Communication

Attenuation of Interleukin 2 Signal in the Spleen Cells of Complex Ganglioside-lacking Mice*

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T cell development and function in complex ganglioside-lacking (GM2/GD2 synthase gene-disrupted) mice were analyzed. GM1, asialo-GM1, and GD1b were representative gangliosides expressed on T cells of the wild type mice and completely deleted on those of the mutant mice. The sizes and cell numbers of the mutant mice spleen and thymus were significantly reduced. Spleen cells from the mutant mice showed clearly reduced proliferation compared with the wild type when stimulated by interleukin 2 (IL-2) but not when treated with concanavalin A or anti-CD3 cross-linking. Expression levels of IL-2 receptor α, β, and γ were almost equivalent, and up-regulation of a chain after T cell activation was also similar between the mutant and wild type mice. Activation of JAK1, JAK3, and SAT5 after IL-2 treatment was reduced, and c-fos expression was delayed and reduced in the mutant spleen cells, suggesting that the IL-2 signal was attenuated in the mutant mice probably due to the modulation of IL-2 receptors by the lack of complex gangliosides.

Gangliosides are enriched in nervous systems of vertebrates, suggesting their importance in the neuronal functions (1). They are also present in cells of the immune system and have been thought to contribute in cell to cell recognition or cellular signal modulation (2). In particular in human T cells, GD31 was induced by various stimulations via up-regulation of GD3 synthase gene (3). Moreover, anti-GD3 monoclonal antibody (mAb) induced T cell activation, suggesting that GD3 could mediate growth signals by binding endogenous ligands (4).

In murine T lymphocytes, a number of ganglioside components were present (5, 6) showing changes depending on the stage of development (7) and differentiation of the cells (8). These results indicate that gangliosides play important roles in the immune system. However, many of these studies were done by the addition of exogenous gangliosides (9) or anti-ganglioside antibody to cultured cells (10) or into experimental animals (11). These studies do not address the functions of endogenously generated gangliosides.

We have established mutant mouse lines that completely lacked GM2/GD2 synthase activity and expression of all complex gangliosides including those of asialo-series (12). They showed mild defects in the nervous system, and severe dysfunction in male genital organ (13). In this study, we analyzed the phenotypic and functional changes in the immune system of the complex ganglioside-lacking mice. Most significantly, it was found that mature T cells from the mutant mice respond very weakly to interleukin 2 (IL-2). We elucidated here that the signaling pathway for IL-2/IL-2 receptor (IL-2R) was largely disrupted by the altered ganglioside arrangement on cell surface. This is the first report to study glycolipid functions by the genetic modification of their carbohydrate structures and showed critical roles for endogenous glycolipids in the transduction of proliferation signals introduced by lymphokines.

EXPERIMENTAL PROCEDURES

Flow Cytometry—For the analysis of IL-2 receptors and gangliosides, spleen cells and thymocytes from 6–8-week-old mice were used. Spleen cells resuspended in phosphate-buffered saline/0.5% bovine serum albumin (BSA) were incubated for 30 min on ice with anti-CD3 monoclonal antibody (mAb) and washed. They were then stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM for CD3 and FITC-conjugated anti-mouse IgG for CD4. After washing, cells were fixed with 1% paraformaldehyde. The samples were analyzed by dual-color flow cytometry using a FACScan, FACSCalibur (Becton Dickinson, San Diego, CA) and analyzed with Cell Quest software (Becton Dickinson).

T Cell Proliferation Assay—Spleen cells from mice (6–8 weeks old) were dispersed and washed twice in plain RPMI 1640 medium at 4 °C. They were adjusted at 3.75 × 10^6/ml in RPMI 1640 containing 10% fetal bovine serum (FBS) and plated into 96-well plates at 100 μl of ascites (these three antibodies were kindly provided by Dr. T. Takeshita at Tohoku University), R24 (anti-GD3 provided by Dr. L. J. Campbell, CA) was used followed by avidin-FITC (EY Laboratories). Additional antibodies were used were FITC anti-rat IgG with nonpanned spleen cells. To detect GM1, biotin-labeled cholera toxin B subunit (List Biological Laboratory, Campbell, CA) was used followed by avidin-FITC (EY Laboratories).

1 Ganglioside nomenclature is based on that of Svennerholm (33).

2 The abbreviations used are: mAb, monoclonal antibody; FBS, fetal bovine serum; IL-2, interleukin 2; IL-2R, IL-2 receptor; FITC, fluorescein isothiocyanate, ConA, concanavalin A.
filter, and then the filter was counted using a liquid scintillation counter (Aloka, Tokyo). To analyze the response to concanavalin A (ConA) stimulation, ConA was added to the spleen cells prepared as described above at 1–5 μg/ml, and proliferation was examined as performed in IL-2 treatment. Proliferation by stimulation with anti-CD3 antibody was examined by cultivating spleen cells in 96-well plates coated by mAb145–5C11 at 10 μg/ml, otherwise as done in IL-2 stimulation.

