

# A Monoclonal Antibody (8H3) that Binds to Rat T Lineage Cells and Augments In Vitro Proliferative Responses

By Yoshihiro Torimoto, Miyuki Kinebuchi, Akihiro Matsuura, Kokichi Kikuchi, and Toshimitsu Uede

From the Department of Pathology, Sapporo Medical College, 060 Sapporo, Japan

## Summary

A murine monoclonal antibody, designated 8H3, recognizes a cell surface antigen expressed exclusively on rat T lineage cells. 8H3 antibody immunoprecipitated 180-, 120-, and 90-kD components from rat thymocytes as well as splenic T cells under nonreducing conditions. 8H3 antibody specifically inhibited the binding of thymocytes to fibronectin. Furthermore, binding of rat thymocytes to immobilized synthetic peptide Gly-Arg-Gly-Asp-Ser-Pro-Cys-BSA was inhibited by 8H3 antibody as was Gly-Arg-Gly-Asp-Ser-Pro-Cys, but not by Gly-Arg-Ala-Asp-Ser-Pro-Lys or Gly-Arg-Gly-Glu-Ser-Pro. Crosslinking of 8H3 antigen on double-negative thymocytes and adult thymocytes, as well as splenic T lymphocytes by 8H3 antibody and F(ab')<sub>2</sub> fragments of goat antibodies to mouse immunoglobulin, led to an increase in the concentration of cytoplasmic free Ca<sup>2+</sup> due to the release of Ca<sup>2+</sup> from intracellular stores as well as the influx of Ca<sup>2+</sup> from extracellular sources. Expression of interleukin 2 receptor and subsequently cell proliferation was observed upon incubation of thymocytes and splenic T cells with 8H3 antibody. Furthermore, 8H3 antibody induced the proliferation of double-negative thymocytes. These data collectively indicate that a cell surface antigen, 8H3, is involved in not only cell adhesion but also involved in the expression of immature as well as mature thymocytes.

Some T cell surface molecules have been shown to be involved in T cell activation and proliferation in human as well as rodent systems (1-6). The antigen-specific CD3/TCR molecule complex has been shown to be a major pathway in T cell activation in humans and mice (1, 2). Recently, two alternative antigen-independent pathways have been reported that involve either the SRBC receptor molecule, T11 (CD2) (3, 4), or the molecule Tp44 (CD28) (5) in humans. In addition, cell surface molecules that anchored via a specific association with phosphatidylinositol have been shown to be involved in T cell activation (6-8). These are the Thy-1 antigen and the T cell activation antigen TAP. Although the roll of those antigens in mature T cell activation has been extensively characterized, less is known about the role of those antigens in immature thymocyte growth and differentiation. Furthermore, natural ligands for most of those T cell surface molecules are not yet identified. Recently, the purified T11 molecules were found to specifically bind to lymphocyte function-associated antigen 3 (LFA-3)<sup>1</sup> (9). The detection and characterization of additional cell surface molecules that are involved in T cell activation are critical for better understanding of T cell activation mechanisms. In this context, our present

findings of a novel rat T cell surface molecule, 8H3, is clearly of interest. Antibody directed against this molecule directly activates immature and mature thymocytes as well as T cells. The immunochemical nature of this antigen is clearly distinct from T3, CD2, Tp44, Thy-1, or TAP. The 8H3 antigen might be involved in the antigen-independent pathway of T cell activation.

## Materials and Methods

**Animals.** Inbred 6-8-wk-old male Lewis and Wistar rats, and BALB/C mice, were purchased from Shizuoka Animal Center (Hamamatsu, Japan).

**Cell Suspensions and Lines.** Single cell suspensions of thymus, spleen, lymph node, or bone marrow cells of rat and mouse origin were prepared as described (10).

**Media.** All cell culture studies were performed in Eagle's MEM (Gibco Laboratories, Grand Island, NY) supplemented with L-glutamine ( $2 \times 10^{-3}$  M), penicillin (100 U/ml), streptomycin (100 µg/ml), 2-ME ( $5 \times 10^{-5}$ ), and 10% FCS (complete medium).

**Production of mAb** Thymocytes were obtained from 8-wk-old Lewis rats. BALB/C mice were injected intraperitoneally three times with  $10^7$  thymocytes. 3 d after the last immunization, spleen cells from the immunized mice were fused with NS/1 myeloma cells in the presence of polyethylene glycol, according to the method of Köhler and Milstein (11). After fusion, cells were resuspended

<sup>1</sup> Abbreviations used in this paper: HAT, hypoxanthine-aminopterin-thymidine; LFA-3, lymphocyte function-associated antigen 3.

in hypoxanthine-aminopterin-thymidine (HAT) containing complete medium at a cell density of  $2 \times 10^5$  spleen cells and were seeded in each well of 96-well plates. Cells were kept in HAT-containing complete medium for 2–3 wk until clones appeared. The hybridomas obtained were screened by indirect immunofluorescence for the production of antibodies to the rat thymocytes, but not to B cells, or bone marrow cells of rat origin. 18 clones were selected for cloning by limiting dilution technique because of antibody reactivities to rat thymocytes. Among these 18 clones, one clone, designated 8H3, was selected and subjected to further study. Antibody subclass determination was by Ouchterlony double immunodiffusion with anti-mouse subclass-specific antisera (Miles Laboratories Inc., Naperville, IL). The 8H3 antibody was found to be IgG1.

