Expression of the Asialo GM$_1$ Determinant on Murine Intestinal Epithelia (43151)

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Abstract. **Murine intestinal epithelial cells were studied by flow cytometric analyses for the expression of lymphocyte-associated membrane antigens. Three lymphocyte antigens were found to be expressed at high density on most nonhematopoietic intestinal epithelial cells. These included major histocompatibility complex class II antigens, the T cell-associated CT carbohydrate determinant, and the asialo GM$_1$ (aGM$_1$) neutral glycolipid. Examination of aGM$_1$ determinant density on epithelial cells, estimated by fluorescence intensity, indicated that aGM$_1$ was expressed at levels equal to those present on lymphoid cells known to be aGM$_1^+$. The potential role for lymphocyte antigenic determinants on nonhematopoietic cells of murine epithelia with respect to local regulation of intestinal lymphocytes is discussed.** [P.S.E.B.M. 1990, Vol 195]

Lymphocytes located within the intestinal epithelium, i.e., intraepithelial lymphocytes (IEL), constitute part of the gut-associated lymphoid tissues. Although the IEL possess properties similar to those of other peripheral lymphocyte populations, it is now becoming evident that the IEL also display characteristics that distinguish them from other peripheral T cells. For example, studies from this and other laboratories demonstrate that murine IEL bear unique differentiation antigens (1–3), and that the IEL possess T cell subsets rarely encountered in peripheral immune compartments (4, 5). The IEL also exhibit endogenous cytotoxic activity acquired *in situ* (1, 6), and a substantial proportion of the IEL utilize a $\gamma\delta$ T cell receptor molecular complex (6, 7). Many unanswered questions remain regarding IEL immunobiology, particularly with respect to mechanisms of IEL activation, immune regulation, and antigen recognition. In general, the role of the IEL in immune protection is uncertain.

Given the organization of the intestinal epithelium, in which the IEL are dispersed among nonhematopoietic epithelial cells, the relationship between IEL and epithelial cells may be an important factor influencing IEL regulation and function. Thus, information pertaining to the nature and composition of intestinal epithelial cells may ultimately shed light on mechanisms of IEL-mediated immunity. In that context, it has been demonstrated that major histocompatibility complex (MHC) class II molecules are expressed on human and murine epithelial cells within the small intestine (reviewed in ref. 8), and that CT T cell-associated carbohydrate determinants are expressed on murine intestinal epithelial tissues (2). Here, we report that in addition to those antigens, the lymphocyte-associated neutral glycolipid, asialo GM$_1$ (aGM$_1$), is also expressed at high density on most epithelial cells within murine small intestine, thereby demonstrating the presence of three lymphocyte-associated antigens on nonhematopoietic components of intestinal epithelia.

Materials and Methods

Mice. BALB/cByJ, C57BL/6, and DBA/2 mice, 8–16 weeks of age, were purchased from The Jackson Laboratory, Bar Harbor, ME. Animals were housed at the University of Tulsa vivarium.

Cells. Primary mixed lymphocyte culture (MLC) cells were prepared by culturing 25 × 10^6 BALB/c responder spleen cells with 25 × 10^6 C57BL/6 mitomycin C-treated splenic stimulator cells in 20 ml of lymphocyte culture media: 90% (v/v) RPMI 1640 (Irvine Scientific, Irvine, CA), 10% heat-inactivated fetal bovine sera (Irvine), 2 m*M* L-glutamine (Irvine), 100

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Received April 3, 1990. [P.S.E.B.M. 1990, Vol 195]
Accepted June 26, 1990.

0037-9727/90/1953-0329$2.00/0
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units/ml penicillin-streptomycin (Irvine), 5×10^{-5} *M* 2-mercaptoethanol (Fisher Scientific, Fair Lawn, NJ), and 5 m*M* Hepes buffer (Fisher).

The origin and derivation of the cytotoxic T lymphocyte (CTL) clone 2H9 has been reported elsewhere (9). Clone 2H9 is a MHC-restricted CTL clone specific for a minor histocompatibility antigen of BALB background.