Preparation of Panning Plates—Anti-mouse IgG (H and L) (Cappel) was coated in 6-cm bacteriological plates (Falcon) at 20 μg/ml in 50 mM Tris-HCl (pH 9.5) for 3 h at room temperature. After washing in saline, the plates were blocked by 0.1% bovine serum albumin in phosphate-buffered saline and then stocked in a freezer.

Immunoprecipitation—Spleen cells from mice of 6–8 weeks old were cultured in 24-well plates at the density of 1 × 10^6/ml/well in RPMI 1640 containing 10% FBS. The cells were cultured for 0, 5, 10, and 30 min after addition of IL-2 (1 unit/ml, Takeda Pharmaceutical Co.) (1 Takeda unit = 383 Japan reference unit). The cells were collected at each time point, and then the pellets were lysed by adding 200 μl of lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM p-aminophenyl-methanesulfonyl-fluoride hydrochloride, aprotinin (10 μg/ml), 1% (v/v) Brij96). After centrifugation at 15,000 rpm, the supernatants were precleared with protein A-Sepharose (Amersham Pharmacia Biotech) and then incubated with 10 μl of antibodies (anti-JAK1 (Q-19), anti-JAK3 (C-21), or anti-STAT5b (C-17)) (Santa Cruz Biotechnology Inc.) and then with 20 μl of protein A-Sepharose for 2 h at 4°C. After washing three times with lysis buffer, the immunoprecipitates were applied to 7.5% gel of SDS-polyacrylamide gel electrophoresis. Proteins were electrically blotted onto polyvinylidene difluoride membranes, and immunoblot was performed by PY-20 (ICN Biomedical Inc.) at 1:2000, anti-JAK1 at 1:4000, anti-JAK3 at 1:4000, and anti-STAT5b at 1:4000 dilution. Second antibodies used were biotinylated anti-mouse IgG for anti-JAK1 and anti-JAK3 and biotinylated anti-rabbit IgG for anti-STAT5b. Antibody binding were detected by ECL detection kit (Amersham Pharmacia Biotech).

Northern Blotting—Spleen cells from mice of 6–8 weeks old were adjusted at 1 × 10^6/ml in RPMI 1640 containing 10% FBS and plated in 24-well plates at 2 × 10^5/well/2 ml. They were cultured for 0, 10, 30, and 60 min in the presence of IL-2 (1 unit/ml). At each time point, cells were collected, and then total RNA was extracted by Trizol (Life Technologies, Inc.) For Northern blot analysis, total RNA (15 μg/lane) was separated in 1.2% agarose-formaldehyde gel and transferred onto a nylon membrane (GeneScreen Plus, NEN Life Science Products). Prehybridization and hybridization was performed with [32P]-Radiolabeled cDNA probes synthesized from c-fos or c-myc containing plasmids as described previously (3). Then the membrane was washed and analyzed by a BioImaging Analyzer BAS-2000 (Fuji, Tokyo).

RESULTS

Ganglioside Expression on T Lymphocytes in the Mutant Mice—GM1, asialo-GM1, and GD1b gangliosides detected in the wild type spleen T cells were completely absent in the mutant spleen cells, as expected from the proposed ganglioside synthetic pathway (Fig. 1, A and B). Instead, GD3 expression was slightly increased in the knock-out mice, probably as a result of precursor accumulation.

Size of Immune Tissues and Numbers of Total Cells and T Cells—Spleens and thymuses from the mutant mice were smaller than the wild type organs (Fig. 1C). Total cell numbers in the spleens or thymuses of the knock-out mice were significantly reduced as shown in Fig. 1C. The ratios of CD3 positive T cells in the whole spleen cells were almost equivalent between the two groups, i.e. 31.8 ± 1.1% and 31.2 ± 1.6% for the wild type and the mutant mice, respectively.

Proliferation of Spleen Cells—To compare their proliferation, spleen cells were stimulated with ConA, anti-CD3 antibody, or IL-2. Those from the mutant mice showed clearly decreased incorporation of [3H]thymidine compared with the wild type when stimulated by IL-2 (Fig. 2) but not when treated with ConA or anti-CD3 cross-linking. Because T cell population in the mutant spleen was equivalent to that of the wild type, the results described above indicate that T lymphocytes in the mutant have defects in IL-2/IL-2R-mediated signaling.