**Antibodies and Reagents.** Murine mAbs R1-3B3, R1-10B5, and RTH-7, which detect rat lymphocyte antigen comparable with human CD5, CD8, and CD4, respectively, were used in this study (12, 13). Therefore, these antibodies were hereafter designated as anti-CD5, anti-CD8, and anti-CD4, respectively. OX-34 antibody, which detects rat lymphocyte antigen equivalent to human CD2 (T11), anti-rat CD2, was obtained from Dr. A. F. Williams (University of Oxford) (14). OX-54 and OX-55, which detect distinct epitopes present on the rat CD2 molecule (15), were also provided by Dr. A. F. Williams. The mAbs R4-8B1 and R1-5B1, which detect rat MHC class I antigen and Thy-1 antigen, respectively, were also used in this study (16). R73, which detects the rat TCR- $\alpha/\beta$  structure, was obtained from Dr. Hunig (Wurzberg, FRG) (17), and anti-rat CD3 antibody was obtained from Dr. Tanaka (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) (18). Anti-rat intercellular adhesion molecule 1 antibody, 1A29, was supplied by Drs. Tamatani and Miyasaka (Tokyo Metropolitan Institute of Medical Science) (19). ART-18 antibody, which detects rat IL-2R (p55) was obtained from Drs. H. Osawa and T. Diamantstein (20). FITC goat anti-rat Ig was purchased from Meloy Laboratories Inc., Springfield, VA, and was absorbed with normal rat serum coupled to Sepharose-2B as described elsewhere (21). FITC goat anti-rat Ig was also purchased from Meloy Laboratories Inc. R2-1A6 antibody, which detects rat macrophages and neutrophils, was also used in this study (22). Antibodies specific to the conserved cytoplasmic portion of the integrin  $\beta$  1 subunit was obtained from Dr. R. Hynes (Massachusetts Institute of Technology, Cambridge, MA). This antibody reacts with  $\beta$  1 proteins in vertebrate, invertebrate, and fungi (23). Purified rat plasma fibronectin was purchased from Biomedical Technologies, Inc., Cambridge, MA. Fibronectin-related peptides, Gly-Arg-Gly-Asp-Ser-Pro-Cys, Gly-Arg-Ala-Asp-Ser-Pro-Lys, and Gly-Arg-Gly-Glu-Ser-Pro, were obtained from Peninsula Laboratories, Inc., Belmont, CA. H-Gly-Arg-Gly-Asp-Ser-Pro-Cys-BSA was obtained from Bachem Fein Chemikalien AG, Bubendorf, Switzerland. Collagen purified from rat tail tendon was purchased from Alfacell Corporation, Bloomfield, NJ. Vitronectin purified from porcine plasma was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Laminin was purchased from Collaborative Research, Lexington, MA. Fura-2 pentaacetoxymethyl ester was purchased from Dojin, Tokyo, Japan.

**Cell Fractionation.** Spleen cells were obtained from Lewis rats, washed several times in HBSS (Gibco Laboratories) containing 3% BSA (B-HBSS), and resuspended at  $1.5 \times 10^7$ /ml in B-HBSS. 3 ml of the suspension was added to each Corning 25-cm<sup>2</sup> plastic tissue culture flask. These were incubated for 1 h at room temperature. After incubation, the nonadherent cells were recovered and resuspended in HBSS containing 10% FCS. The nonadherent cells were further fractionated into a T cell-enriched fraction by using

a nylon wool column (24). The purity of T cells was >96% as judged by FACS (FACStar; Becton Dickinson & Co., Sunnyvale, CA) using anti-CD5 antibody. Double-negative thymocytes were enriched by the panning method as described previously (25). Briefly, thymocytes were obtained from day 18 fetuses of Wistar rats. Thymocyte cell suspension was incubated with anti-CD8 and anti-CD4 antibody for 1 h at 4°C. After washing, cells were loaded onto F(ab')<sub>2</sub> fragments of goat anti-mouse Ig-coated culture plates. After a 1-h incubation at 4°C, nonadherent cells were recovered. This panning procedure was repeated twice, and nonadherent cells were recovered and used as double-negative thymocytes. The purity of double-negative cells was >95% as judged by FACStar analysis using anti-CD8 and anti-CD4 antibodies.

