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Antibody against Ganglioside GD1c Containing NeuGcα2-8NeuGc Cooperates with CD3 and CD4 in Rat T Cell Activation[1]

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Gangliosides have long been implicated in T cell activation. GD1c with two *N*-glycolylneuraminic acids [GD1c(NeuGc,NeuGc)] is the predominant ganglioside in rat T cells. In the present study, the anti-GD1c(NeuGc,NeuGc) mAb, AC1, which binds to the NeuGcα2-8NeuGcα2- sequence, was found to enhance Con A-activated cellular proliferation at a concentration at which AC1 alone did not activate the cells. The potentiation by AC1 was observed more consistently and effectively in the cellular activation elicited by cross-linking of anti-CD3 and anti-CD4, rather than in the cell growth induced by immobilized anti-CD3 alone. Moreover, the combination of immobilized anti-CD4 and soluble AC1 had a remarkable mitogenic effect. In addition, we have demonstrated the existence of a 100 kDa protein in rat T cell lysates which reacts with AC1 on Western blots, and this interaction is abolished by sialidase-treatment of the membrane. Pronase treatment of the T cells, which rendered the 100 kDa protein undetectable on Western-blotting, reduced the number of AC1-positive cells by 40-50% on flow cytometry. On the other hand, all cells became AC1-negative after sialidase treatment. These findings indicated that AC1 reacts with both GD1c(NeuGc,NeuGc) and the 100 kDa glycoprotein on rat T cells. Taken together, these results predict the presence of a novel regulatory mechanism of T cell activation involving CD4 and the NeuGcα2-8NeuGcα2- sequence.

Key words: CD4, GD1c, glycoprotein, *N*-glycolylneuraminic acid, T cell activation.

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Gangliosides, the sialylated glycosphingolipids that are present on the outer leaflets of plasma membranes, have long been known to be potential modulators of lymphocyte activation (*1*). More than a decade ago, the B subunit of cholera toxin was shown to activate rat thymocytes, the immature T cells, by specifically binding to ganglioside GM1 on the cells (*2*). In humans, stimulation of T cells with a mAb against GD3, which is expressed on a portion of CD4 and CD8 T cells (*3-6*), is known to induce cellular proliferation, cytotoxicity, expression of surface markers, and production of cytokines (*3, 7-9*). These studies implied that gangliosides are involved in the signal transduction pathway of T cell activation, and that ligands for the oligosaccharide moieties of gangliosides are potentially engaged in regulation of the T cell function. Another example of ganglioside regulation of T cell activation was the finding that exogenously added gangliosides elicit down-modulation of CD4 in rat, mouse and human T cells (*10*). CD4 is a membrane glycoprotein and cooperates with the T cell receptor (TCR)-CD3 complex in the activation of helper T cells (*11, 12*). Exogenously added GM1 was subsequently shown to trigger the dissociation of tyrosine kinase $p56^{lck}$ from the cytoplasmic tail of CD4, leading to CD4 internalization and degradation in human T cells (*13, 14*). As GM1 was reported to be virtually undetectable in the gangliosides from human lymphocytes (*15*), a major part of this effect of ganglioside on CD4 maybe being related to the ceramide moiety. The detergent-insoluble glycosphingolipid-enriched complex (DIG) (*16*), composed of a glycosphingolipid core, cholesterol, and GPI-anchored proteins, and associated with non-receptor kinases like src-family kinases, has been reported to occur in many types of cells (*16, 17*). The DIG domain containing GM3 ganglioside, CD4 and $p56^{lck}$ kinase was recently found in human lymphocytes (*18*). Exogenous gangliosides may possibly exert their effects by affecting the organization of this domain.

In previous studies, we identified the predominant ganglioside in rat T cells and thymocytes as GD1c having two *N*-glycolylneuraminic acids (NeuGcα2-8NeuGcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′ceramide) (*19, 20*).

