A Cytotoxic Antibody Recognizing Lacto-N-fucopentaose I (LNFP I) on Human Induced Pluripotent Stem (hiPS) Cells*

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Background: Carbohydrate epitopes are often used as markers for characterization of hiPS cells.
Results: A mouse IgG1 antibody (R-17F) was raised using hiPS cells as an antigen.
Conclusion: R-17F recognizes lacto-N-fucopentaose I on glycolipid and exhibits a cytotoxic effect on hiPS/ES cells.
Significance: R-17F may be beneficial for safer regenerative medicine by eliminating residual undifferentiated hiPS/ES cells, which are a risk factor for carcinogenesis.

We have generated a mouse monoclonal antibody (R-17F, IgG1 subtype) specific to human induced pluripotent stem (hiPS)/embryonic stem (ES) cells by using a hiPS cell line as an antigen. Triple-color confocal immunostaining images of hiPS cells with R-17F indicated that the R-17F epitope was expressed exclusively and intensively on the cell membranes of hiPS cells and co-localized partially with those of SSEA-4 and SSEA-3. Lines of evidence suggested that the predominant part of the R-17F epitope was a glycolipid. Upon TLC blot of total lipid extracts from hiPS cells with R-17F, one major R-17F-positive band was observed at a slow migration position close to that of anti-blood group H1(O) antigen. MALDI-TOF-MS and MS analyses of the purified antigen indicated that the presumptive structure of the R-17F antigen was Fuc-Hex-HexNAc-Hex-Hex-Cer. Glycan microarray analysis involving 13 different synthetic oligosaccharides indicated that R-17F bound selectively to LNFP I (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc). A critical role of the terminal Fucα1-2 residue was confirmed by the selective disappearance of R-17F binding to the purified antigen upon α1-2 fucosidase digestion. Most interestingly, R-17F, when added to hiPS/ES cell suspensions, exhibited potent dose-dependent cytotoxicity. The cytotoxic effect was augmented markedly upon the addition of the secondary antibody (goat anti-mouse IgG1 antibody). R-17F may be beneficial for safer regenerative medicine by eliminating residual undifferentiated hiPS cells in hiPS-derived regenerative tissues, which are considered to be a strong risk factor for carcinogenesis.

Because of their pluripotency and indefinite growth capability, hiPS/ES cells exhibit great potential both as a resource for functional studies of early human development and as a renewable source of cells for use in regenerative medicine and transplantation. To make use of these cells, the expression and chemical structural complexity of their cell surface antigens have to be defined. For this purpose, the identification of novel cell surface markers is highly advantageous. Among others, carbohydrate-recognizing antibodies have been used as pivotal tools for monitoring the changes of cell surface glycan structures and also for identification of specific glycans on pluripotent stem cells with high sensitivity and strict specificity (1–3). These marker antibodies for hiPS/ES cells include TRA-1-60 (4), TRA-1-81 (4), GCTM29 (5, 6), and GCTM343 (5), which recognize keratan sulfate, and SSEA-3 (7) and SSEA-4 (8, 9), which recognize Galβ1-3GalNAcβ1-3Galβ1-4Glcβ1-1-Cer and NeuAcα2-3Galβ1-3GalNAcβ1-3Gal(α1-4)Galβ1-2glc(1-4)Galβ1-1-Cer, respectively. However, the abbreviations used are: hiPS, human induced pluripotent stem; ES, embryonic stem; HES, human ES; 7-AAD, 7-amino-actinomycin D; ADHP, N-a-monoaceetyl-N-(9-anthrecnethyl) methyl-1,2-dihexadecyl-sn-glycer-3-phosphoethanolamine; Cer, ceramide; QIT, quadrupole ion trap; Fuc, fucose; Hex, hexose; HexNAc, N-acetylhexosamine; EC, embryonal carcinoma; hEC, human EC; HPTLC, high performance thin layer chromatography; NGL, neoglycolipid; PFA, paraformaldehyde; LNFP, lacto-N-fucopentaose; DPDP, 1-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol-HCl; MBL, mannann-binding lectin; MPβ, mannann-binding protein; JCRB, Japanese Collection of Research Bioresources. All of the sugar residues have the D-configuration except fucose, which has the L-configuration.

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these antibodies have the common potential drawback that they do not distinguish between hiPS/ES cells and human embryonal carcinoma (hEC) cells. hiPS/ES cells and hEC cells have some common properties, but hEC cells are the stem cells of teratocarcinomas and the malignant counterparts of ES cells derived from the inner cell mass of blastocyst-stage embryos. Therefore, it would be highly beneficial for basic research and also for practical purposes if we had antibodies that distinguish hiPS/ES cells from hEC cells.

Recently, we generated two hybridomas using a hiPS cell line (Tic) (10, 11) as an antigen. These hybridomas produced antibodies that specifically bound to hiPS/ES cells but exhibited little or no binding to hEC cells (12). Interestingly, both of these antibodies were found to be carbohydrate-recognizing antibodies of the IgG1 subtype. One of these antibodies, designated as R-10G, gave a single diffuse band in a high molecular mass region over 250 kDa on Western blot after SDS-PAGE. The following studies indicated that R-10G recognizes a type of keratan sulfate lacking oversulfated structures expressed on podocalyxin molecules on hiPS/ES cells (12). This specificity of R-10G is clearly distinct from those of commercially available anti-keratan sulfate antibodies, including 5D4, which recognizes a highly sulfated keratan sulfate (13), and is rather similar to those of anti-TRA-1-60 (4) and anti-TRA-1-81 (4) antibodies in that all of these antibodies interact with keratan sulfate on hiPS/ES cells.

In the present study, we have characterized the second antibody, R-17F, a subclone of hybridoma 17 that was described in our previous paper (12). Upon immunohistochemical assaying involving flow cytometry and laser confocal scanning microscopy, R-17F exhibited stronger interactions with hiPS/ES cells than R-10G. However, on Western blot after SDS-PAGE, R-17F gave only a faint band, suggesting that the predominant portion of the R-17F antigen is a lipid-linked molecule. In addition, R-17F was found to exhibit a cytotoxic activity toward hiPS/ES cells, suggesting that R-17F can possibly eliminate residual undifferentiated hiPS cells in hiPS-derived regenerative tissues, which are considered to be a strong risk factor for carcinogenesis (3, 14). Thus, R-17F should be useful as a molecular probe for investigating the roles of glycans on the surface of hiPS/hES cells and also beneficial as a practical tool for the evaluation and standardization of hiPS cells toward safer regenerative medicine.

