-1$^+$ thymocytes, perhaps under the inductive influence of thymic epithelial cells. Although it has been presumed that bone marrow and fetal liver stem cells are precommitted to become the T cell lineage (9, 22, 23), it has not been known whether T cell progenitor cells in bone marrow or fetal liver have their own specific markers distinct from other lymphoid cell lineages. Thus, this new differentiation antigen, asialo GM$_1^+$, might prove to be a good marker if the few asialo GM$_1^+$ cells found in the fetal liver or bone marrow are commit-
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Figure 4. Electron micrograph of lymphoid cells from the thymus of CBA/N at 13 gestational days. The cells were incubated with anti-asialo GM1 by the indirect immunoperoxidase technique. Reaction products are present on the cell surface as the dark fine deposits (single arrow). Moderately abundant cytoplasm of the cell show many polysomes and a few endoplasmic reticula. Asialo GM1 negative cell possessing no reaction products on the cell surface is found at the right bottom. Magnification ×3300. Staining with lead salt was omitted.

We have reported that this anti-asialo GM1 reacts selectively with the blast cells of human acute lymphoblastic leukemia (27). This antiserum also stained AKR thymic leukemia cells. Furthermore, murine tumor cell lines of lymphoid origin, such as YAC-1, RL2-1, AKSL-2, and GRSL, react with anti-asialo GM1 (data not shown). If early human fetal thymocytes also react with this antiserum, as we have shown for 13-day mouse fetal thymocytes, asialo GM1 would be a clear example of an oncofetal antigen: one that is present on fetal and adult transformed cells but not on normal nontransformed adult cells. It would be interesting in this context to find out if asialo GM1 is found on human as well as mouse fetal thymocytes of comparable gestational stages.

The reaction of membrane antigens of cell subpopulations with anti-BAT (25), thereby demonstrating antibody activity against various glycolipid molecules (16), provided the initial clue that prompted our focusing on glycolipids as markers of lymphoid cells. Other glycolipid molecules also may provide new markers for studying the differentiation of T cells.

Quantitative changes in Lyt-1, Lyt-2, Lyt-3, and Thy-1 antigens on T lineage cells during ontogeny and in different adult lymphoid organs also give useful insights into T cell differentiation (11, 28, 29).


Acknowledgments. We are grateful to Dr. J. Ledbetter for supplying fluorescein-conjugated monoclonal antibody, to Dr. T. Tokuhisa, Mr. T. F. Gadus, and Ms. S. Scaling for their excellent technical assistance, and to Mr. A. Akatsuka for assistance in electronmicroscopic studies.

REFERENCES