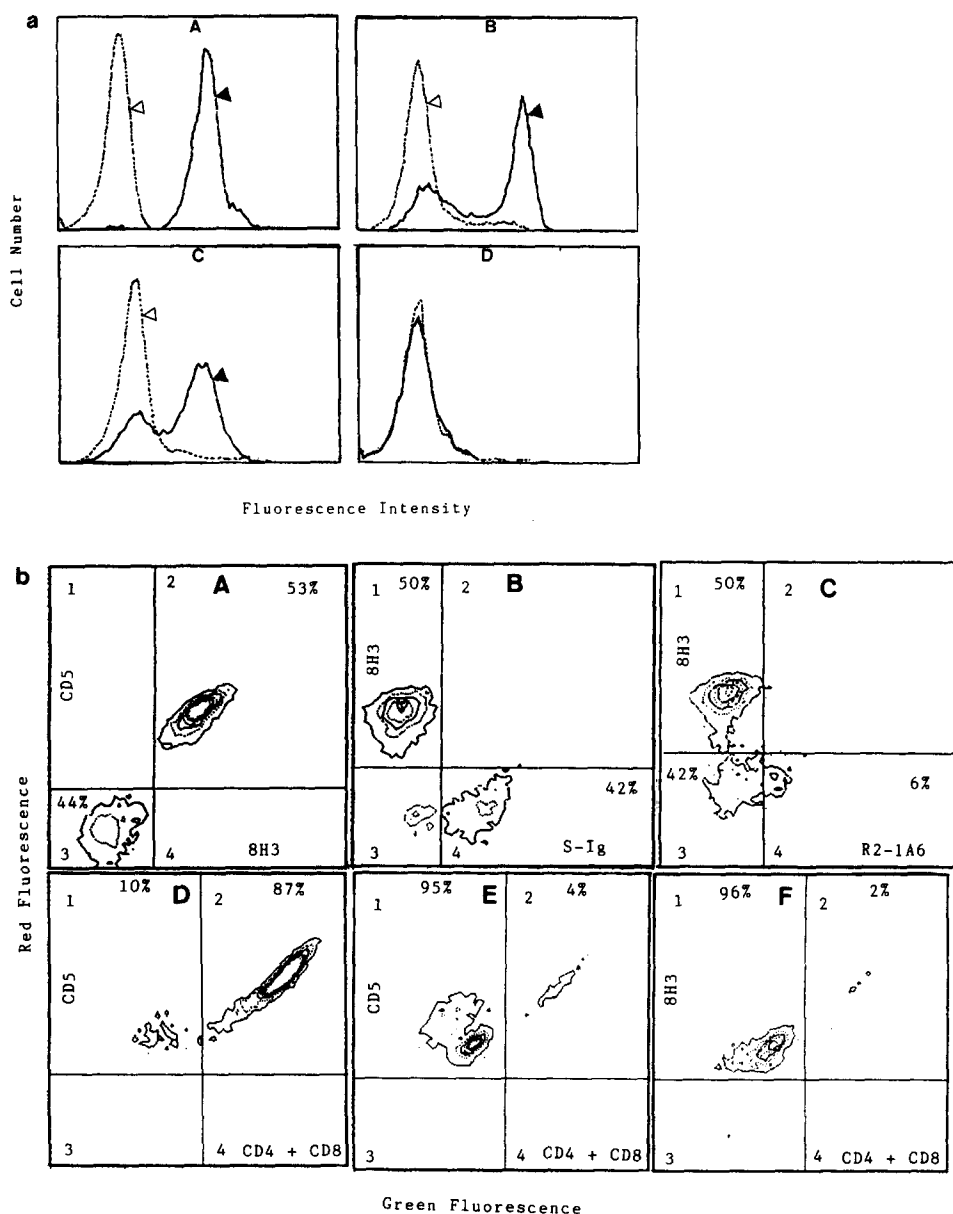
**Thymocyte Binding Assay.** The binding assay was performed according to the method described by Cardarelli and Pierschbacher (26), with some modification. Briefly, culture dishes (60 × 15 mm) (3002; Falcon Labware, Oxnard, CA) were coated with the indicated proteins by incubating the protein solutions at various concentrations in the dishes overnight at 4°C. Unbound proteins were removed from the dishes by washing with PBS (150 mM NaCl/10 mM sodium phosphate, pH 7.4). The dishes were then treated with DME (5 ml per dish) containing BSA (2.5 mg/ml) for 2 h at 37°C. Thymocytes were suspended in DME plus BSA (2.5 mg/ml) at a cell density of  $5 \times 10^6$ /ml, and contaminating nonlymphoid cells were eliminated by passing the G-10 column twice. Thymocytes were resuspended in the same medium at a cell density of  $10^6$ /ml. 4 ml of thymocyte cell suspension containing  $4 \times 10^6$  cells was added to the protein-coated dishes, and the dishes were incubated at 4°C for 90 min on an orbital shaker at 30 rpm. In some experiments, the thymocyte binding assay was done in dishes precoated with 10  $\mu$ g/ml of fibronectin or 200  $\mu$ g/ml of synthetic peptide. Unbound cells were collected by two gentle washes in PBS, and the cell number was counted by an automated cell counter (Coulter Electronics Inc., Hialeah, FL). Attached cells were removed by treated plates with 0.025% trypsin for 5 min at room temperature with vigorous pipetting according to the method described by Ignatz and Massague (27). All assays were done in triplicate, and the data were expressed as the mean  $\pm$  SEM. According to this procedure, the sum of unbound and bound cells was almost equal to the total cell number added to the dishes. Furthermore, specificity of thymocyte binding to fibronectin was confirmed by the method described by Cardarelli and Pierschbacher (26). Accordingly, bound thymocytes to fibronectin-coated dishes were specifically detached by adding 1 mg/ml Gly-Arg-Gly-Asp-Ser-Pro peptide for 10 min.

**Fluorographic Analysis.** Various lymphoid cells were aliquoted into volumes containing  $10^6$  cells and were pelleted for staining. Various mAbs were added to the cells in a volume of proper dilution for the mAb and incubated for 45 min at 4°C. For the detection of B cells, various cells were stained with FITC goat anti-rat Ig for 45 min at 4°C. Cells were washed twice and diluted appropriately for use in the FACStar analysis. Cells treated with normal mouse Ig and then with FITC goat anti-mouse Ig were used as control in FACStar analysis. In some experiments, cells were simultaneously stained with two antibodies. First, cells were treated with 10  $\mu$ g/ml of biotinylated anti-CD5 antibody at 4°C for 30 min. After washing, cells were incubated with an appropriate dilution of PE-conjugated avidin (Avidin-PE; Becton Dickinson Monoclonal Center, Mountain View, CA) at 4°C for 30 min. After washing, cells were then incubated with FITC-conjugated anti-CD8, anti-CD4, 8H3, or FITC goat anti-rat Ig. Samples were analyzed by FACStar. Green fluorescence (515–530-nm wave length) from FITC

and red fluorescence (630-nm wave length) from PE were detected independently and displayed as dot plots or contour map in the logarithmic scale of fluorescence intensity.

**Immunochemical Characterization of 8H3-defined Antigen.** Rat thymocytes or purified rat splenic T cells were also externally labeled by  $^{125}\text{I}$  by using lactoperoxidase (Sigma Chemical Co., St. Louis, MO) according to the method described previously (28). After iodination, the cell membranes were disrupted by adding lysis buffer (0.05 M Tris HCl buffer, pH 7.4, containing 0.14 M NaCl, 0.5% NP-40, 1 mM PMSF, 1  $\mu\text{g}/\text{ml}$  pepstatin, 0.05% sodium azide, 8 mM iodoacetamide, 0.2 TIU/ml aproptinin, and 5 mM EDTA for 1 h at 4°C and were dialyzed against 0.05 M Tris HCl buffer, pH 7.4, containing 0.1% NP-40 at 4°C for 24 h. Aliquots of the labeled material were then preabsorbed with 1/10 (vol/vol) of normal mouse Ig coupled to Sepharose-4B beads. Preabsorbed material was then incubated with 1/10 (vol/vol) of the 8H3 antibody coupled

to Affi Gel for 4 h at room temperature. The beads were then washed with a buffer containing 0.5 M Tris HCl, pH 7.4, 0.14 M NaCl, 0.1% NP-40 (washing buffer). After extensive washing, the beads were extracted with an SDS-PAGE sample buffer (10% SDS, 10% glycerol, 0.01% bromophenol blue, and 62.5 mM Tris HCl, pH 7.0) for 30 min at room temperature. Then, the SDS-PAGE was performed under reducing or nonreducing conditions. Proteolytic peptide mapping of 8H3 antigen was carried out according to the method of Cleaveland et al. (29). The SDS-PAGE was done under nonreducing condition, and the bands of 180,000, 120,000 and 90,000 were located by alignment with autoradiographic exposures and cut out. The gel slices were then placed in the sample wells of the second slab's stacking gel. The wells were filled with SDS sample buffer, and the samples were allowed to equilibrate for 120 min. Protease solution in SDS sample buffer containing 20% glycerol, 0.05% bromophenol blue, and 0.5  $\mu\text{g}$  of *Staphylococcus*