Experimental Procedures

Materials

**Antibodies**—Anti-TRA-1-60 (clone TRA-1-60, mouse IgM), anti-TRA-1-81 (clone TRA-1-81, mouse IgM), anti-SSEA-4 (clone MC813, mouse IgG3), and anti-Lewis a (clone 7LE, mouse IgG1) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). Anti-SSEA-3 (clone MC-631, rat IgM) and anti-Lewis x/SSEA-1 (clone MC-480, mouse IgM) were obtained from R&D Systems (Minneapolis, MN). Anti-blood group H1(O) antigen (anti-H type 1) (clone 17-206, mouse IgG3, ascites) was obtained from Abcam (Cambridge, UK). The IgG3 antibody content of the ascites was estimated with a mouse immunoglobulin quantification kit (FastELISA kit) obtained from RD Biotech (Besançon, France) according to the manufacturer’s protocol using an IgG3 antibody (anti-sialyl Lewis a monoclonal antibody, clone 1H4, obtained from Seikagaku Biobusiness (Tokyo, Japan)) as a standard. Anti-blood group H2(O) antigen (anti-H type 2) (clone BRIC231, mouse IgG1) was obtained from AbD Serotec (Oxford, UK). Anti-globo H (clone MBr1, mouse IgM) was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY). Mouse IgG1 isotype control antibody (clone 15H6, IgG1κ) was obtained from Beckman Coulter, Inc. (Fullerton, CA), and anti-human mannan-binding lectin/protein (anti-MBL/MBP) (clone HYB 131-01, mouse IgG1) was provided by BioPorto Diagnostics (Gentofte, Denmark). Anti-human iPS/ES antibodies R-10G (mouse IgG1) and R-17F (mouse IgG1) were prepared as described previously (12). The following fluorophore-conjugated antibodies were used as secondary antibodies for visualizing the (primary) antibodies described above. Alexa Fluor 647-conjugated chicken anti-mouse IgG, Alexa Fluor 488-conjugated goat anti-mouse IgG, Alexa Fluor 488-conjugated goat anti-mouse IgG1, Alexa Fluor 488-conjugated goat anti-mouse IgG3, and Alexa Fluor 488-conjugated goat anti-mouse IgM were obtained from Invitrogen. Alexa Fluor 555- conjugated goat anti-rat IgM was obtained from Abcam. DyLight 649-conjugated goat anti-mouse IgG3 (H+L) polyclonal antibody preadsorbed (DyLight 649) was obtained from BioLegend (San Diego, CA). HRP-conjugated rabbit anti-mouse Ig was obtained from Agilent Technology (Santa Clara, CA), HRP-conjugated goat anti-rat IgM was obtained from Thermo Fisher Scientific. Biotinylated goat anti-mouse IgG (H+L) was obtained from KPL, Inc. (Gaithersburg, MD). Biotinylated goat anti-rat IgM was obtained from Abcam. Non-labeled goat anti-mouse IgG1 was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL).

**Synthetic Oligosaccharides**—Lacto-N-tetraose, lacto-N-fucopentaose I (LNFP I), lacto-N-fucohexaose I (Lewis b), and lacto-N-neotetraose were obtained from ProZyme (Hayward, CA). Lacto-N-fucopentaose II (LNFP II, Lewis a) was obtained from Seikagaku Biobusiness. Blood group H antigen pentaoe type 2, blood group H antigen tetraose type 1, blood group H antigen tetraose type 2, blood group H antigen tetraose type 4, blood group H antigen triose type 1, blood group H antigen triose type 2, and blood group H antigen triose type 5 were obtained from Elichyl (Crolles, France). Gb5/globopentaose and biotinylated lacto-N-fucopentaose I were provided by Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

**Cells and Cell Culture**—Human iPSC cell lines Tic (JCRB1331) and Squizzy (JCRB1329) were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank, National Institutes of Biomedical Innovation, Health and Nutrition (Osaka, Japan). These cells were generated from MRC-5, a human embryonic lung fibroblast cell line (15), by transfection of four defined factors (Oct3/4, Sox2, KLF4, and c-Myc) (16). 201B7 was provided by the Center for iPS Cell Research and Application, Kyoto University (Kyoto, Japan). Human ES cell line H9 (WA09) was obtained from the Wisconsin International Stem Cell Bank, WiCell (Madison, WI), and human ES cell line KhES-3 was provided by the Institute for Frontier Medical Sciences, Kyoto University (Kyoto, Japan). Tic cells were maintained in knock-out serum replacement-based medium as...
described previously (12) (hiPS culture medium) on mouse embryonic fibroblasts in 25-cm² flasks (Corning, Inc.) at 37 °C/5% CO₂ and transferred to a growth factor-defined serum-free culture medium, hESF9 (17), for 2 days before harvest as described previously (12). The human embryonal carcinoma cell line 2102Ep was a generous gift from Prof. Peter Andrews (University of Sheffield) to the National Institute of Biomedical Innovation. NCR-G3 (JCRB1168) was obtained from JCRB. MRC-5 (JCRB9008) was obtained from JCRB. H9, KhES-3, and 2102Ep were cultured only at the National Institute of Biomedical Innovation following the Guidelines for Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Furthermore, the Independent Ethics Committee of the National Institute of Biomedical Innovation approved the study.

**Generation of Monoclonal Antibody R-17F**

Immunization of mice with a hiPS cell line (Tic) and subsequent screening of hybridomas were described in our previous work (12). Briefly, freeze-thawed Tic cells in phosphate-buffered saline (PBS) were mixed with Freund’s complete adjuvant and used to immunize C57BL/6 mice intraperitoneally or subcutaneously. Prime screening of a total of 960 hybridomas revealed 29 hybridomas that exhibited reactivity to surface antigens on Tic cells. For these hybridomas, a second screening was carried out to exclude hybridomas that exhibited reactivity to a human EC cell line, 2102Ep, and a human fibroblast cell line, MRC-5. By this procedure, two hybridomas, numbers 10 and 17, were obtained. Clone R-10G was a subclone obtained from hybridoma 10 as described previously (12). R-17F was a subclone obtained in this study from hybridoma 17.

**Immunocytochemistry**

Imaging analysis, flow cytometry, and laser confocal scanning microscopy were carried out by the indirect immunofluorescence technique using the corresponding subtype-specific fluorophore-conjugated secondary antibodies except for double staining of R-10G and R-17F. In the latter case, the direct immunofluorescence technique was used because R-10G and R-17F staining of R-10G and R-17F. In the latter case, the direct immunofluorescence technique were carried out by the indirect immunofluorescence microscopy.

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**Imaging Analysis with an InCell Analyzer**—Cells seeded in 24-well plates were fixed in 4% paraformaldehyde (PFA) and then incubated with primary antibodies (R-17F (10 μg/ml), R-10G (10 μg/ml), TRA-1-60 (2 μg/ml), TRA-1-81 (2 μg/ml), SSEA-3 (5 μg/ml), and SSEA-4 (2 μg/ml)) at 4 °C overnight, followed by incubation with Alexa Fluor 647-conjugated chicken anti-mouse IgG antibodies (far red). The cells were imaged using an InCell analyzer 2000 (GE Healthcare, Buckinghamshire, UK) and quantitated using Developer Toolbox version 1.8 (12).

**Laser Confocal Scanning Microscopy**—Cells were seeded with mouse embryonic fibroblasts onto a gelatin-coated 4-well plastic chamber (Millipore EZ slides (Millipore, BillERICA, MA)). After a 3–5-day culture in the hiPS culture medium, cells were fixed in 4% PFA and washed with 1% BSA-PBS. Then the cells were incubated with each primary antibody (R-17F, SSEA-3, and SSEA-4) at 4 °C overnight. After washing, the cells were incubated with an appropriate secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse IgG1 antibody (green) for R-17F, Alexa Fluor 555-conjugated goat anti-rat IgM antibody (orange) for SSEA-3, and DyLight 649-conjugated goat anti-mouse IgG3 (H+L) polyclonal antibody, preadsorbed (blue) for SSEA-4) at room temperature for 1 h for visualization. For double staining with SSEA-3 and SSEA-4 antibodies and triple staining with R-17F, SSEA-3, and SSEA-4 antibodies, cells were co-incubated with the respective pairs of primary antibodies, followed by incubation with the corresponding secondary antibodies. For double staining of R-17F and R-10G (both IgG1 subtype), R-17F and R-10G (40 μg for each) were directly conjugated with Alexa Fluor 555 (orange) and Alexa Fluor 488 (green), respectively, using an APEX antibody labeling kit according to the manufacturer’s protocol (Thermo Fisher Scientific). Tic cells in a plastic chamber were fixed in 4% PFA and then stained with the fluorophore-conjugated R-17F and R-10G (8 μg/150 μl/well for each) at 4 °C overnight. For nuclear and chromosome counterstaining, the cells were fixed with 0.1% Triton X-100, 4% PFA at room temperature for 10 min, followed by staining with TO-PRO3 (1:500 in PBS; Invitrogen), and monitored under a confocal laser-scanning microscope (Olympus, Tokyo, Japan).