Isolation of Intestinal IEL and Epithelial Cells. Methods used to isolate IEL and epithelial cell populations have been described previously by the authors (1, 5, 10). Murine intestinal epithelia were twice extracted by 20-min treatment in phosphate-buffered saline (Ca^{2+}, Mg^{2+} free) containing 1 m*M* EDTA (Sigma Chemical Co., St. Louis, MO) and 1 m*M* dithiothreitol (Bio-Rad Laboratories, Richmond, CA). Isolated cells were rapidly filtered through nylon-wool to remove cell sheets, mixed in 40% isotonic Percoll (Sigma), and centrifuged for 20 min at 600*g*. This resulted in two cell fractions: a pelleted portion, which contained lymphocytes and some epithelial cells, both with high overall viability (>95%); and an epithelial fraction with lower viability, which migrated above Percoll.

Antibodies and Flow Cytometry. Antibodies used in this study were: M1/89.18.7HK and M1/9.3.4HL.2 (anti-common leukocyte) (11), 2.43 and 3.155 (anti-CD8) (12), RL172.4 (anti-CD4) (13), MK-D6 (anti-I-A^d) (14), CT1.1 (anti-CT1) (2), anti-aGM_1 (anti-aGM_1) (Wako Chemicals, Dallas, TX), SH34 (anti-aGM_1) (15), and normal rabbit and normal rat sera used for control reactivity (Zymed Laboratories, South San Francisco, CA). For direct staining (MK-D6 and CT1.1), monoclonal antibodies were affinity purified from culture supernatants and coupled to fluorescein isothiocyanate. For indirect staining, cells were reacted for 30 min at 4°C with one of the antibodies listed above, washed by centrifugation, and reacted with fluorescein isothiocyanate (FITC)- or phycoerythrin-conjugated goat anti-rat immunoglobulin (Southern Biotechnology, Birmingham, AL), with $F(ab')_2$ fragment goat anti-mouse IgM (Jackson ImmunoResearch, West Grove, PA), or with goat anti-rabbit immunoglobulin (Zymed) as required. Antigen expression on IEL and epithelial cells was analyzed at the University of Tulsa flow cytometry core facility using an Epics 751 flow cytometer (Coulter Electronics, Hialeah, FL) interfaced to an MDADS II computer. IEL and epithelial cells were discriminated based on forward angle and 90° light scatter; populations for study were circumscribed in bitmaps according to the presence or absence of the T200 common leukocyte antigen. Nine contour levels from 2 to 512 events per pixel were used for dual-color analyses.

Results

Composition of Cell Populations Used in this Study. Cells that migrated below 40% Percoll were characterized according to relative cell size (forward angle light scatter) and granularity (90° light scatter). Two discrete populations, both with high viability, which consisted of small agranular lymphocytes (Fig. 1A) and a population of larger more granular cells, were present (Fig. 1B). Between those two groups, IEL and epithelial cells were easily differentiated based on the presence or absence of T200 expression. That marker is a reliable indicator of hematopoietic cell lineages given that T200 is expressed on all murine hematopoietic cells except erythrocytes (11). Moreover, in bone marrow radiation chimeras, the T200 marker has been shown to effectively differentiate IEL from intestinal epithelial cells (2). Of cells that migrated below 40% Percoll, $T200^+$ cells were predominantly small to medium in size, with a few $T200^+$ large cells (Fig. 2B), generally with low relative granularity (Fig. 2D). In contrast, $T200^-$ cells were primarily large in size (Fig. 2B) with medium to high granularity (Fig. 2D). Based on those criteria, cells located in population A (Fig. 1) were typical murine IEL, whereas those in population B (Fig. 1) consisted primarily of epithelial cells. The overall proportion of those two cell populations in 40% Percoll-fractionated BALB/c isolates was 70 to 80% $T200^+$ IEL, and 20 to 30% $T200^-$ epithelial cells, both with high viability (>95%).

Asialo GM_1 is Expressed on a Minor IEL Subset and on Most Epithelial Cells. Typical phenotypic profiles of IEL and epithelial cell populations in 40% Percoll-fractionated preparations are shown in Figure 3. IEL were predominantly $T200^+$ (93%) and $CD8^+$ (74%), with a minor $CD4^+$ (28%) fraction (Fig. 3, A–C). That phenotype is consistent with previous IEL studies from this (1, 5) and other (2, 4, 6, 7) laboratories, demonstrating a preference among IEL for $CD8^+$ lymphocytes. aGM_1 expression on $T200^+$ IEL represented 3 to 15% of that population (Fig. 3D).