**Figure 1.** (a) Cytofluorographic analysis of rat thymocytes (A), spleen cells (B), lymph node cells (C), and bone marrow cells (D) stained with 8H3 (◀), or normal mouse Ig as a control (◁). (b) Two-color analysis of rat lymphoid cell populations. Spleen cells (A, B, and C), fetal thymocytes obtained from day 18 fetuses (D), and double-negative cell-enriched cells (E and F) were stained with different antibodies. Antibodies used in each instance are labeled on the graph. Quadrants were set with the use of background levels for FITC-labeled antibody and PE-conjugated avidin. Different quadrants (1, 2, 3, and 4) on each panel represent single-positive green (4) or red fluorescent cells (1), or double-positive (2) and double-negative cells (3). Percentage of positive cells is marked in each quadrant.

*aureus* V8 protease (Miles Laboratories, Inc.) was laid over the samples. The gels were further equilibrated for 120 min and electrophoresis was begun. After electrophoresis, the gels were dehydrated by using a gel dryer. The radioactivity in the slab gel was visualized by autoradiography.

**Determination of  $[Ca^{2+}]_i$ .** To load cells with the calcium-specific Fura-2, cells ( $10^8$ /ml) were incubated at 37°C for 30 min with 1  $\mu$ M Fura-2 pentaacetoxymethyl ester. The Fura-2-loaded cells were washed and resuspended in HEPES-buffered 0.9% saline (pH 7.4) containing 1 mM  $CaCl_2$ , 0.5 mM  $MgCl_2$ , 5 mM KCl, and 1 mM  $Na_2HPO_4$ . The fluorescence of the cell suspension ( $10^7$ /ml) was monitored with a fluorescence spectrophotometer (650-10S; Hitachi, Japan), and  $[Ca^{2+}]_i$  was calculated by the method of Grynkiewicz et al. (30).

## Results

**Distribution of a Cell Surface Antigen Defined by 8H3 Antibody.** To determine which type of cell expresses the antigenic determinant recognized by 8H3, the reactivity of this antibody with various rat lymphoid cell populations was examined. Cytofluorographic analysis of rat lymphoid cells stained with 8H3 antibody is depicted in Fig. 1 *a*. The results show that the antigen is present on 96% of thymocytes (A), 54% of spleen cells (B), and 57% of mesenteric lymph node cells (C), but <1% of bone marrow cells (D). As shown in Fig. 1 *b*, two-color analysis of spleen cells clearly demonstrated that cells bearing 8H3 antigen also express a T cell marker such as R1-3B3 (CD5 equivalent) (A). In addition, cells bearing 8H3 antigen are distinct from B cells (B), as well as neutrophils and macrophages that are stained by R1-1A6 antibody (C). On day 18, fetal thymocytes contained ~10% of double-negative cells (D). Double-negative thymocytes were enriched up to 95% (E). Those double-negative thymocytes express 8H3 antigen (F).

We have also examined the reactivity of 8H3 with various rat normal tissues by using an immunoperoxidase staining method. We found that 8H3 antibody did not react with any of the normal tissue tested, such as kidney, brain, liver, pancreas, and lung.

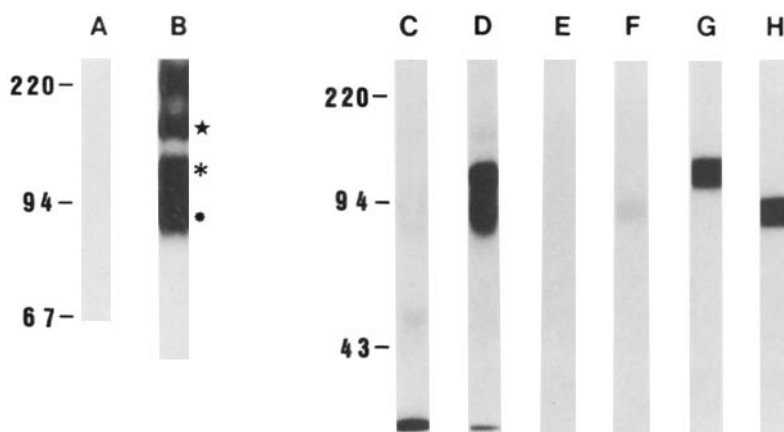
**Biochemical Characterization of 8H3-defined Antigen.** The  $^{125}I$  surface-labeled rat thymocytes and splenic T cells were

examined, purified by immunoabsorption, and analyzed by SDS-PAGE. As shown in Fig. 2, the 8H3 antibody immunoprecipitated 180-, 120-, and 90-kD components under non-reducing conditions (B) and 120-, and 90-kD components under reducing conditions (D). 8H3 antigen obtained from spleen T cells exhibited the same biochemical nature as thymocytes did (data not shown).

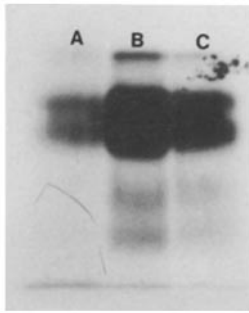
To clarify which component of 8H3 antigen is actually recognized by 8H3 antibody, each component was eluted from SDS-PAGE gels that were run under nonreducing conditions. Then, each component was applied to 8H3-coupled Affi Gel, and immunopurified antigen was re-analyzed by SDS-PAGE. All three components were affinity purified by 8H3 antibody. To further characterize the immunochemical nature of these three bands, proteolytic peptide mapping analysis was performed. As shown in Fig. 3, 180-kD (lane A), 120-kD (lane B), as well as 90-kD (lane C) bands gave identical peptide mapping patterns with two major bands being 56 and 40 kD, and two minor bands being 30 and 20 kD.