**Flow Cytometry**—hiPS/ES cells (0.5–1 × 10⁶ cells), which had been harvested either by treatment with 0.1% EDTA-4Na/PBS at 37 °C for 2 min or by incubation in 0.25% trypsin/EDTA (Invitrogen) at 37 °C for 5–15 min, were incubated with each primary antibody (R-17F (2 μg/ml), R-10G (10 μg/ml), SSEA-4 (10 μg/ml), and TRA-1-60 (2 μg/ml)) in 1% BSA-PBS (100 μl) at 4 °C for 45 min. After washing with 1% BSA-PBS, the cells were incubated with a secondary antibody (100 μl) (Alexa Fluor 488-conjugated goat anti-mouse IgG1 (green) for R-17F and R-10G, Alexa Fluor 488-conjugated goat anti-mouse IgG3 for SSEA-4, and Alexa Fluor 488-conjugated goat anti-mouse IgM for TRA-1-60) at 4 °C for 1 h in a light-protected state. After washing with 1% BSA-PBS, cell pellets were resuspended in the same buffer (1 ml), filtered with 5-ml cell strainer round-bottom cap tubes (BD Falcon Biosciences, Lexington, TN), and then analyzed with a BD FACSCalibur (BD Biosciences) or BD LSRII (BD Biosciences) analyzer.

**SDS-PAGE and Western Blot**

SDS-PAGE and Western blot were carried out as described previously (12). Tic cell lysates (10 μg of protein) were resolved by electrophoresis on 4–15% gradient SDS-polyacrylamide gels under reducing conditions. The resolved proteins were transferred to Immobilon transfer membranes (Merck Millipore) and then incubated with R-10G (3 μg/ml) at 4 °C overnight, followed by incubation with Alexa Fluor 647-conjugated chicken anti-mouse IgG antibodies (far red). The cells were imaged using an InCell analyzer 2000 (GE Healthcare, Buckinghamshire, UK) and quantitated using Developer Toolbox version 1.8 (12).

**Flow Cytometry for Cytotoxic Activity of R-17F**

hiPS/ES cells (1 × 10⁵ cells), which had been washed with 1% BSA-PBS, were incubated with R-17F in 1% BSA-PBS (100 μl)
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at 4 °C for 45 min. After washing by centrifugation, the pellets were suspended in 1.0 ml of the same buffer, followed by the addition of 5 μl of 7-amino-actinomycin D (7-AAD) Viability Staining Solution (50 μg/ml; eBioscience Inc., San Diego, CA) as a viability probe. After incubation for 5 min, the cells were analyzed by flow cytometry.

Effect of a Secondary Antibody on the R-17F-induced Cytotoxicity toward hiPS Cells

Tic cells (1.7 × 10^5 cells) were incubated with 1.0 μg of R-17F in 1% BSA-PBS (100 μl) at 4 °C for 45 min. After washing by centrifugation, the pellets were incubated in 100 μl of the same buffer containing increasing amounts (0.025, 0.05, and 0.1 μg) of non-labeled goat anti-mouse IgG1 at 4 °C for 30 min. After incubation with 5 μl of 7-AAD, the cells were analyzed by flow cytometry.

Effect of R-17F on the Growth of the Tic Cell Colonies in Culture

Tic cells were cultured in plastic chambers with the iPS culture medium for 2 days until small sized colonies were formed. Then the medium was changed to hESF9 medium (200 μl) containing 100 μg of R-17F or R-10G, followed by incubation for 3 days. At every 24 h, cell growth was monitored under a phase-contrast microscope. The control contained no antibody.

Effect of a Secondary Antibody on the R-17F-induced Cytotoxicity toward tic Cells

After a 5-day culture in the hiPS culture medium, Tic cells (3.0 × 10^6 cells) were cultured in freshly prepared cultured medium containing 20 μM D-PDMP (a glucosylceramidase synthase inhibitor (18); Matreya LLC, Pleasant Gap, PA) for 96 h. Then the cells were harvested, and the epitope profiles were analyzed by flow cytometry after incubation with each primary antibody (R-17F, SSEA-4, and TRA-1-60), followed by treatment with the corresponding secondary antibodies (Alexa Fluor 488-conjugated goat anti-mouse IgG1, IgG3, and IgM antibodies).

Trypsin Digestion

Tic cells (1 × 10^6 cells), which had been harvested with 0.1% EDTA-PBS, were suspended in 1% BSA-PBS (1 ml). After washing by centrifugation, the pellets were incubated in 0.25% trypsin/EDTA-PBS (250 μl) at 37 °C for 15 min under shaking. The trypsin digestion was stopped by the addition of soybean trypsin inhibitor (1.0 mg/ml; Sigma-Aldrich). The cells were analyzed for epitope profiles by flow cytometry after incubation with each primary antibody (R-17F, R-10G, SSEA-4, and TRA-1-60), followed by treatment with the corresponding Alexa Fluor-conjugated secondary antibodies (Alexa Fluor 488-conjugated goat anti-mouse IgG1, IgG3, and IgM antibodies).

Isolation of R-17F Lipid Antigen by TLC—

Tic cells (1.7 × 10^5 cells) were cultured in freshly prepared cultured medium containing 100 μg of R-17F in 1% BSA-PBS (100 μl) at 4 °C for 45 min. After washing by centrifugation, the pellets were incubated in 1% BSA-PBS (1 ml). After washing by centrifugation, the pellets were incubated in 100 μl of the same buffer containing increasing amounts (0.025, 0.05, and 0.1 μg) of non-labeled goat anti-mouse IgG1 at 4 °C for 30 min. After incubation with 5 μl of 7-AAD, the cells were analyzed by flow cytometry.

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Isolation of R-17F Antigens from hiPS Cells

Preparation of Total Lipids—Tic cells (4.5 × 10^7 cells) were suspended in 4.5 ml of CHCl₃/MeOH/H₂O (1:2:0.8, v/v/v) and sonicated in a sonic bath for 5 min, and then incubated at 37 °C for 1 h with shaking. After centrifugation at 1000 × g for 10 min, the supernatant was withdrawn and then transferred to a conical bottom glass centrifuge tube (first extract). To each pellet, 3 ml of CHCl₃/MeOH/H₂O (1:2:0.8, v/v/v) was added, and the suspension was incubated at 37 °C for 2 h with shaking. After centrifugation, the supernatant was withdrawn and combined with the first extract (total extract). This procedure was repeated once more for an equal number of Tic cells (4.5 × 10^7 cells), and the pooled extracts were combined (total lipids). The total lipids were dissolved in 400 μl of CHCl₃/MeOH/H₂O (65:25:40, v/v/v) and stored at 4 °C.

TLC Analysis—Total lipids corresponding to 6.0 × 10^5 cells were applied to an HPTLC silica gel 60 aluminum sheet (10 × 10 cm, Merck Millipore) using a sample applicator (Linomat 5, CAMAG, Muttenz, Switzerland). The TLC plate was developed with a developing solvent, CHCl₃/MeOH/H₂O (65:25:4, v/v/v), in a developing chamber (CAMAG) until the solvent front reached 6 cm above the origin. In some experiments, to improve the separation efficiency, the dried plate was redeveloped with the same developing solvent until the solvent front reached 8.5 cm above the origin, followed by third development with the same solvent (the three-time TLC development method). After drying and spraying the HPTLC plate with primuline reagent (0.001% primuline in acetone/H₂O (4:1, v/v)), all lipids, including glycosphingolipids, were visualized using a UV transilluminator (365 nm) (DTB-20MP, ATTO Co., Tokyo, Japan).