As expected, the distribution of lymphocyte antigens among epithelial cells differed from IEL in that

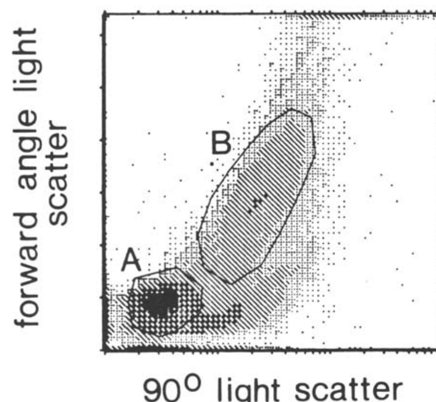

Figure 1. Characterization of cell populations in 40% Percoll-fractionated intestinal cell isolates based on cell size (forward angle light scatter, linear scale) and granularity (90° light scatter, log scale).

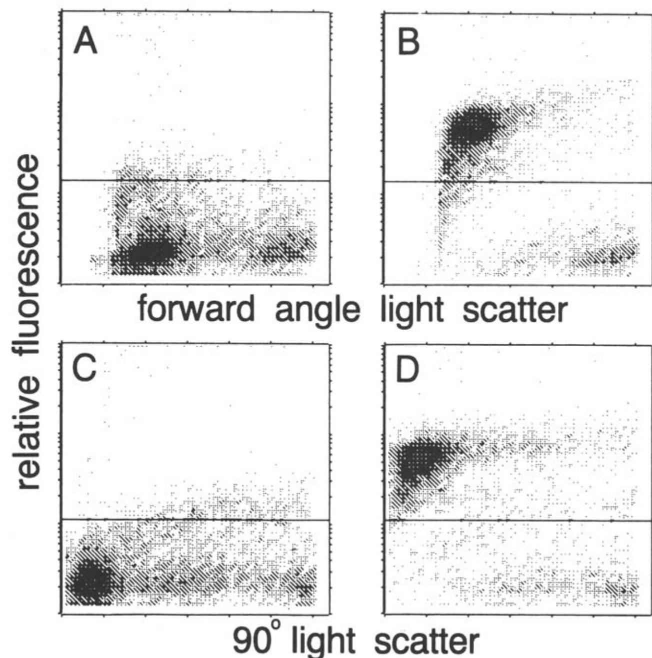

Figure 2. Identification of hematopoietic (IEL) and nonhematopoietic (epithelial) cell populations in 40% Percoll-fractionated intestinal cell isolates based on forward angle and 90° light scatter (linear scale), and according to expression (log fluorescence) of the T200 common leukocyte antigen. Background fluorescence is demarcated in scattergrams by horizontal lines: 94% (A) and 96% (C) of the cells were unreactive in control samples. Overall, 76% (B) and 80% (D) of the cells were $T200^+$.

epithelial cells were predominantly $T200^-$ (76%), $CD4^-$ (85%), and $CD8^-$ (83%) (Fig. 3, E–G). $T200^+$ cells (24%) associated with the epithelial fraction presumably represent a subset of large granular IEL. However, the most notable distinction between IEL and epithelial cells was the expression, at high density, of the aGM_1 determinant on nonhematopoietic ($T200^-$) epithelial cells (82% aGM_1^+) (Fig. 3H). To confirm the specificity of anti-aGM_1 antisera used in the above experiment (Fig. 3D, and H), aGM_1 expression on IEL and epithelial cells was evaluated using anti-aGM_1 monoclonal antibody SH34 (15). That reagent reacted with 10% of IEL (Fig. 4A) and 87% of epithelial cells (Fig. 4B), in a pattern typical of aGM_1 expression observed in Figure 3, D and H.

To more critically evaluate the expression and distribution of aGM_1 on hematopoietic and nonhematopoietic cells of the intestinal epithelium, dual-color fluorescence analyses were done using anti-T200 and anti-aGM_1 antibodies. For that, bitmaps were drawn to include *both* IEL and epithelial populations providing combined analyses of those cell groups. As shown in Figure 5A, there was a sharp demarcation between aGM_1 and T200 expression on cell populations isolated from BALB/c mice such that aGM_1 expression was predominantly associated with $T200^-$ intestinal epithelial cells. This also was true for strains of mice other than BALB/c in that aGM_1 was expressed on intestinal epithelial cells, though not on most IEL, from C57BL/6 mice (Fig. 5B) and DBA/2 mice (Fig. 5C).