**Binding of Rat Thymocytes to Fibronectin Was Inhibited by 8H3 Antibody.** Rat thymocytes specifically bound to fibronectin, but not to laminin, collagen type I, or vitronectin. The binding of thymocytes was dependent on the concentration of fibronectin. When dishes were precoated with 10  $\mu$ g/ml of protein solutions, and  $4 \times 10^6$  thymocytes were applied to each dish, bound thymocytes to BSA, fibronectin, laminin, collagen, and vitronectin were  $2.1 \pm 0.2 \times 10^4$  (unbound cell number,  $38.8 \pm 1.2 \times 10^5$ ),  $40.8 \pm 1.5 \times 10^4$  ( $34.2 \pm 0.6 \times 10^5$ ),  $2.8 \pm 0.5 \times 10^4$  ( $37.9 \pm 1.1 \times 10^5$ ),  $2.4 \pm 0.4 \times 10^4$  ( $38.4 \pm 1.3 \times 10^5$ ), and  $2.1 \pm 0.2 \times 10^4$  ( $38.6 \pm 1.5 \times 10^5$ ), respectively.

Data were summarized in Fig. 4. The binding of thymocytes to fibronectin was inhibited by Gly-Arg-Gly-Asp-Ser-Pro-Cys, but not by Gly-Arg-Ala-Asp-Ser-Pro-Lys or Gly-Arg-Gly-Glu-Ser-Pro (Fig. 4 B). It should be noted that binding was also inhibited by 8H3 antibody and not by the isotype-matched control antibody, anti-CD5 antibody. It should be noted that none of the other antibodies tested (anti-CD4, CD8, ICAM-1, class I, CD3, CD2, and TCR) failed to inhibit the binding of thymocytes to fibronectin (Fig. 4 C). Next, we tested whether rat thymocytes bound to an im-



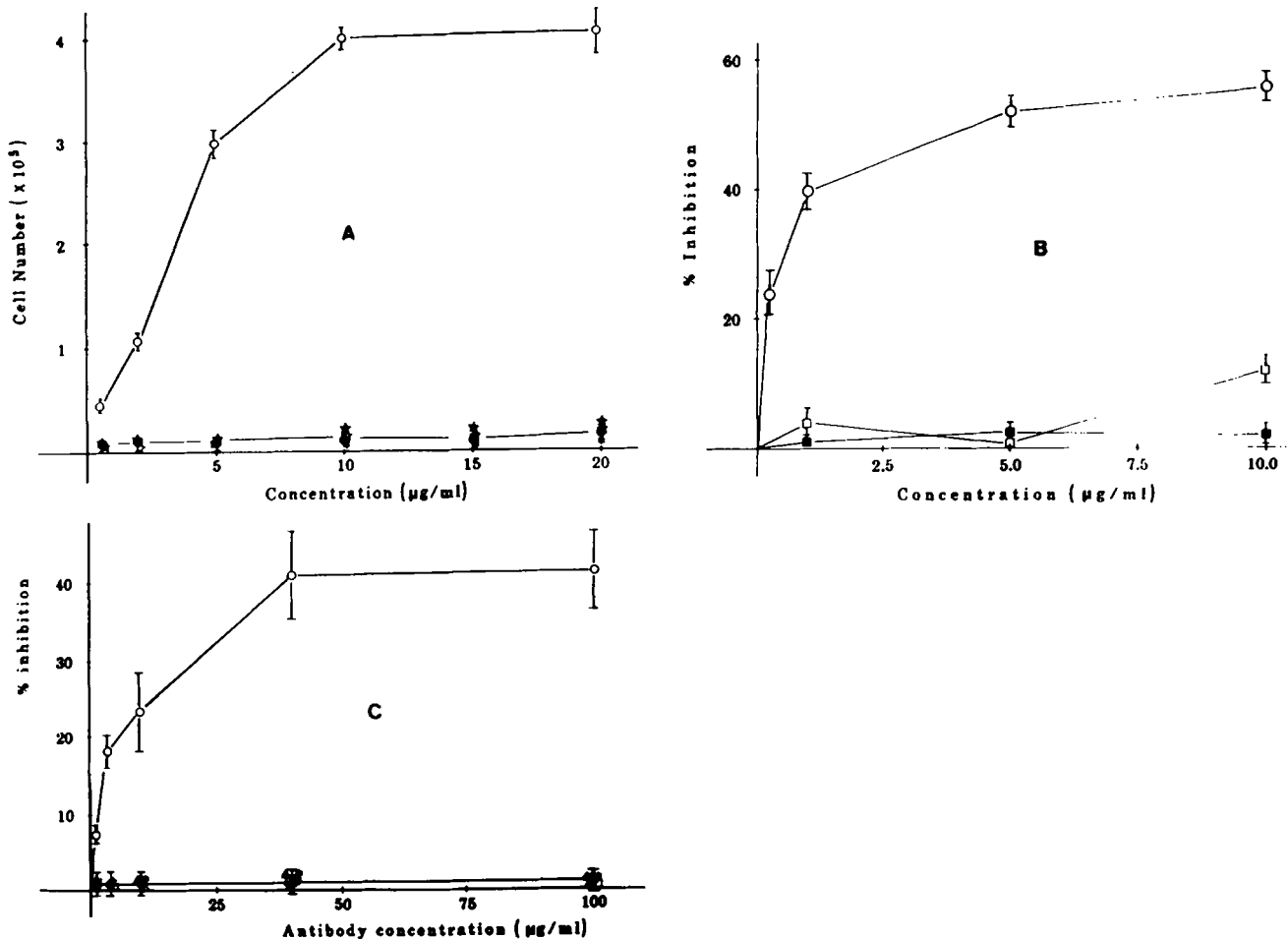
**Figure 2.** Immunoblot analysis of 8H3 antigen. 8H3 antigen immunoprecipitated by 8H3 antibody-coupled (lanes B and D) or isotype-matched antibody-coupled (lanes A and C) Affi Gel from thymocytes was analyzed by SDS-PAGE under nonreducing (lanes A and B) or reducing (lanes C and D) conditions. Gels corresponding to 180- (★), 120- (\*) and 90-kD (•) components were cut, antigens were eluted, and re-applied to 8H3 antibody-coupled Affi Gel. Bound fractions were analyzed by SDS-PAGE under reducing conditions. All three components were affinity purified by 8H3 antibody as shown in lanes F (180 kD), G (120 kD), and H (90 kD). Lane E represents the unbound fraction, and is used as a control.