TLC Immunoblot (Far-Eastern Blot)—The HPTLC plates were dipped in a blot solvent (isopropyl alcohol, 0.2% CaCl₂, methanol (40:20:7, v/v/v)) for 15 s and then placed on a glass fiber filter (ATTO Co.), followed by covering with a PVDF membrane (Clear Blot Membrane-P, 0.2 mm, ATTO Co.), a PTEE membrane (ATTO Co.), and a glass fiber filter according to the method described previously (19, 20). This assembly was transferred to a TLC thermal blotter (ATTO Co.) and then heated at 180 °C for 30 s at a pressure level of 8. The PVDF membranes were washed with H₂O three times for 5 min and then with 3% BSA-PBS for 1 h, followed by incubation with R-17F (1 μg/ml) or another primary antibody in 1% BSA-PBS overnight at 4 °C. After washing with PBS, each membrane was incubated with appropriate biotinylated secondary antibodies (e.g. biotinylated goat anti-mouse IgG (H+L) antibodies (0.1 μg/ml) for R-17F) for 1 h at room temperature, followed by streptavidin-HRP (55 ng/ml; Pierce) for 1 h at room temperature. Bands were developed using SuperSignal West Pico chemiluminescent substrate (Pierce) and quantified with a LuminolImage Analyzer, Las 4000 mini.

Isolation of R-17F Lipid Antigens by TLC—The total lipids corresponding to 4.0 × 10^7 cells in 180 μl of CHCl₃/MeOH/H₂O (65:25:4, v/v/v) were applied to an HPTLC silica gel 60 F254 MS-grade glass plate (10 × 10 cm; Merck) as a 66-mm width spot in the middle of the origin and then developed by the three-time TLC development method described above. Both the right and left ends (3-mm width) of the sample lane were cut off and then subjected to TLC blot and immunostaining with R-17F. Then silica gel corresponding to the visualized R-17F band was scraped off, and the antigens were extracted with 3 ml of CHCl₃/MeOH/H₂O (65:25:4, v/v/v) under sonication for 3 min at room temperature, followed by overnight incubation at 4 °C. The extract was applied to a Glass SPE cartridge (GL Science Inc., Tokyo, Japan), and the filtrate was collected in a con-
ical bottom glass centrifuge tube and dried under a stream of N₂ gas. The dried materials were dissolved in 150 μl of CHCl₃/MeOH/H₂O (65:25:4, v/v/v) and stored at 4 °C (purified R-17F antigen).

**MALDI-TOF MS Analysis of the Isolated R-17F Lipid Antigen**

MS analysis was performed with a MALDI-QIT-TOF mass spectrometer (AXIMA Resonance; Shimadzu/Kratos) in the positive ion mode. Ionization was performed with a 337-nm pulsed N₂ laser. Helium and argon gas were used for ion cooling and collision-induced dissociation, respectively. For sample preparation, a 1-μl aliquot of the purified R-17F antigen solution was deposited onto the MALDI target plate with a glass capillary (Hirschmann Laborgeräte), and 0.5 μl of a 2.5-dihydroxybenzoic acid (Shimadzu GLC) solution (5 mg/ml in 50% acetonitrile including 0.05% trifluoroacetic acid) was immediately added to the analyte. The solution on the target plate was completely dried, and then the plate was introduced into the instrument. The instrument was calibrated using an external standard peptide mixture of angiotensin II ([M + H]⁺, 1046.54) and human ACTH peptide fragment ([M + H]⁺, 2465.20). All of the MS signals of the analyte were assigned as sodium-adducted ions.

**Glycan Microarray Analysis Using Fluorescent Neoglycolipids (NGLs)**

Glycan microarray analysis was carried out as described by Fukui et al. (21). Fluorescence-labeled NGLs were prepared by conjugation of oligosaccharides with N-aminoacetyl-N-(9-anthracenyl methyl)-1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine (ADHP) (ADHP-conjugated NGLs) according to the method of Stoll et al. (22). After conjugation at 60 °C for 3 days in the presence of tetrabutylammonium cyanoborohydride, the NGLs produced were purified by HPTLC, dissolved in 400 μl of CHCl₃/MeOH/H₂O (25:25:8, v/v/v), and stored at -20 °C (purified R-17F Blot Transfer Medium Purple Nitrocellulose, 0.45 wide spot) was printed onto a nitrocellulose membrane (Trans-Blot Transfer Medium Purple Nitrocellulose, 0.45 μm; Bio-Rad) with a Linomat V sample applicator. After blocking with 4% BSA-PBS overnight at 4 °C, the nitrocellulose membranes were overlaid with 40 μl/cm² R-17F (2 μg/ml in 0.1% BSA-PBS) and incubated for 2 h at room temperature. After washing with PBS, the membranes were incubated with 40 μl/cm² of HRP-conjugated rabbit anti-mouse Ig and 40 ng/ml HRP-conjugated goat anti-rat IgM corresponding to the isotype of the primary antibody used.

**Fucosidase Digestion of the R-17F Antigen**

Digestion of the purified R-17F antigen (corresponding to 4.6 × 10⁶ cells) with α1–2 fucosidase (4.7 milliunits; New England BioLabs, Inc., Ipswich, MA) and with α1–3/4 fucosidase (12.5 microunits; Takara Bio Inc., Shiga, Japan) was carried out at 37 °C overnight as described previously (12) except for the addition of sodium taurocholate (2 mg/ml) to the incubation medium. The digests were subjected to TLC, followed by Far-Eastern blot as described above.

**Comparison of Tic Cell Death Mediated by R-17F and by UV Irradiation-induced Apoptosis**

**Confocal Scanning Microscopy**—For UV irradiation-induced apoptosis, Tic cells cultured in Petri dishes containing the hiPS culture medium (1 ml) were irradiated with SpectroLinker XL-1000 UV cross-linker (Spectronics Corp. Westbury, NY) at 2.0 J/cm² and then cultured in the hiPS culture medium at 37 °C/5% CO₂ for 24 h. After washing in PBS, the collected cells were incubated with SYTOX Green nucleic acid stain (1 μM in 1% BSA-PBS; Life Technologies, Inc.) at room temperature for 30 min. After washing in 1% BSA-PBS, the cells were transferred to chamber slides and fixed with 0.5 ml of 4% PFA for 30 min. Then the cells were incubated with TO-PRO-3 for 1 h and visualized under a laser confocal scanning microscope. For R-17F-mediated cell death, Tic cells in single cell suspension in 1% BSA-PBS were incubated with R-17F (10 μg/100 μl of medium) at 4 °C for 45 min. After washing, the cells were incubated with SYTOX Green nucleic acid stain and then subjected to TO-PRO-3 staining, followed by visualization under a laser confocal scanning microscope as described above.

**Apoptosis Ladder Detection Assay**—Tic cells in a single cell suspension in 1% BSA-PBS (1 × 10⁵ cells) were either incubated with R-17F (10 μg/100 μl 1% BSA-PBS) at 4 and 37 °C for 1–5 h or submitted to UV irradiation as described above. After washing with 1% BSA-PBS, the cells were treated with an Apoptosis Ladder Detection kit Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan), which involves DNA extraction, 1.5% agarose gel electrophoresis in tris-acetate-EDTA buffer, and DNA staining with SYBR Green, followed by viewing with a Lumino-Image Analyzer, Las 4000 mini.