In the experiments mentioned above, aGM_1 expression was evaluated on epithelial cells that comigrated with IEL in Percoll preparations. In order to evaluate the overall distribution of aGM_1 on epithelial cells, aGM_1 expression was examined on non-Percoll-fractionated isolates of intestinal epithelial cells. For this, cells were isolated from the small intestine as described in Materials and Methods, except that, after extraction and dissociation of the epithelial layer, cells were filtered through a 60-μm nylon screen and stained and analyzed without Percoll fractionation. As shown in Figure 5D, the majority of unfractionated cells from BALB/c mice were epithelial in nature (79% $T200^-$), of which approximately half were aGM_1^+ (39%). Roughly one fifth of the total cells were IEL (21% $T200^+$). Moreover, the demarcation between T200 and aGM_1 expression remained evident, similar to that in 40% Percoll-fractionated isolates, except that among unfractionated isolates only about half of the epithelial cells were aGM_1^+.

Relative Expression of aGM_1 on Small Intestine

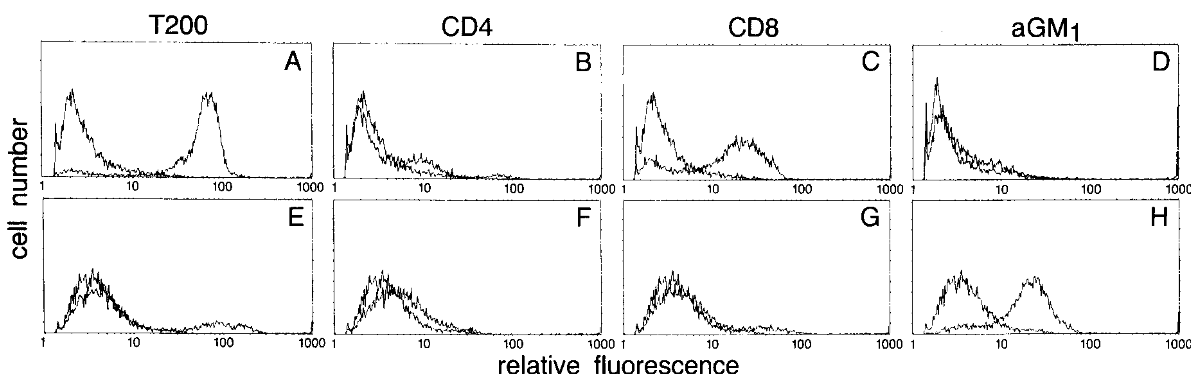

Figure 3. Expression of T200, CD4, CD8, and aGM_1 on BALB/c intestinal IEL (A–D) and epithelial (E–H) cell populations as defined in Figure 1, A and B, respectively. Left peaks in each histogram indicate background staining due to FITC-conjugated antibody to rat or rabbit immunoglobulins; right peaks indicate reactivity of a primary antibody plus FITC-conjugated anti-immunoglobulin.

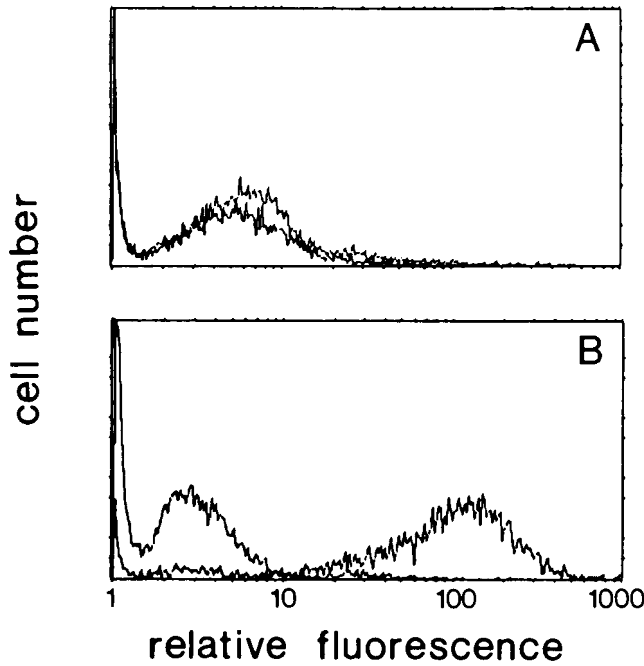

Figure 4. Reactivity of anti-aGM$_1$ monoclonal antibody, SH34, on Percoll-fractionated intestinal IEL (A) and intestinal epithelial cells (B).