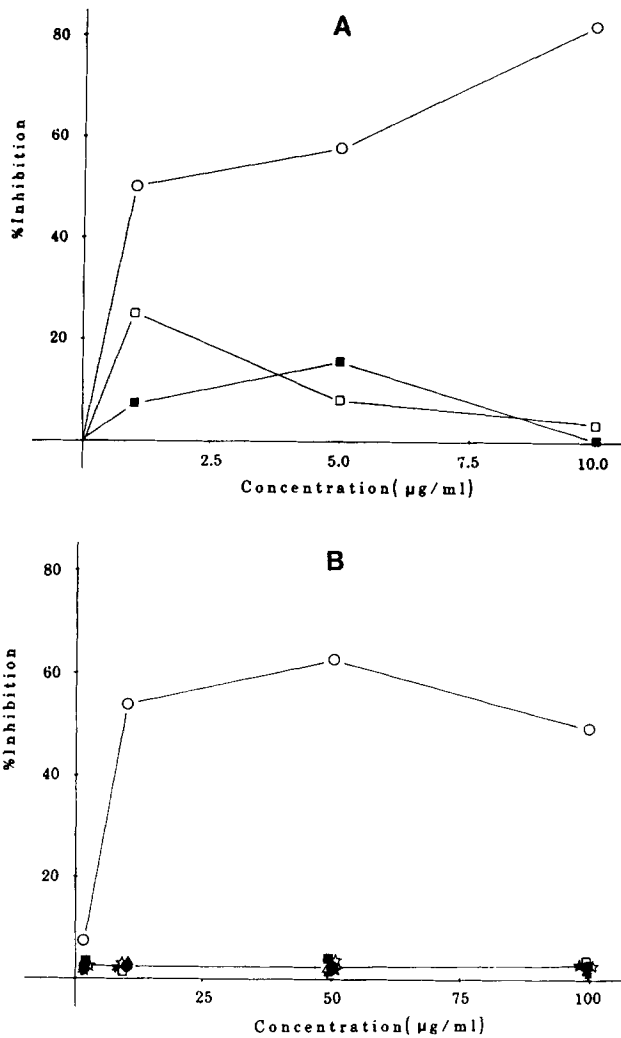
**Figure 3.** Peptide mapping analysis. Rat thymocytes were labeled by  $^{125}\text{I}$ . Antigens defined by 8H3 antibody were purified from lysate of  $^{125}\text{I}$ -labeled thymocytes by immunoabsorption. Antigens were then analyzed by SDS-PAGE. Appropriate bands corresponding to 180 kD (lane A), 120 kD (lane B), and 90 kD (lane C) in SDS-PAGE gels were cut out and digested by enzyme treatment as described. The digested antigens were analyzed by SDS-PAGE.

mobilized synthetic peptide, Gly-Arg-Gly-Asp-Ser-Pro-Cys-BSA, and found that thymocytes bound to the synthetic peptide in a dose-dependent manner (data not shown). The binding of rat thymocytes to immobilized synthetic peptide

was specifically inhibited by Gly-Arg-Gly-Asp-Ser-Pro-Cys, but not by Gly-Arg-Ala-Asp-Ser-Pro-Lys or Gly-Arg-Gly-Glu-Ser-Pro (Fig. 5 A). Furthermore, this binding was also inhibited by 8H3 antibody, but not by isotype-matched control antibody, anti-CD5, or various mAbs (Fig. 5 B).  $[\text{Ca}^{2+}]_i$  are increased in T cells by 8H3 antibody when 8H3 antigen is crosslinked by  $\text{F}(\text{ab}')_2$  fragments of goat anti-mouse Ig antibody. We examined the possibility that 8H3 antibody might stimulate  $[\text{Ca}^{2+}]_i$  in rat thymocytes, splenic T cells, fetal thymocytes, and double-negative thymocytes as assessed by an increase in the fluorescence of cells loaded with Fura-2. By itself, 8H3 antibody was without effect. After cells were exposed to 8H3 antibody, however, the addition of  $\text{F}(\text{ab}')_2$  fragments of goat anti-mouse Ig antibody caused a prompt increase in  $[\text{Ca}^{2+}]_i$  (Fig. 6). When extracellular  $\text{Ca}^{2+}$  was abrogated by the addition of 10 mM EGTA, the increase in



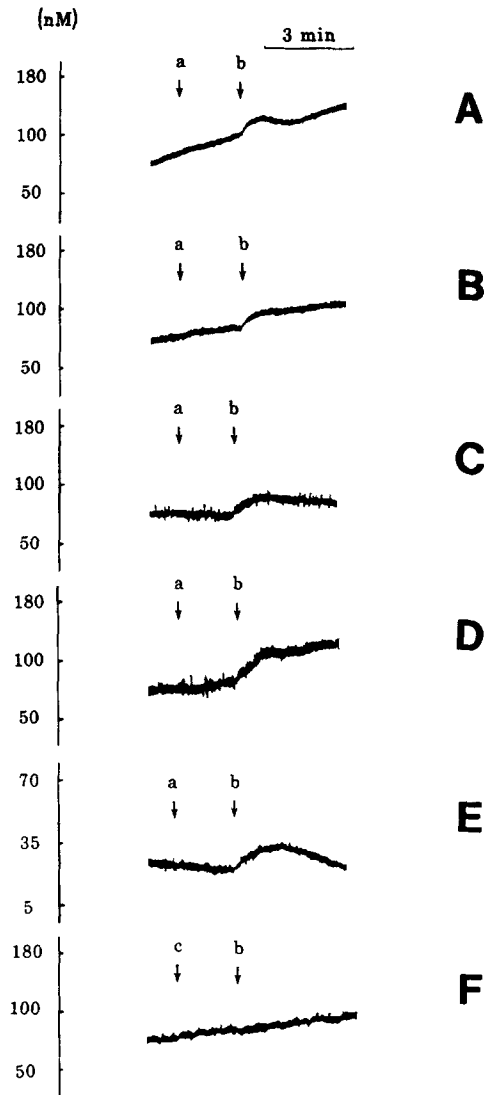
**Figure 4.** (A) Binding of rat thymocytes to fibronectin. Culture dishes (3002; Falcon Labware) ( $60 \times 15$  mm) were coated with various concentrations of fibronectin (O), laminin ( $\star$ ), collagen ( $\star$ ), vitronectin ( $\bullet$ ), or BSA ( $\ast$ ).  $4 \times 10^6$  rat thymocytes were applied to each dish and incubated for 90 min at  $4^\circ\text{C}$ . Bound cells were counted by a cell counter (Coulter Electronics Inc.). Experiments were done in triplicate, and the data were expressed as a mean  $\pm$  SEM. (B and C); Binding experiments were done in the presence of various inhibitors under the same condition as described in A. Dishes were coated with  $10 \mu\text{g/ml}$  of fibronectin. (B) Thymocytes were added to the plate in the presence or absence of inhibitors such as Gly-Arg-Gly-Asp-Ser-Pro-Cys (O), Gly-Arg-Ala-Asp-Ser-Pro-Lys ( $\square$ ), or Gly-Arg-Gly-Glu-Ser-Pro ( $\blacksquare$ ). (C) Thymocytes were added to the plate in the presence of 8H3 antibody (O), isotype-matched antibody, R1-3B3 ( $\bullet$ ), R73 ( $\Delta$ ), anti-CD4 ( $\star$ ), anti-CD8 ( $\square$ ), anti-class I ( $\blacksquare$ ), anti-Thy-1 ( $\blacktriangle$ ), anti-CD3 ( $\star$ ), or anti-ICAM-1 ( $\bullet$ ) antibody. After a 90-min incubation at  $4^\circ\text{C}$ , bound cells were counted. All experiments were done in triplicate, and data were expressed as percent inhibition  $\pm$  SEM.