**Transmission Electron Microscopic Analysis**—Tic cells in a single cell suspension in 1% BSA-PBS (1 × 10⁵ cells) were either incubated with R-17F (100 μg/ml in 1% BSA-PBS) at 4 °C for 45 min or submitted to UV irradiation as described above. After washing in PBS, the precipitated cells were prefixed with 2% glutaraldehyde and 2% PFA in 0.1 M phosphate buffer, pH 7.4, left on ice for 2 h, and then embedded in 1% agarose on ice for 30 min. After washing with phosphate buffer, the samples were postfixed with 1% OsO₄ on ice for 2 h and left in phosphate buffer at 4 °C overnight. Then the samples were dehydrated with 50, 70, 80, 95, and 100% ethanol, which was replaced sequentially with ethanol/QY-1 (1:1), QY-1, and QY-1/EPON (1:1) for 7 h and QY-1/EPON (1:3) overnight. Then the samples were thermopolymerized, and ultrathin sections were cut to the thickness of 60 nm using a Leica UC6 ultramicrotome, doubly stained with uranyl acetate and lead citrate, and then observed under an electron microscope (H-7650, Hitachi Ltd., Tokyo, Japan).
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Results

Binding Properties of R-17F toward hiPS/ES/EC Cells—We examined the binding activity of R-17F toward several hiPS/ES/EC cell lines using an InCell analyzer 2000. R-17F bound very effectively to three different hiPS cell lines (Tic cells (71 ± 7%), mean percentage binding in four experiments ± S.D.), 201B7 cells (100 ± 0%), and Squeaky cells (94 ± 7%)) and also to two human ES cell lines (H9 cells (86 ± 6%) and KhES-3 cells (99 ± 1%) (data not shown)). Under the same conditions, R-10G, TRA-1-81, TRA-1-60, SSEA-3, and SSEA-4 antibodies also exhibited high binding activities of 64 – 88%, 96 – 98%, 94 – 99%, 64 – 72%, and 85 – 99%, respectively, toward these five types of hiPS/ES cells, as shown in our previous work (12). R-17F did not bind to an EC cell line, 2102Ep, significantly (5 ± 3%), as was expected from the screening strategy described above. R-17F was determined to be a member of the IgG1 subtype family.

Further evidence indicating the high potential of R-17F as a marker antibody for undifferentiated hiPS was provided by flow cytometry of R-17F binding to Tic cells. As shown in Fig. 1A, upon incubation of R-17F with Tic cells at 4 °C for 45 min, a sharp symmetrical peak was observed with a high level of fluorescence. This profile was similar to that of TRA-1-60, the most commonly used hiPS/ES marker antibody. R-10G gave a broad and low peak, reflecting considerable heterogeneity in the R-10G epitope distribution between individual hiPS cells (12). SSEA-4 showed a profile similar to that of R-10G.

As shown in Fig. 1B, binding of R-17F and R-10G to Tic cells was investigated by laser confocal scanning microscopy using Alexa Fluor 488-conjugated R-10G (green) and Alexa Fluor 555-conjugated R-17F (orange) in combination with DNA staining (TO-PRO3, blue) as a control. R-17F stained almost all of the cells in the colonies in orange (a), which turned violet in the R-17F/TO-PRO3-merged images (d), indicating that most of the hiPS cells in the colonies expressed the R-17F epitope. In contrast, R-10G stained the central area of the colonies strongly but the peripheral areas only weakly (b and e). Upon double staining with R-17F and R-10G (f), most of the cells were colored orange (R-17F only) or yellow (R-17F/R-10G, mixed colors), and a few cells were colored green (R-10G only), indicating that the R-17F epitope was expressed almost ubiquitously all over the hiPS cells.

Interestingly, as shown in Fig. 1C, upon SDS-PAGE/Western blot of Tic cell lysates, R-10G gave a very strong band corresponding to >300 kDa, whereas R-17F gave only a very faint band at around 250 kDa when equal amounts of R-10G and R-17F antibodies were used. This finding was apparently inconsistent with the extremely strong cell staining capability of R-17F, as described above. This inconsistency may be explained by assuming that the R-17F epitope is a lipid-linked molecule, in contrast to the R-10G epitope, which is a protein-linked glycosaminoglycan (keratan sulfate), as revealed in our previous study (12).

Then we examined the localization of the R-17F epitope on hiPS (Tic) cells under a laser confocal scanning microscope in comparison with glycolipid (globosides)-recognizing hiPS/ES marker antibodies, SSEA-3 and SSEA-4. As shown in Fig. 1D (a), R-17F evenly stained the entire surface of the cell membranes, but no visible staining was observed in the cytoplasm. SSEA-3 (b) and SSEA-4 (c) antibodies also stained the cells on the cell membranes, but the staining was not quite as even as that with R-17F. Upon merging the staining images with the SSEA-3 and SSEA-4 antibodies, substantial areas of epitopes appeared to be co-localized (d), this being consistent with their close structural relationship as globosides. Interestingly, in the triple merged image of R-17F, SSEA-3, and SSEA-4 staining (e), a significant portion of the cell membrane network turned white in color, suggesting that these three epitopes were co-localized in comparable numbers in close vicinity on the cell membranes. However, there were also a significant number of cells that were stained green, indicating that the R-17F epitope is distinct from SSEA-3 and SSEA-4 and that the R-17F epitope appears to be a more ubiquitously distributed constituent of hiPS/ES cell membranes.

In order to examine the association of the R-17F antigen with a membrane lipid, the trypsin sensitivity of the R-17F epitope was examined. As shown in Fig. 1E (a–d), upon treatment of Tic cells with 0.25% trypsin at 4 °C for 15 min, the fluorescent levels of protein-linked epitopes, R-10G epitope (b) and TRA-1-60 (d), decreased dramatically (60.4 and 45.8%, respectively, as calculated for the median values of the peaks). On the other hand, the fluorescent level of a lipid-linked epitope, SSEA-4 (c), increased significantly (39.4%) for some unknown reason, and that of the R-17F epitope (a) also increased slightly (3.2%). These results suggested a membrane lipid-linked nature of the R-17F epitope. In the next experiments, the effect of a glycosylceramide synthase inhibitor, d-PDMP (18), on the fluorescent level of the R-17F epitope was examined. Tic cells, which had been cultured with or without d-PDMP for 96 h, were analyzed by flow cytometry. As shown in Fig. 1E (e–g), upon d-PDMP treatment, the expression of the TRA-1-60 epitope (glycoprotein) did not change as expected but somehow increased slightly (6.5%) (e). On the other hand, the expression of SSEA-4 (a cell membrane glycolipid) decreased dramatically (72.1%) (f), and that of the R-17F epitope was similarly suppressed (56.7%) (g). Assuming that the levels of fluorescence correlated with the number of epitopes expressed on the surface of Tic cells, these results suggested that the predominant part of the R-17F epitope on the Tic cell is a glycolipid.

Isolation and Characterization of the R-17F Antigen from Tic Cells—On the basis of the above results, we tried to isolate the R-17F antigen from Tic cells. First, we examined the R-17F/TLC immunostaining profile of the Tic cell total lipids by means of a Far-Eastern blot. As shown in Fig. 2A, lane 1, upon development on an HPTLC silica gel plate with a developing solvent, CHCl3/MeOH/H2O (65:25:4, v/v/v), the total lipids were resolved into several major lipid components, including phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM). In contrast, the R-17F-positive band was detected at a slow migration position, where no visible primuline-positive lipid component was detected (lane 2), indicating that the R-17F antigen was a minor component of the membrane lipids. However, upon Far-Eastern blot with anti-H type 1 antibody (17-206), one major band was observed close to that of the R-17F antigen (lane 3). In addition, on the lectin blot
with a fucose-binding lectin, biotinylated *Aleuria aurantia* lectin, the R-17F antigen seemed to co-migrate with a major fucose-carrying component in the Tic cells (lane 4). On the other hand, neither anti-Lewis a antibody (7LE) nor anti-Lewis x antibody (MC-480) showed any detectable signals under the conditions tested (data not shown).