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Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; DIG, detergent-insoluble glycosphingolipid-enriched complex; FITC-, fluorescein isothiocyanate-conjugated; mAb, monoclonal antibody; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; PBS, phosphate-buffered saline; PE-, phycoerythrin-conjugated; PVDF, polyvinylidene fluoride; TCR, T cell receptor. GD1c, sialylα2-8sialylα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′ceramide; GD3, sialylα2-8sialylα2-3Galβ1-4Glcβ1-1′ceramide. Other gangliosides are designated according to Svennerholm (*41*). In the case of gangliosides containing NeuGc, the sialic acid-species is indicated in parenthesis after the abbreviation.

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Then, we generated the mAb, AC1, by immunizing mice with this ganglioside (*21*). NeuGcα2-8NeuGcα2- is considered to be critical for the recognition of AC1, because AC1 also bound to GD1b(NeuGc,NeuGc), which includes the NeuGcα2-8NeuGcα2- sequence linked to the internal galactose of an asialo-GM1 backbone, albeit weaker than to GD1c(NeuGc,NeuGc), and the other gangliosides containing NeuAcα2-8NeuAcα2-, NeuAcα2-, and NeuGcα2- as sialic acid residues, did not react with AC1 (*21*). AC1-positive cells are detected in a portion of CD4 T cells and CD4-single positive mature thymocytes in rats (*21*).

In the present study, we examined the effect of AC1 on rat T cell activation and showed that AC1 modulates this event in a very unique way. Furthermore, we reexamined rat T cell lysates by Western-blotting using AC1 in this study. We previously observed no specific band reacting with AC1 (*21*), when the blotted membranes were washed once with 2 M urea/1% Triton X-100 after antibody treatment in order to reduce non-specific binding according to Davis and Bennett (*22*). In the present study, a 100 kDa protein specifically recognized by AC1 was identified when no urea/Triton X-100 wash was performed. The protein has been shown to be detected by AC1 through its sialic acid residues and accounts for about 40-50% of the AC1-staining in rat T cells. Taken together, our findings predict the existence of a novel regulatory mechanism of T cell activation which involves CD4 and glycoconjugates with NeuGcα2-8NeuGc residues.

MATERIALS AND METHODS

Materials—The following materials were purchased: Triton X-100 from Wako (Osaka); aprotinin, leupeptin, PMSF, sodium orthovanadate, and sialidase (from *Vibrio cholera*, type III, and from *Clostridium perfringens*, type X) from Sigma; *Streptomyces griseus* pronase from Calbiochem, CA; and DNase I (bovine pancreas) from Boehringer Mannheim.

Antibodies—The mAb, AC1, directed against GD1c(NeuGc,NeuGc) (mouse IgG3, κ) was established in our laboratory (*21*). Anti-rat CD3 (G4.18, mouse IgG3), PE-anti rat CD4, FITC-anti rat CD8, and FITC-anti mouse IgG3 were purchased from Pharmingen (San Diego, CA); anti-rat CD4 (W3/25, mouse IgG1, and MRC OX-35, mouse IgG2a) and CD11b (MRC OX-42) from Serotec (Oxford, UK); anti-rat B cell (RLN-9D3) from Caltag Lab. (Burlingame, CA); and horseradish peroxidase-conjugated anti mouse IgG from Sigma.

Preparation and Culture of Rat T Cells—T cells were prepared from the spleens of male Wistar rats, which were supplied by Clea Japan (Tokyo). Splenocytes were treated with an ammonium chloride/EDTA solution to eliminate red blood cells (*23*) and then fractionated on a column packed with nylon wool (Wako Pure Chemicals, Osaka) according to Julius *et al.* (*24*). T cells were obtained in the column pass-through fraction. Except where specifically mentioned, these cells were used as T cells. To obtain further purified cells, T cells were incubated with mAbs against B cells and CD11b (a marker for macrophages, dendritic cells, and granulocytes) for 30 min at room temperature, washed once, and then treated with Dynabeads M-450 sheep anti mouse IgG (Dynal Inc., Great Neck, NY) for 30 min. Dynabeads-adsorbed cells were excluded with a magnet according to the manufacturer's protocol. The purity of the T cells was assessed by flow cytometry using PE-anti-CD4 and FITC-anti-CD8 mAbs. All cultures were performed in complete medium, which consisted of RPMI 1640 (Nissui Pharmaceutical, Tokyo) supplemented with 10% FCS, 12 mM Hepes, 0.05 mM 2-mercaptoethanol, 2 mM sodium pyruvate, penicillin, and streptomycin.