**Figure 5.** The inhibition of thymocyte binding to synthetic peptide containing RGD sequences. Plates were pre-coated with 200 µg/ml of Arg-Gly-Asp-Ser-Pro-Cys-BSA overnight at 4°C. After washing, thymocyte binding to synthetic peptide-coated plates was determined in the presence of (A) Gly-Arg-Gly-Asp-Ser-Pro-Cys (O), Gly-Arg-Ala-Asp-Ser-Pro-Lys (■), or Gly-Arg-Gly-Glu-Ser-Pro (□), or (B) 8H3 antibody (O), isotype-matched antibody, (●), or various antibodies as indicated in Fig. 4 C.

[Ca<sup>2+</sup>]<sub>i</sub> in response to crosslinked 8H3 antigen was comparable. This result indicates that the increase in [Ca<sup>2+</sup>]<sub>i</sub> is due largely to the release of Ca<sup>2+</sup> from intracellular stores. The increase in [Ca<sup>2+</sup>]<sub>i</sub> could be also induced by an equimolar F(ab')<sub>2</sub> preparation of 8H3 antibody, crosslinked by F(ab')<sub>2</sub> fragments of goat anti-mouse Ig antibody (data not shown). No increase in [Ca<sup>2+</sup>]<sub>i</sub> was observed, with or without F(ab')<sub>2</sub> fragments of goat anti-mouse Ig antibody in response to other mAbs that bind to rat T cells such as R1-3B3 (CD5 equivalent) or RTH-7 (CD4 equivalent) antibody.

**Proliferation of Rat T Lineage Cells Induced by 8H3 Antibody.** When thymocytes were cultured with immobilized 8H3 antibody, no cell proliferation was observed, while splenic



**Figure 6.** Crosslinking of 8H3 antigen induces a [Ca<sup>2+</sup>]<sub>i</sub> response. Fura-2-loaded thymocytes (A, E, and F), splenic T cells (B), fetal thymocytes of day 18 fetuses (C), or double-negative thymocytes derived from day 18 fetuses (D) were stimulated by 8H3 antibody (a) and goat anti-mouse Ig (b), or CD4 antibody (c) in the presence (e) or absence (A, B, C, D, and F) of 10 mM EGTA.

T cells proliferated considerably under the same condition (Table 1). Thymocyte proliferation was induced by immobilized 8H3 antibody only when exogenous IL-2 was added to the culture. Thymocytes do express IL-2R upon stimulation by immobilized 8H3 antibody (data not shown). To determine the functional importance of 8H3 antigen on triggering T cell activation and proliferation, the efficacy of 8H3 antigen in inducing T cell proliferation was compared with anti-rat TCR antibody R73. In the presence of exogenous IL-2, R73 induced thymocyte proliferation. However, the intensity of cell proliferation was weak compared with that induced by 8H3 antibody plus IL-2. In fetal thymocytes, similar findings were noted, as 8H3 antibody was a more potent

**Table 1.** Proliferation of T Cells and Thymocytes Induced by 8H3 Antibody

	Day	Medium	8H3	R73	IL-2	8H3 + IL-2	R73 + IL-2
Thymocyte	2	396 ± 87*	402 ± 93	188 ± 43	1,872 ± 208	12,072 ± 3,725	2,317 ± 128
	3	126 ± 48	208 ± 50	118 ± 65	2,419 ± 284	16,242 ± 3,896	4,988 ± 540
	4	91 ± 20	234 ± 80	201 ± 85	1,724 ± 280	14,007 ± 2,459	6,082 ± 437
Splenic T cell	2	1,840 ± 165	4,192 ± 845†	4,025 ± 198†	9,572 ± 3,190	ND	ND
	3	1,832 ± 403	6,278 ± 398†	6,624 ± 508†	18,452 ± 4,118	62,581 ± 3,974	95,982 ± 4,180
	4	2,078 ± 516	5,848 ± 514†	9,084 ± 443†	24,310 ± 1,188	59,004 ± 4,683	125,431 ± 5,033
Fetal thymocyte	2	2,850 ± 184	3,804 ± 271	2,604 ± 189	9,366 ± 1,742	22,407 ± 4,241	9,538 ± 2,007
	3	1,506 ± 96	3,470 ± 304	1,448 ± 107	10,223 ± 1,168	35,427 ± 3,827	12,623 ± 4,103
	4	2,432 ± 284	4,011 ± 419	2,607 ± 245	9,210 ± 2,041	31,620 ± 2,714	14,527 ± 3,115
Double-negative thymocyte	2	2,160 ± 92	6,518 ± 419†	2,034 ± 103	5,842 ± 307	9,842 ± 603	6,042 ± 316
	3	1,550 ± 83	5,890 ± 713†	1,845 ± 124	6,256 ± 748	10,965 ± 1,651	6,842 ± 963
	4	1,470 ± 175	6,721 ± 506†	1,866 ± 176	64,459 ± 609	10,425 ± 2,172	7,267 ± 1,032

Adult thymocytes, splenic T cells, and fetal thymocytes obtained from day 18 fetus, or double-negative thymocytes were cultured in 96-well plates precoated with 50 µg/ml antibody for 2–4 d at a cell density of  $2 \times 10^5$ /well in the presence or absence of 300 pM IL-2.