Then we tried to purify the R-17F antigen by preparative TLC under different experimental conditions and finally suc-
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FIGURE 2. Isolation and characterization of the R-17F antigen from hiPS cells. A, purification of the R-17F antigen by preparative TLC. a, TLC analysis of Tic cell total lipids. The total lipids were resolved on an HPTLC silica gel 60 aluminum sheets with a solvent, CHCl3/MeOH/H2O (65:25:4, v/v/v). Lane 1, all lipids, including glycosphingolipids, were stained with primuline and visualized using a UV transilluminator (365 nm). The major lipid components of Tic cells are shown on the left: phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM). Lane 2, Far-Eastern blot with R-17F; a single (probably doublet) R-17F antigen band (indicated by an arrowhead). Lane 3, Far-Eastern blot with anti-H type 1; a single major band at a position close to that of the R-17F antigen and several other minor bands at upper positions. Lane 4, biotinylated A. aurantia lectin (AAL) blot; a single major band at a position close to that of the R-17F antigen and several other minor bands at upper positions. B, TLC analysis of Tic-cell total lipids separated by the three-time TLC development method. Lane 5, primuline staining indicates the major lipid components. Lane 6, Far-Eastern blot with R-17F. A single (probably triplet) R-17F antigen band (indicated by an arrowhead). Lane 7, Far-Eastern blot with anti-SSEA-3 antibody. A single major band at the position of SSEA-3 and a second band at the position of SSEA-4, B, fucosidase digestion of the R-17F antigen. The R-17F antigen was treated with α-1–2 fucosidase (4.7 milliunits) and with α-1–3/4 fucosidase (12.5 microunits) at 37 °C overnight, and the digests were subjected to TLC, followed by a Far-Eastern blot with R-17F. Lane 1, no fucosidase (control). Lane 2, α-1–2 fucosidase-treated. Lane 3, α-1–3/4 fucosidase-treated.

In our control experiments, the α1–3/4 fucosidase was shown to release α1–2- and α1–4-linked fucose residues from ADHP-conjugated Lewis b oligosaccharides under the experimental conditions used (data not shown). These results confirmed the presence of a fucose residue at the non-reducing end of the R-17F antigen and also indicate that an α1–2-linked fucose residue but not an α1–3/4-linked one is critically important for the antigenic and epitopic activities. The results obtained in the TLC blot experiments described above suggested that the R-17F antigen isolated in this study is in a highly purified state, which allowed the detailed structural study by MALDI-TOF-MS.

Because the structural characterization of glycosphingolipids on a TLC plate using MALDI-QIT-TOF MS has been reported, the applicability of the MSn being described in terms of the quality and sensitivity of the analysis (23), the purified antigen was subjected to analysis by MALDI-QIT-TOF MS. The MS spectrum of the R-17F antigen is shown in Fig. 3A. All of the MS signals can be classified into four groups (Groups A, B, C, and D). In each group, five major signals were detected at m/z values differing by 26 or 28, which probably indicate five different alkyl chain lengths in the ceramide portion. Glycan components in Group D were investigated by MS2 and MS3 analysis of the five major precursor ions of the MS spectrum. Typical MS2 and MS3 spectra of group D (m/z 1506 [M + Na]+ in D-e) are shown in Fig. 3B. A series of mass differences of fragment ions, 146, 162, 203, and 162, were thought to represent typical glycan units and sequences, which represent Fuc, Hex, HexNAc, and Hex, in that order. To obtain more structural information, MS4 analysis was performed for the m/z 833 fragment ion observed on MS2. As shown in Fig. 3B, the experiment revealed another 162 difference from the precursor, which indicated the elimination of a hexose. The glycan structure deduced on MS4 analysis was Fuc-Hex-HexNAc-Hex-Hex. Given that the two units from the last of the sequence are Hex-Hex, the m/z 671 fragment ion observed on MS2 possibly indicates a ceramide portion of the antigen, because Gal-Glc has been recognized to be the core sequence for glycosphingolipids, and the last Glc is attached to the ceramide. The 26 or 28 difference seen in the MS spectrum is supportive of this assumption. The same results were obtained for the m/z 1452 fragment ion [M + Na]+ (D-c in Fig. 3D) and the m/z 1396 one [M + Na]+ (D-a, in Fig. 3F) observed on each MS2 and MS3 analysis. In the MS2 spectrum of the m/z 1480 fragment ion [M + Na]+ (D-d in Fig. 3C) and the m/z 1424 one [M + Na]+ (D-b in Fig. 3E), the fragment ions resulting from the elimination of glycan were detected as well as in the other MS2 spectra of Group D, although the ceramide ion [Cer + Na]+ could not be detected. To summarize the results of MS4 analysis, Group A corresponds to the presumptive structure of Hex-Hex-Cer, Group B corresponds to HexNAc-Hex-Hex-Cer, Group C corresponds to Hex-HexNAc-Hex-Hex-Cer, and Group D corresponds to Fuc-Hex-HexNAc-Hex-Hex-Cer.

These results indicated that the antigen was purified to homogeneity in terms of glycan structure, although the ceramide portion probably consists of five molecular species differing in their ceramide structure. Fuc-Hex-HexNAc-Hex-Hex-Cer (Group D) represents the intact molecular ion of the isolated antigen.
The binding specificity of R-17F was studied by glycan microarray analysis on nitrocellulose membranes using 13 synthetic standard neoglycolipids, which had been prepared by conjugation of the oligosaccharides with ADHP. As shown in Fig. 4, lane 2 (see also Table 1), R-17F exhibited a strong fluorescent signal to Fuc(1→2)Gal(1→3)GlcNAc(1→3)Gal (spot 5), followed by Fuc(1→2)Gal(1→3)GlcNAc(1→3)Gal (spot 7), whereas it did not show any detectable signals to other oligosaccharides, including Fuc(1→2)Gal(1→3)GlcNAc (spot 10). Because the reducing end sugar of the these oligosaccharides was conjugated with ADHP and no longer had a sugar ring structure, these results indicated that R-17F recognized the Fuc(1→2)Gal(1→3)GlcNAc(1→3)Gal structure most effectively, and Fuc(1→2)Gal(1→3)GlcNAc may be the minimum required structure for recognition.

One critical feature for the R-17F recognition is the Fuc(1→2)Gal structure at the non-reducing end of the glycan, which is shared by all anti-blood group H(O) antigens (spots 4–12). However, as for spot 4, Fuc(1→4) substitution of the GlcNAc residue in the Fuc(1→2)Gal(1→3)GlcNAc structure appears to block the R-17F binding. Another critical feature is the linkages between Gal and GlcNAc residues. In the case of the active spots (spots 5 and 7), the linkage was type 1 (1→3), whereas for the inactive spots (spots 6 and 8) the linkage was type 2 (1→4). Thus, R-17F selectively recognized the type 1 core structure.
spot 3, the anti-H type 2 antibody (BRIC231) recognized spots 6 and 8 (lane 4), the anti-globo H antibody (MBR1) recognized spot 9, and the anti-SSEA-3 antibody recognized spot 13 (lane 6), as expected under the same conditions used (2 μg/ml). The anti-H type 1 antibody (17-206) gave no visible signal under the same conditions (2 μg/ml), but at a higher dose (30 μg/ml), signals were detected at spots 5 and 7. Overall, it may be reasonable to propose that the complete epitope structure of R-17F is Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4).