Expression of IL-2 Receptors—Expression levels of IL-2 receptors were analyzed by flow cytometry. All three components of IL-2 receptor, α, β, and γ, were equally detected by specific monoclonal antibodies between the wild type and the mutant mice (Fig. 3). After culture with IL-2, their expression patterns were also similar. When they were cultured in the presence of ConA, marked up-regulation of IL-2Rα chain was observed in both groups (Fig. 3). Thus, there are no marked differences in the expression levels of IL-2 receptors and in the up-regulation of α chain.

Activation of JAK-STAT System with IL-2—To analyze the activation of the JAK/STAT pathway, the phosphorylation patterns of JAK1, JAK3, and STAT5 proteins that are the representative signal molecules responsible for transmission of the IL-2 signal were examined. JAK1 and JAK3 were phosphorylated at 5 min after IL-2 treatment in the wild type mouse T
cells, and the activation level was sustained for 10 and 30 min for JAK1 and JAK3, respectively (Fig. 4). On the other hand, JAK1 was scarcely phosphorylated, and JAK3 phosphorylation detected at 5 min quickly returned to the basal line in the mutant mice. STAT5 was also phosphorylated at 5 min after IL-2 stimulation and kept the activated form even after 30 min in the wild type. In contrast, the mutant spleen cells showed delayed response and already declined at 30 min after IL-2 treatment (Fig. 4C).

c-myc and c-fos Gene Expression during IL-2 Stimulation—c-fos/c-jun, c-myc, or bcl2 has been reported to be involved in the cell proliferation by IL-2 (14). We analyzed the regulation of c-fos and c-myc gene transcription when stimulated with IL-2. mRNA of c-fos in the wild type mice was readily detected by Northern blotting 10 min after IL-2 stimulation and then gradually decreased. In the mutant mouse, c-fos mRNA levels reached the plateau at 30 min and soon fell (Fig. 5A). On the other hand, c-myc mRNA levels exhibited very similar patterns in the mutant and wild type mice.

**DISCUSSION**

Ganglioside GM2/GD2 synthase gene knock-out mice showed defects in ganglioside expression on T cells in accord with the proposed pathway of ganglioside synthesis (15). The comparative study of the immune system using these mutant mice, as well as the wild type mice, is expected to unambiguously elucidate the actual roles of gangliosides in vivo. In fact, results obtained here clearly showed important roles of gangliosides in the regulation of IL-2/IL-2R-mediated signals in a straightforward manner.

In many studies, the roles of glycolipids in the immune system have been analyzed by observing the effects of exogenous gangliosides, and they exhibited suppressive effects on T cell functions such as CD4 internalization (9) and reduced response to mitogens or allogeneic antigens (16). Actually, gangliosides were reported to bind to IL-2 (17) and thereby inhibit T cell proliferation (18). These immunosuppressive effects of gangliosides have been considered to explain immunosuppression in cancer patients (19). However, results obtained in these experiments do not seem likely to reflect the real effects of endogenously generated gangliosides.

Compartmentalization through protein-protein or protein-lipid interactions has now been recognized as a fundamental mechanism for efficient and organized cell signaling (20, 21). Some membrane components are apparently organized into glycolipid-enriched membrane domains or detergent-insoluble glycolipid-enriched domains (22) known as rafts. These rafts are enriched in sphingolipids, cholesterol, glycosylphosphatidylinositol-anchored proteins (23), and a variety of signaling molecules. Recently, dynamic T cell receptor recruitment to such a microdomain upon T cell activation has been reported in a T cell line (24, 25) and in thymocytes (26). These data suggest that gangliosides expressed on T cells associate with receptors and signaling molecules to transduce the IL-2 signals at such a microdomain, although it is yet unclear that IL-2 receptors are enriched in glycolipid-enriched membrane domains.

Defects in the IL-2 signal transduction found in the mutant mice T cells should take place due to lack of GM1/GD1b/GA1 and may be due to serious structural defects of IL-2 receptors. In particular, the configuration of β and γ chains may be affected because it has been reported that JAK1 and JAK3 are differentially associated with IL-2Rβ and IL-2Rγ, respectively (27). Among many signaling molecules, JAK3 is reported to mainly act in cell proliferation and to activate STAT5 (28) and induce c-fos expression (29). Reduced activation of JAK3 and JAK1 followed by reduced activation of STAT5 and reduced c-fos induction upon IL-2 stimulation may explain the suppressed proliferation of the mutant T cells. The fact that all mutant mice deficient for IL-2Rβ/IL-2Rα, except for those deficient in IL-2Rγ, show deregulated T cell activation and autoimmune
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