\* Each sample was tested in triplicate. Data were based on three experiments, and values represent mean ± SEM.

† Proliferative responses significantly different from medium control ( $p < 0.01$ ).

stimulator. Although R73, as well as 8H3, induced splenic T cell proliferation in the absence of exogenous IL-2, R73 was more efficient than 8H3. Double-negative thymocytes were enriched up to 95% from day 18 rat fetuses. In contrast to adult and fetal thymocytes, the proliferation of double-negative thymocytes was observed upon incubation with immobilized 8H3 antibody alone, whereas R73 alone failed to stimulate the significant proliferation of double-negative thymocytes.

## Discussion

Various T cell surface antigens were known to be involved in T cell activation. However, ligands for most of those T cell molecules are not yet identified. Only LFA-3 was found as a ligand for CD2 (T11 antigens). We described here that rat thymocytes specifically bound to fibronectin, as well as to synthetic fibronectin peptide Gly-Arg-Gly-Ser-Pro-Cys-BSA. This binding was specifically inhibited by 8H3 antibody (although we tested variety of mAbs), as well as Gly-Arg-Gly-Asp-Ser-Pro-Cys, but not by synthetic peptides in which one of the essential Arg-Gly-Asp sequences was replaced by Ala or Glu. Therefore, it is likely that 8H3 antigen may recognize the tripeptides Arg-Gly-Asp present in fibronectin. Functional data strongly suggested the possibility that 8H3 antibody recognizes one of the T cell-specific integrin molecules. Furthermore, the SDS-PAGE analysis of 8H3 antigen exhibited multiunit complexes, revealing the similarity to chicken integrin complex (31). It was assumed that the 120-component and 90-kD components represent the  $\alpha$  chain and  $\beta$  chain of integrin, respectively. However, anti- $\beta$ -1 antisera failed to react with either of the three components detected by 8H3 antibody (data not shown). In addition, all three com-

ponents eluted from the SDS-PAGE gel rebounded to 8H3 antibody, indicating the possibility that all the bands are different forms of the one-gene products. To test this possibility, we performed peptide mapping analysis of three components and found that the same mapping patterns were observed in all three components. Although data are not shown, we performed an immunoprecipitation study under different conditions, since the formation of the  $\alpha$  and  $\beta$  chain complex might be disrupted by the inadequate pH or the absence of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (32). Furthermore, we used chemical crosslinking reagents to study whether 8H3 antigen would be coprecipitated with other components. However, we could not detect any associated components to 8H3 antigen. Thus, biochemical aspects of 8H3 antigen clearly suggested that 8H3 antigen is not an integrin. Purification and sequencing of the  $\text{NH}_2$ -terminal amino acid sequence of 8H3 antigen may clarify the molecular nature of 8H3 antigen.

In this report, we determined the role of 8H3 antigen on the proliferation of early fetal thymocytes, since the distribution pattern of 8H3 antigen was very similar to that of CD2 antigen, as opposed to CD3/TCR, whose expression is limited to a fraction of thymocytes (33). Early fetal thymocytes express the T11 antigen, which lacks the expression of CD3/TCR. Anti-T112 plus anti-T113 stimulation of early thymocytes caused  $[\text{Ca}^{2+}]_i$  mobilization and increased IL-2R expression, and led to thymocyte proliferation if exogenous IL-2 was provided (34). T cell activation protein (TAP) is expressed on 30–40% of double-negative thymocytes and early fetal thymocytes (8). Anti-TAP mAb induces the proliferation of immature, double-negative thymocytes in the presence of IL-2 and PMA. Although  $T_{p44}$  is also expressed on a subpopulation of thymocytes, the role of the  $T_{p44}$  molecule in early

thymocyte activation and proliferation is not well understood. A new murine T cell-activating molecule, thymocyte-activating molecule (THAM), has been recently described (35). THAM was found to consist of two polypeptides, 110 and 128 kD under nonreducing conditions. Crosslinking of THAM induced the proliferation of both double-negative and mature thymocytes in the presence of either PMA or IL-1 and IL-2. Furthermore, TCR-mediated proliferation was prominent in peripheral T cells. However, the significant proliferation of fetal as well as double-negative thymocytes was not observed by R73 stimulation under the condition we used. In contrast to these observations, rat double-negative thymocytes exhibited a proliferative response upon treatment with immobilized 8H3 antibody, even in the absence of exogenous IL-2. The proliferation detected with double-negative thymocytes was not due to the contamination of mature thymocytes, because adult and fetal thymocytes did not show

proliferative response upon crosslinking of 8H3 antigen if exogenous IL-2 was not provided.

The cell proliferation induced by crosslinking of 8H3 antigen is specific, because treatment of adult thymocytes with 8H3 antibody and subsequent crosslinking of antigen by goat anti-mouse Ig triggers the mobilization of  $[Ca^{2+}]_i$  from extracellular as well as intracellular sources. The mobilization of  $[Ca^{2+}]_i$  was also observed in peripheral T cells, double-negative thymocytes, and fetal thymocytes. These results suggested that double-negative thymocytes are responsive to at least some form of receptor-mediated transmembrane signaling before surface expression of CD3/TCR. Taken together, it is possible that 8H3 antigen represents a unique T cell-specific molecule that is expressed on only immature as well as mature T cells in rats, and is involved in thymocyte and peripheral T cell activation and proliferation.

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Address correspondence to Toshimitsu Uede, Department of Pathology, Sapporo Medical College, S-1, W-17, Chou-ku, Sapporo 060, Japan.

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