Cytotoxic Effect of R-17F toward hiPS/ES Cells—The most interesting property of R-17F was the cytotoxic effect toward hiPS/ES cells. Upon incubation of the Tic cell suspension with R-17F, followed by incubation with an Alexa Fluor-conjugated second antibody, a large proportion of the cells was recovered in the dead cell gate on flow cytometry. This initial finding prompted us to study this interesting property in more detail using 7-AAD as a viability probe. As shown in Fig. 5A (a), upon incubation of Tic cells with increasing amounts of R-17F at 4 °C for 45 min, the percentage of viable cells decreased dose-dependently. Thus, the percentages of viable cells were 60.4 ± 16.7% at 0.2 μg R-17F/100 μl, 43.8 ± 5.9% at 1.0 μg, 20.2 ± 8.7% at 5.0 μg, and 12.3 ± 3.5% at 10.0 μg. Approximately 90% of the cells were killed upon the addition of R-17F under the conditions where the effect of the isotype (IgG1)-matching control antibody (anti-MBL/MBP) was not significant (16% decrease). This R-17F-induced cytotoxicity was not complement-mediated, because no serum component was present in the assay medium. As shown in (b), upon incubation of R-17F-sensitized cells (with 1.0 μg of R-17F) with a small amount (0.025–0.1 μg) of the secondary antibody (goat anti-mouse IgG1 antibody), the cytotoxic effect of R-17F was enhanced markedly in a dose-dependent manner, and more than 90% of the cells were killed upon the addition of 0.1 μg of secondary antibody. This cytotoxic reaction of R-17F proceeded rapidly; cell death occurring almost spontaneously (c), and was less sensitive to the temperature change (d), the viability at 4 °C being 30.7 ± 3.4% and that at 37 °C 32.5 ± 11.9%. In addition, this cytotoxic effect appears to be specific to R-17F, because other hiPS/ES cell marker antibodies, including TRA-1-60, TRA-1-81, SSEA-4, and R-10G, did not show significant cytotoxicity (e). Then we extended these studies to other hiPS/ES cells. As shown in Fig. 5B, upon flow cytometry after incubation of R-17F (0.4 μg) with another hiPS cell line (201B7) (b) and two hES cell lines (H9 and KhES-3) (c and d) for 45 min at 4 °C, R-17F bound to these cells even better than to Tic cells (a), suggesting the generality of the recognition of hiPS/ES cells by R-17F. Similarly, the cytotoxic activity of R-17F appeared to be effective on other hiPS/ES cells (e). Upon incubation with 0, 2.5, 5.0, and 10.0 μg of R-17F for 45

![FIGURE 4. Binding specificity of R-17F studied with glycan microarrays.](image)

Each spot (spots 1–13) contained 5 pmol of ADHP-conjugated glycan, the structures of which are shown in Table 1. Antibodies tested are as follows: anti-Lewis a (lane 1), R-17F (lane 2), anti-H type 1 (lane 3), anti-H type 2 (lane 4), anti-globo H (lane 5), and anti-SSEA-3 (lane 6).

**TABLE 1**

| Spot | ADHP-conjugated NGLs | Alias name | Structure of oligosaccharides | Note |
|------|---------------------|------------|--------------------------------|------|
| 1    | Lacto-N-tetraose    | LNT        | Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc |      |
| 2    | Lacto-N-neotetraose | LNT         | Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc |      |
| 3    | Lacto-N-fucopentaose II | LNFP II | Gal(β1-3)(Fuc(α1-4))GlcNAc(β1-3)Gal(β1-4)Glc | Lewis a |
| 4    | Lacto-N-difucohexose I | LNDFH I | Fuc(α1-2)Gal(β1-3)(Fuc(α1-4))GlcNAc(β1-3)Gal(β1-4)Glc | Lewis b |
| 5    | Lacto-N-fucopentaose I, H type 1 pentaose | LNFP I | Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc |      |
| 6    | H type 2 pentaose   | LnNFP I    | Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc |      |
| 7    | H type 1 tetraose   | Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal |      |
| 8    | H type 2 tetraose   | Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3)Gal |      |
| 9    | H type 4 tetraose   | Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal |      |
| 10   | H type 1 triose     | Fuc(α1-2)Gal(β1-3)GlcNAc |      |
| 11   | H type 2 triose     | Fuc(α1-2)Gal(β1-4)GlcNAc |      |
| 12   | H type 5 triose     | Fuc(α1-2)Gal(β1-4)Glc |      |
| 13   | Globohexaose, Gb5   | Gal(β1-3)GlcNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc | SSEA-3 |
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min at 4 °C, 201B7 cells, H9 cells, and KhES-3 cells were killed dose-dependently.

In the next experiment, we examined the cytotoxic effect of R-17F toward colony Tic cells on culture because hiPS/ES cells usually multiply as colonies on culture and not as a single cell suspension. Tic cells were cultured in the chamber slides for 2 days with iPS culture medium until small colonies were formed. Then the medium was changed to hESF9 medium (200 μl) containing 100 μg of R-10G or 100 μg of R-17F or no antibody (0 h), and the colonies were incubated for another 3 days. At every 24 h, cell growth was monitored under a phase-contrast microscope. As shown in Fig. 5C, Tic cells grew rapidly with a doubling time of

![Figure 5](image-url)

**FIGURE 5. Cytotoxic effect of R-17F on hiPS/ES cells.** A, the cytotoxic effect of R-17F on Tic cells was assayed by flow cytometry using 7-AAD as a viability probe. a, effect of R-17F treatment on the viability of Tic cells. Red circles, percentages of viable cells after incubation with increasing amounts of R-17F at 4 °C for 45 min. Blue circles, effects of the isotype (IgG1)-matching control antibody. Data are the averages ± S.D. of four independent experiments. b, effect of the secondary antibody on the cytotoxic effect of R-17F. Red circles, percentages of viable cells after subsequent incubation of the R-17F (1.0 μg)-sensitized Tic cells (1.5 × 10^5 cells) with increasing amounts (0.025–0.1 μg) of the secondary antibody (goat anti-mouse IgG1 antibody). Blue circles, effects of the isotype (IgG1)-matching control antibody. Red circles, percentages of viable cells. Blue circles, effects of the isotype (IgG1)-matching control antibody. Data are the averages ± S.D. of three independent experiments. c, effect of R-17F on the growth of Tic cells in cultured colonies. Tic cells were cultured in chamber slides with the iPS culture medium for 2 days until small sized colonies were formed. Then the medium was changed to hESF9 medium (200 μl) containing 100 μg of R-10G or 100 μg of R-17F or no antibody (0 h), and the colonies were incubated for another 3 days. At every 24 h, cell growth was monitored under a phase-contrast microscope.
about 1 day without an antibody (control) throughout this period. In contrast, in the presence of R-17F, significant cell multiplication was not observed, and instead the colonies had withered significantly by day 3. In the presence of R-10G, however, Tic cells grew as rapidly as in the control, despite the fact that R-10G recognizes and binds to Tic cells as effectively as R-17F. These results suggested that the cytotoxic effect of R-17F occurs not only for cells in suspension but also for cells forming colonies (adherent undifferentiated hiPS cells). At the same time, these results indicated that this cytotoxic activity is specific (i.e. it depends on the epitope molecule on the surface of iPS cells that R-17F binds to).

Cytotoxic Activity of R-17F Is Not Mediated by Apoptosis—In order to elucidate the mechanism by which R-17F kills iPS/ES cells, morphological changes of Tic cells induced by R-17F were monitored by phase-contrast microscopy in comparison with the effect of R-10G (non-cytotoxic antibody). As shown in Fig. 6A, upon incubation of the free Tic cell suspension with R-10G (2 µg/200 µl of 1% BSA-PBS) for 45 min at 4 °C, and morphological changes of Tic cells were monitored under a phase-contrast microscope. The arrow in the close-up view indicates a swelling phenomenon. In the presence of R-17F and by UV irradiation-induced apoptosis, Tic cells, which had been incubated with R-17F (10 µg/100 µl of medium) at 4 °C for 45 min, were incubated with SYTOX Green (viability staining dye) and then subjected to TO-PRO-3 staining, and Tic cells, which had been irradiated with a UV cross-linker at 2.0 J/cm², were cultured for 24 h, stained with SYTOX Green, and then subjected to TO-PRO-3 staining. Arrows, slight cell shrinkage and nuclear condensation. c, control cells (with no treatment). DNA fragmentation analysis by agarose gel electrophoresis. Tic cells in single cell suspension were either incubated with R-17F (10 µg/100 µl of 1% BSA-PBS) at 4 °C (lanes 1–3) or 37 °C (lanes 4–6) for 1 h (lanes 1 and 4), 3 h (lanes 2 and 5), or 5 h (lanes 3 and 6) or submitted to UV irradiation (lane 8). Lane 7, control. The molecular mass markers are shown on the left of lanes 1 and 7.