Proliferation Assay—T cells were activated by culturing with Con A (10 μg/ml) or immobilized antibodies in flat-bottom microtiter plates. The antibodies were immobilized by coating the plates with 100 μl of anti-CD3 (0.5 μg/ml), anti-CD4 (W3/25, 10 μg/ml), or AC1 (20 μg/ml) for 2 h at 37°C, and then washed twice with PBS. When anti-CD3 and anti-CD4 were used in combination, the plates were first coated with anti-CD3, and then after one wash, were coated with anti-CD4 mAb (*25*). Cell proliferation was measured after two days using a Cell Proliferation ELISA, BrdU kit (Boehringer Mannheim, Tokyo). The reaction of anti-BrdU-conjugated peroxidase was stopped with sulfuric acid and the absorbance was measured at 450 nm, with reference to 630 nm.

Lysis and Western Blotting—Rat splenic T cells were lyzed in ice-cold 0.5% Triton X-100 lysis buffer (0.5% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 1 mM sodium orthovanadate, 5 mM EDTA, 50 mM Tris-HCl, and 150 mM NaCl), and the supernatants were collected after centrifugation for 10 min at 12,000 × *g* at 4°C. The lysates were subjected to SDS-PAGE and then electroblotted onto polyvinylidene fluoride (PVDF) membranes (ATTO, Tokyo). The membranes were blocked, incubated with AC1 or control IgG3 at 2 μg/ml for 1 h, washed, and then treated with horseradish peroxidase-conjugated anti mouse IgG for 1 h. In some experiments, the membranes were additionally washed after the first mAb-treatment with 2 M urea-1% Triton X-100 for 3 min. After washing, the membranes were developed using the enhanced chemiluminescence (ECL) system (Amersham).

Enzymatic Hydrolysis of Cells or PVDF Membranes—T cells were treated with 0.04% pronase in PBS containing 100 μg/ml DNase I according to Suzuki *et al.* (*26*). After quenching the enzyme activity with FCS and washing, the cells were analyzed by flow cytometry or lysates were prepared. Sialidase treatment was carried out by treating cells with sialidase from *V. cholera* or *C. perfringens* in PBS (2.5 U/1 × 10^7 cells/0.25 ml) for 1 h at 37°C, after which the sialidase activity was quenched by the addition of FCS, and then the cells were washed. PVDF membranes were hydrolyzed with *V. cholera* sialidase at 50 mU/ml in 0.05 M sodium acetate (pH 5.5)-9 mM $CaCl_2$ for 5 h at 37°C (*20*) before staining with AC1.

Flow Cytometry—T cells were analyzed using a FACSCalibur as described previously (*21*).

RESULTS

Enhancement by AC1 of Mitogen-Induced T Cell Activation—As shown in Fig. 1, soluble AC1 at 20 μg/ml did not activate the T cells prepared from a rat spleen with a nylon wool column (around 85% pure), and immobilized AC1 was also ineffective. On the other hand, AC1 at the same dose markedly enhanced Con A-induced T cell proliferation (Fig. 1). AC1 at 10 μg/ml was less effective than at 20 μg/

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ml (data not shown). Control IgG3 had no effect on the proliferation of Con A-stimulated T cells (Fig. 1). Soluble AC1 and immobilized AC1 up to 40 μg/ml were not mitogenic toward T cells (data not shown). These results were reproducible in several experiments, and suggested that AC1 may cooperate with other activation signals rather than evoke T cell activation by itself.