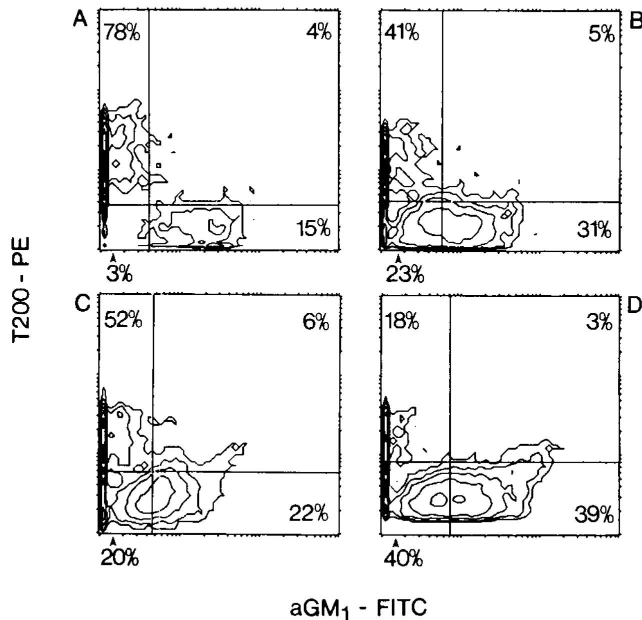

Figure 5. T200 and aGM$_1$ dual-color fluorescence analyses of combined IEL and epithelial cell populations (A plus B, Fig. 1) present in 40% Percoll-fractionated preparations from BALB/c (A), C57BL/6 (B), and DBA/2 (C) mice. T200 and aGM$_1$ dual-color analyses of non-Percoll-fractionated intestinal cell isolates from BALB/c mice (D).

Epithelia. The level of expression of aGM$_1$ on epithelial cells was compared with that of lymphoid cell populations generally regarded to express aGM$_1$ (16–18). Cells used for comparison included (i) alloreactive MLC cells 1 week after primary *in vitro* stimulation and (ii) a murine MHC-restricted CTL clone (2H9) (9). As shown in Figure 6A and B some primary MLC cells, and the majority of cloned CTL, expressed aGM$_1$ at equivalent levels of fluorescence intensity. By comparison, aGM$_1$ expression on intestinal epithelial cells was as great as, or slightly higher than, that of lymphoid cells as estimated by fluorescence intensity (Fig. 6C). It should be noted, however, that epithelial cells are two to three times larger than IEL (see Fig. 1, forward scatter). Thus, some increase in fluorescence intensity could be due to greater surface area of epithelial cells.

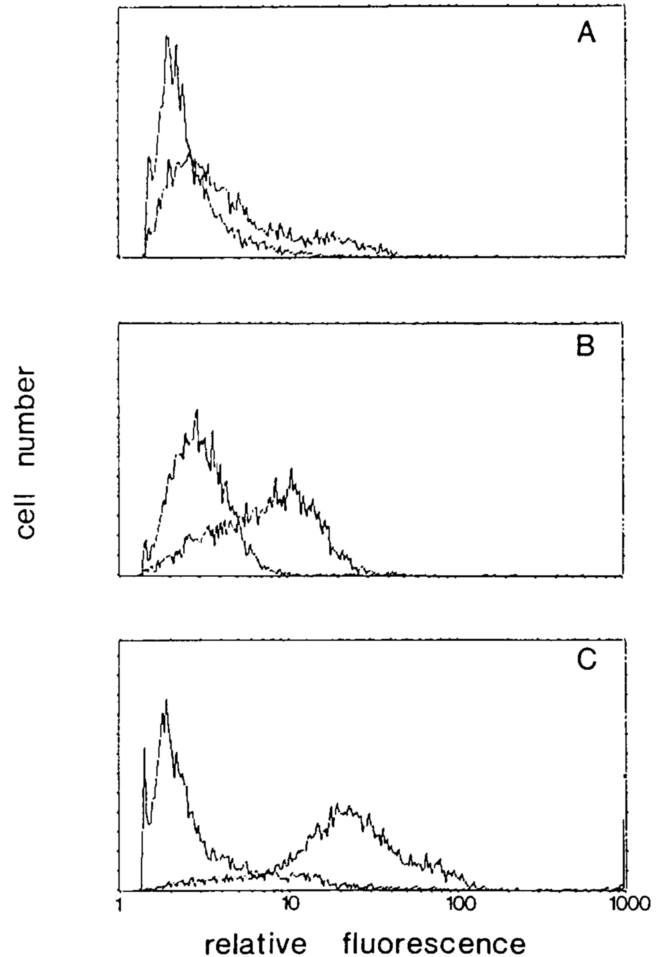

Figure 6. Relative expression of aGM$_1$ on primary MLC cells (A), murine CTL clone 2H9 (B), and BALB/c Percoll-fractionated intestinal epithelial cells (C).