Then we compared the modes of cell death mediated by R-17F and by UV irradiation-induced apoptosis. As shown in Fig. 6B, incubation of Tic cells in a single cell suspension with R-17F at 4 °C for 45 min resulted in the loss of membrane integrity, as was shown on SYTOX Green-positive staining. On the other hand, some of the UV-irradiated cells showed signs of apoptosis (i.e. slight shrinkage and nuclear condensation and fragmentation). These results suggest that R-17F-mediated cell death was not associated with apoptosis. This observation was further confirmed by a DNA fragmentation assay by agarose gel electrophoresis, as shown in Fig. 6C. UV-irradiated Tic cells showed a faint DNA ladder, which is a hallmark of apoptotic cells (25), whereas R-17F-treated cells did not show significant DNA degradation. In addition, as shown in Fig. 7, the morphological study involving electron microscopy indicated that R-17F-treated cells showed morphological changes caused by damage to the plasma membrane and mitochondria, which are hallmarks of necrotic cells (24, 26). These results suggested that the cytotoxic activity of R-17F is not mediated by apoptosis but is probably evoked by necrosis, although the detailed mechanism of this interesting phenomenon remains to be studied.

Discussion

Over the last several years, we have attempted to raise new mouse monoclonal antibodies that can specifically recognize
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hiPS/ES cells because most of the conventional hiPS/hES marker antibodies recognize hEC cells as well as hiPS/hES cells. Thus, we immunized mice with a hiPS cell line (Tic), and the hybridomas obtained (in 960 wells) were subjected to differential screening, in which hybridomas that were positive as to hiPS cells and negative as to a hEC cell line, 2102Ep, were selected. By this procedure, we succeeded in the cloning of two hybridomas, R-10G and R-17F (12). Most notable is that both antibodies were directed against carbohydrates, a proteoglycan and a glycolipid, despite that no screening process for carbohydrate recognition was involved. These results suggested that carbohydrates on the surface of these cells play some important roles in distinguishing two closely related undifferentiated pluripotent cell types, hiPS/ES cells and hEC cells.

The R-17F antigen, which had been extracted from Tic cells with CHCl3/MeOH (2:1, v/v) and purified to homogeneity by preparative TLC, was shown to be Fuc-Hex-HexNAc-Hex-1 structure) to the ganglio-series during hES cell differentiation has been reported (27). Similarly, the lacto-N-fucopentaose I structure has been shown to be a characteristic feature of undifferentiated hiPS/ES cells by extensive structural studies on hiPS/ES cell glycolipids (28, 29), and this was supported by lectin array (30) and glycan array (31) studies. In addition, R-17F binds dose-dependently to chemically synthesized biotinylated LNFP I on avidin-coated plates, so we have developed an ELISA system to quantitate the binding activity of every lot of R-17F antibodies (data not shown).

The binding specificity of R-17F appears to be similar to those of the anti-H type 1 antibody (17–206), which was raised against a human colon cancer cell line, SW-403, and the anti-SSEA-5 antibody, which was raised against hES cells (35). With respect to anti-H type 1 antibodies, 10 antibodies are listed in a database, “GlycoEpitope” (36), which compiles carbohydrate-recognition antibodies. These antibodies are classified as one group because of their common requirement of Fuc(1–4)Glc for the recognition. However, regarding the precise mutual relationship of these antibodies, much remains to be elucidated, including the complete epitope structure of each antibody, the carrier molecules of the epitope, the mechanism of the glycan and antibody interaction, the issue of cross-reactivity with other glycan structures, and so on.
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The most interesting characteristic of R-17F is its cytotoxic activity toward hiPS/ES cells. This cytotoxic effect is concentration-dependent and complement-independent and proceeds very rapidly even at low temperature (4 °C). In addition, incubation of iPS cells in the presence of a Rho-associated kinase inhibitor, Y-27632 ((R)-(−)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide-2HCl) (Sigma-Aldrich Inc.), did not affect the cytotoxic effect of R-17F (data not shown), indicating that the R-17F cytotoxicity was mediated by a Rho-associated kinase inhibitor-insensitive process (37). Furthermore, the cytotoxic effect of R-17F was enhanced markedly when R-17F-sensitized cells were incubated with a secondary antibody (anti-mouse IgG antibody), suggesting that the immune complexes formed between R-17F and the antigen on the surface of the iPS/ES cells were clustered by the secondary antibody, which induced the following downstream reactions. Based on these results, together with morphological observations, it may be inferred that the cytotoxic effect of R-17F is mediated by necrosis and not by apoptosis, although elucidation of the molecular mechanism of cell death induced by R-17F awaits further studies.

A similar type of cytotoxic activity toward hiPS/ES cells was reported by Dr. Choo’s group for mAb84, which was raised against human ES cell line HES-3 (38, 39). However, R-17F is distinct from mAb84 in several basic properties. R-17F antigen is a glycolipid, whereas mAb84 antigen is a membrane glycoprotein, podocalyxin-like protein-1 precursor (PCLP1) (38). R-17F belongs to the IgG1 subtype, whereas mAb84 is an IgM protein. To the best of our knowledge, a carbohydrate-recognizing antibody that has cytotoxic activity toward hiPS/ES cells has not been reported before. Interestingly, however, when we examined the cytotoxic effect of the anti-H type 1 antibody (17-206) toward Tic cells under our assay conditions, the antibody exhibited an appreciable level of cytotoxic activity, which was almost comparable with that of R-17F (data not shown). In view of the overlapping binding specificity between these two antibodies, these results may not be surprising, although the final identification of the active factor(s) in the anti-H type 1 antibody reagent was precluded due to coexistence of the IgG3 antibody and ascites components in the reagent and consequently in the assay medium.

It should be noted that the self-renewal and pluripotency of hiPS/ES cells make them an attractive target for cell replacement therapies. However, the tumorigenic property of stem cells is a major obstacle to their full clinical application (3, 14). Therefore, removal of undifferentiated hiPS/ES cells from preparations of differentiated cells is considered to be a critical step toward safer regenerative medicine. With this background, the development of R-17F, a new marker antibody with cytotoxic activity, will lead to important progress for safer regenerative medicine.

Finally, the predominant part of the R-17F epitope on Tic cells was identified as LNFP I on a membrane glycolipid. However, the association of the R-17F epitope with membrane glycoproteins on the hiPS/ES cell surface cannot be ruled out because we detected a trace of R-17F staining on a Western blot of Tic cell lysates.

Author Contributions—T. K. and N. K. conceived and coordinated the study and wrote the paper. K. Kawabe and M. K. F. generated monoclonal antibodies. S. M. and M. N. performed immunohistochemical studies. S. M., K. Kawabata, and T. Y. performed flowcytometric analysis. H. N. and T. T. performed purification of the R-17F antigen. T. O., Y. Y., and S. N. performed MALDI-TOF MS analysis. H. N., H. T., and Y. T. performed glycan microarray analysis. S. M. carried out cytotoxic assays. All authors reviewed the results and approved the final version of the manuscript.

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