Effect of AC1 on T Cell Proliferation Induced by Ligation of CD3 or CD4—We then examined whether or not AC1 augments the main T cell activation pathway that came into operation on TCR-CD3 complex ligation. Figure 2 shows representative results of several experiments. As indicated in the figure, however, AC1 at 20 μg/ml hardly affected the T cell proliferation elicited by immobilized anti-CD3 mAb. Although the addition of AC1 augmented the anti-CD3-induced T cell growth in one of six experiments (data not shown), it did not show any effect in the other five experiments.

Since cross-linking of anti-CD3 and anti-CD4 was reported to jointly activate T cells in human and mouse (*25, 27-29*), the effect of AC1 on this pathway was then examined. As shown in Fig. 2, co-immobilized anti-rat CD3 (G4.18) and anti-CD4 (W3/25) were also more effective in potentiating the growth of T cells than immobilized anti-CD3 alone. Furthermore, AC1 had a consistent enhancing effect on this synergistic activation (Fig. 2). Control IgG3 had no effect on the co-stimulated T cell proliferation in any of the experiments. In addition, while immobilized anti-CD4 alone showed only a weak mitogenic effect, co-stimulation with immobilized anti-CD4 and soluble AC1 remarkably enhanced cellular proliferation, as shown in Fig. 2. Control IgG3 had no additive effect on anti-CD4-stimulated T cells (data not shown). While another anti-CD4 mAb, OX-35, cooperated with anti-CD3 (G4.18) in T cell activation, AC1 did not affect the proliferative response caused by this cross-linking, nor did it induce cellular proliferation in the presence of OX-35 (data not shown).

To reduce the effects of contaminating cells, the same experiments were repeated using further purified T cells (98% pure) (Fig. 3). In two experiments, AC1 was confirmed to have a prominent enhancing effect on T cell proliferation induced by cross-linking of anti-CD3 with anti-CD4. Representative results are shown in Fig. 3. In these experiments, AC1 also augmented the cell growth elicited by immobilized anti-CD3 alone, although the increase was less than in cells co-stimulated with anti-CD3 and anti-CD4. Surprisingly, the combination of immobilized anti-CD4 and soluble AC1, neither of which is mitogenic alone, was clarified to have a strong proliferative effect. Control IgG3 had no additive effect on immobilized anti-CD4-stimulated T cells (data not shown).

Detection of AC1-Binding Protein by Western-Blotting—Western blot analysis of AC1-reactive proteins in rat T cell lysates revealed the presence of a 100 kDa protein which was detected by AC1 but not by control IgG3 (Fig. 4A, lanes 1 and 2). The interaction between AC1 and the 100 kDa protein was shown to be prevented in the presence of 2 M urea-1% Triton X-100 (Fig. 4A, lane 3). As shown in Fig. 4B, lane 1, the 100 kDa protein did not react with AC1 after sialidase treatment of the blotted proteins. Therefore, the protein appeared to be a glycoprotein containing the NeuGcα2-8NeuGcα2- sequence. The protein did not correspond to any of the abundant bands detected on staining of

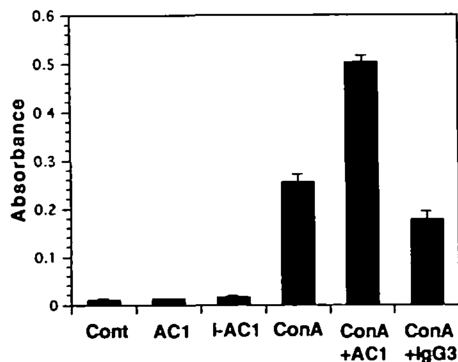

Fig. 1. **Potentiation by AC1 of the mitogenic response of T cells to Con A.** T cells prepared from rat spleen with a nylon wool column (1×10^5 cells/well) were cultured with soluble AC1 (20 μg/ml), or immobilized AC1 (i-AC1, with 100 μl of 20 μg/ml), or stimulated with Con A (10 μg/ml) in the presence or absence of soluble AC1 (20 μg/ml) or the same amount of control IgG3. After 2 days, the uptake of BrdU was measured. The values represent the means$\pm$SE for 6 wells per group.