Co-Expression of Three Lymphocyte-Associated Antigens on Intestinal Epithelial Cells. Murine intestinal epithelial cells have been shown to express Ia (8) and CT (2) determinants. Based on that, we sought to compare the expression of Ia, CT, and aGM$_1$ on intestinal epithelial cells. As shown in Figure 7, the majority of epithelial cells from Percoll-fractionated BALB/c small intestine expressed I-A^d, CT1, and aGM$_1$ determinants, demonstrating the co-expression of three lymphocyte-associated antigens on nonhematopoietic cells of murine small intestine epithelia.

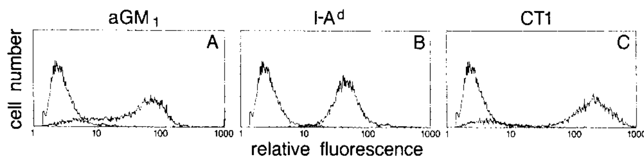

Figure 7. Expression of aGM_1 (A), I-A^d (B), and CT1 (C) on BALB/c intestinal epithelial cells. Background fluorescence, including that for direct staining (MK-D6 and CT1.1), consisted of reactivity due to FITC-conjugated antibody to rabbit immunoglobulin.

Discussion

The findings reported here demonstrate the shared expression of three lymphocyte-associated antigens on nonhematopoietic cells of murine intestinal epithelia. These antigens include MHC class II molecules, the CT determinant, and the aGM_1 glycolipid. The presence of the aGM_1 determinant on epithelial cells is of interest because of the wide, yet selective, distribution of that marker on cells within the murine immune system. On hematopoietic cells, aGM_1 is present on some splenic and lymph node cells (17), on alloreactive CTL (18, 19), on natural killer cells (20), and on murine fetal thymocytes (21). That the expression of aGM_1 on immune cells is under regulatory control is demonstrated by its appearance on selected lymphocyte populations during differentiation and/or activation (19, 20). Functionally, it has been suggested that the aGM_1 determinant is involved in transmembrane signaling (22), or that it serves as a cell adhesion molecule on effector cells (19). In the context of the latter, antibody to the aGM_1 determinant has been shown to block lytic activity of alloreactive CTL (19). However, in addition to cells of the immune system, the aGM_1 determinant is also selectively expressed on certain nonimmunologic tissues, most notably on cells of the central nervous system. Glycosphingolipids have been reported on cells of the intestinal epithelium (23), however earlier studies did not take into account the distribution of such antigens on specific intestinal cell populations.

The functional role of lymphocyte antigens on nonhematopoietic epithelial cells remains to be determined. Clearly, MHC class II molecules on epithelial cells can function as antigen presentation structures for IEL (24–26). That the aGM_1 determinant on intestinal epithelia may be immunologically relevant is suggested by similarities noted between events that influence class II expression and glycolipid biosynthesis on intestinal epithelia. For example, expression of MHC class II antigens on epithelial cells of rats and mice can be enhanced by enteric antigen exposure (8). Likewise, biochemical studies of intestinal glycoconjugates indicate that the biosynthesis of fucoglycolipids within the intestinal epithelium occurs in germ-free mice upon conventionalization with intestinal microflora (27), suggesting a correlation between foreign antigen exposure and biochemical modifications of intestinal glycolipids. Thus, aGM_1 determinants may serve to enhance communication between intestinal lymphocytes and epithelial cells, similar to the role proposed for aGM_1 between cells of the immune system (19, 20, 22).

The selective expression of lymphocyte antigens outside the immune system may provide important clues toward understanding mechanisms of IEL activation, regulation, and differentiation. In that context, there is now increasing evidence suggesting that local microenvironmental factors play a critical role in IEL maturation and differentiation (28). Understanding the involvement of lymphocyte antigens with epithelial cells should help to further elucidate how the IEL participate, mechanistically, in immune protection and/or disease states at the level of the gut epithelium.

This work was supported by NIH Grant DK35566 and by a grant from the Oklahoma Centers of Excellence.

The authors wish to thank Dr. L. Lefrancois for providing the CT1 hybridoma line and Dr. T. Higgins for providing the SH34 hybridoma line. We also thank Dr. K. Miller for helpful discussions.

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