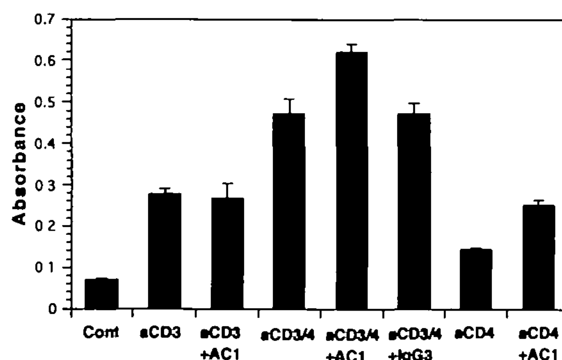

Fig. 2. **Potentiation by AC1 of the T cell proliferation induced by cross-linking of anti-CD3 and CD4 (W3/25) mAb, as well as W3/25-stimulated T cell growth.** T cells prepared with a nylon wool column were cultured at 2×10^5 cells/well with immobilized anti-CD3 and/or anti-CD4 mAb in the presence or absence of AC1 (20 μg/ml) or the same amount of control IgG3. After 2 days, cell proliferation was measured. The values represent the means$\pm$SE for 6 wells per group.

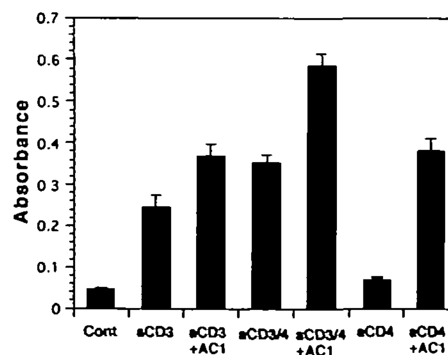

Fig. 3. **Effect of AC1 on the proliferation of purified T cells.** T cells further purified to 98% purity by an immuno-magnetic method were cultured, and then their proliferation was measured by the same method as that described in Fig. 2.

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the blotted membrane with Coomassie Brilliant Blue (data not shown).

Reactivity of AC1 with T Cells after Pronase or Sialidase Treatment of the Cells—To examine the contributions of GD1c(NeuGc,NeuGc) and the 100 kDa glycoprotein to the binding of AC1, T cells were hydrolyzed with pronase or sialidase, and then expression of the AC1 antigen was analyzed by flow cytometry. When T cells were treated with pronase, the AC1-reactive 100 kDa glycoprotein was completely absent on Western blots (Fig. 4A, lane 4). The hydrolysis of cells with 0.04% pronase also completely prevented CD4 and CD8 expression, as indicated by the flow cytometry shown in Fig. 5. There was a 40–50% reduction in AC1-positive cells (Fig. 5). Treatment of cells with 0.1% pronase gave virtually identical results (data not shown). On the other hand, treatment of T cells with *C. perfringens* sialidase rendered all the cells AC1-negative (Fig. 5). The cells also became completely AC1-negative after treatment with *V. cholera* sialidase (data not shown). Control cell preparations treated under the same conditions but without enzymes exhibited very similar staining patterns to those of non-treated cells (data not shown). These data indicated that about 50-60% of the AC1 staining is due to GD1c(NeuGc,NeuGc), the rest being due to the 100 kDa glycoprotein.

Taken together, these findings predict the existence of a unique regulatory mechanism of T cell activation in which GD1c(NeuGc,NeuGc) and/or the novel 100 kDa glycoprotein cooperate with CD4 in the interaction with ligands and the subsequent signal transduction.

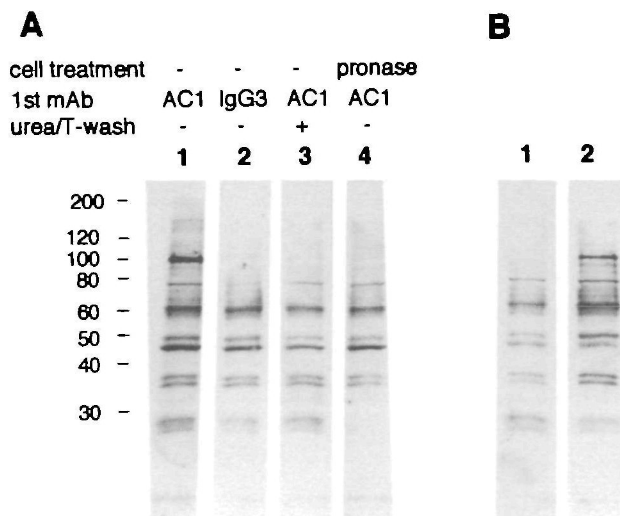

Fig. 4. **Western-blotting analysis of rat T cell lysates using AC1.** T cell lysates (7.2 μg protein/lane) were separated on 10% polyacrylamide mini-slab gels with SDS, and then electroblotted onto PVDF membranes. The AC1-binding protein was detected as described under "MATERIALS AND METHODS." (A) Lysates were prepared from untreated (lanes 1-3) or 0.04% pronase-treated (lane 4) T cells, and then analyzed using AC1 (lanes 1, 2, and 4) or control IgG3 (lane 2) as the first antibody. The membrane shown in lane 3 was washed with urea/Triton X-100 after AC1-treatment. (B) Lysates of T cells treated with (lane 1) or without (lane 2) sialidase were analyzed using AC1 as the first antibody.

DISCUSSION

The present study has demonstrated that the mAb, AC1, which recognizes the NeuGcα2-8NeuGcα- sequence, enhances the growth of activated T cells, and has a marked effect on the activation induced by the cross-linking of anti-CD3 and anti-CD4. Moreover, co-stimulation with immobilized anti-CD4 and soluble AC1, neither of which is mitogenic alone, has been shown to effectively evoke T cell activation. These interactions may represent a unique regulatory pathway of T cell activation involving CD4 and glycoconjugates with the NeuGcα2-8NeuGcα- sequence. Recent studies have clarified that the signal transmitted through CD4 plays an important role in the phenotype development of Th1 or Th2 from naive CD4 T cells upon activation (*30, 31*). As AC1 synergistically acts with CD4 in cellular activation, it can be assumed that the glycoconjugates with the NeuGcα2-8NeuGcα- sequence play a role not only in activation but also in phenotype development. While AC1 augmented the anti-CD3-activated proliferation of 98%-purified T cells (Fig. 3), it barely had an effect on the CD3-induced growth of around 85%-purified T cells

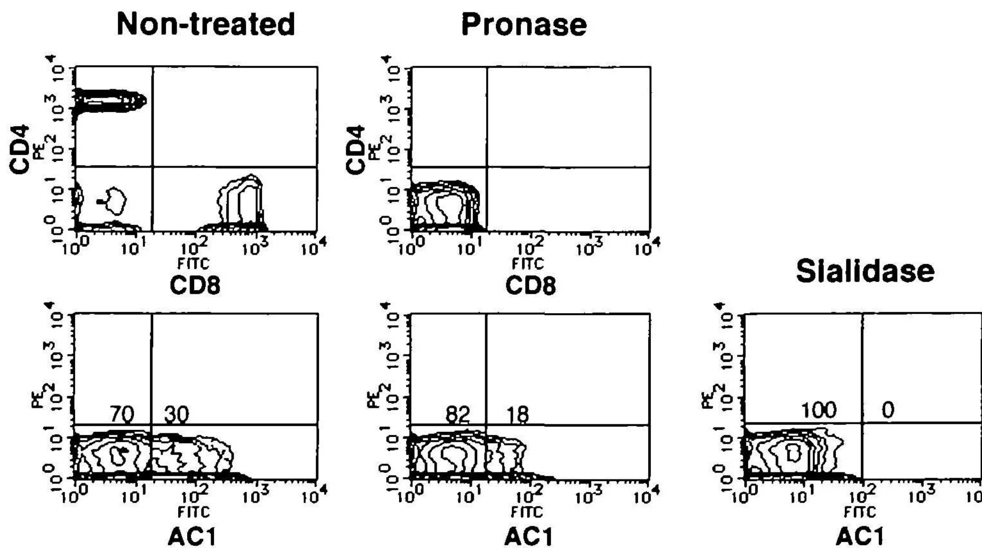

Fig. 5. **Expression of AC1-positive cells among rat T cells after pronase or sialidase treatment.** T cells were hydrolyzed with pronase (0.04%) or sialidase, and then AC1-positive cells were detected by flow cytometry using AC1 and FITC-anti IgG3 mAb (bottom figures). For control staining to set the quadrant marker, cells were stained with control IgG3 and FITC-anti IgG3. The expression of CD4 and CD8 was also assessed in non-treated and pronase-treated cells using PE-anti-CD4 and FITC-anti-CD8 (upper figures).

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(Fig. 2). Contaminating cells in the 85% preparation may inhibit the additional effect of AC1 on the anti-CD3-driven growth. Similar results were reported for anti-GD3-induced proliferation of human CD8 T cells, where CD16$^+$ NK cells selectively inhibited the growth through an unknown mechanism (*8*).

We also examined whether or not AC1 interacts with any rat T cell glycoproteins, since in a recent study, Sato *et al.* (*32*) demonstrated the occurrence of oligo-(NeuGcα2-8) chains in mammalian glycoproteins, using an anti-oligo/poly-NeuGc mAb in conjunction with chemical and biochemical methods. Using their mAb, the existence of oligo-NeuGc residues as glycoproteinaceous components was found in a number of rat tissues including spleen (*32*). In the present study, AC1 bound to a 100 kDa glycoprotein in a sialic acid-dependent manner, although the interaction was not strong enough to resist urea/Triton X-100 treatment. Moreover, both the 100 kDa glycoprotein and GD1c(NeuGc,NeuGc) were deduced to be recognized by AC1 on rat T cell membranes. To determine the roles of these two molecules in ligand recognition and signal transduction, it is necessary to further characterize the 100 kDa glycoprotein, which is presumed to contain NeuGcα2-8NeuGcα2- or oligo/polyNeuGc residues in its saccharide moiety.

The ligand for the GD1c(NeuGc,NeuGc) or NeuGcα2-8NeuGcα2- sequence has not been identified yet. A number of diverse sialic acid-binding proteins have been reported in animals, plants, and microorganisms (*33*), including immunoglobulin superfamily lectins CD22, sialoadhesin, and CD33, which are related to leukocyte functions in mammals (*34-36*). We therefore predict that there will be specific ligands for the NeuGcα2-8NeuGcα- sequence as well. It is also mandatory to characterize these ligands to clarify the physiological roles of GD1c(NeuGc,NeuGc) and the 100 kDa glycoprotein in rat T cells.

There is some intriguing evidence regarding the association of cell surface gangliosides and kinases currently emerging. The physical association of α-galactosyl derivatives of GD1b ganglioside with Src family tyrosine kinase p53/56^{lyn} and a serine kinase was demonstrated on rat basophilic leukemia RBL-2H3 cells by Minoguchi *et al.* (*37*) by coimmunoprecipitation with a mAb against the GD1b-derivatives. Subsequently, it was reported that GM1 directly binds to Trk, the high-affinity tyrosine kinase-type receptor for nerve growth factor (NGF), and strongly enhances neurite outgrowth and neurofilament expression in rat PC12 cells (*38*). In recent studies, the association of GD3 with Lyn in rat brain (*39*), and GM3 with c-Src and the low molecular weight G-protein, Rho, in human B16 melanomas (*40*) were observed in the DIG domain. Our findings here further emphasize the problem of how gangliosides and glycoproteins having common oligasaccharide chains, like GD1c(NeuGc,NeuGc) and the 100 kDa glycoprotein, share roles in ligand recognition and signal transduction.

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