MONOCLONAL ANTIBODIES THAT RECOGNIZE THE PRODUCT CONTROLLED BY A GENE IN THE *I-J* SUBREGION OF THE MOUSE *H-2* COMPLEX

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The product of gene(s) in the *I-J* subregion of the mouse *H-2* complex that are expressed on allotype-specific and antigen-specific suppressor T cells has been defined (1; reviewed in 2). The soluble factors derived from antigen-specific suppressor T cells have also been found to bear the *I-J* subregion gene product (3–8). However, it has not been determined whether the I-J product on the antigen-specific suppressor T cells is the same as that on the antigen-specific suppressor T cell factor. Moreover, recent studies have shown that conventional anti-I-J alloantisera detect the cell surface markers on some helper T cells (9), concanavalin A (Con A)[1]-induced T cells (10) and some antigen-presenting cells (11). Tada et al. (9), especially, have demonstrated that the absorption of anti-I-J antisera with Lyt-1-positive splenic T cells resulted in the loss of ability to remove the helper (Th2) T cell function, whereas the absorbed anti-I-J still preserved the capacity to eliminate the suppressor activity. Thus, they proposed that the I-J product expressed on the antigen-specific suppressor T cells is distinct from that on the helper T cells. Therefore, it seems most likely that two or more genes rather than a single gene are accommodated in the *I-J* subregion.

A major difficulty in the interpretation of the results from these experiments as mentioned above is the following: I-J products have been detected by alloantisera raised by the combination of the intra-*H-2* recombinant strains, i.e., B10.A(3R) and B10.A(5R) or B10.HTT and B10.S(9R) pairs, possibly with an unknown and unequal region in the extra-*H-2* on the 17th chromosomal segment introduced from either parent for raising the recombinant strains. It is therefore possible that conventional anti-I-J alloantisera contain antibodies against the *I-J* subregion gene products as well as the products encoded by genes located somewhere in the extra-*H-2* on the 17th chromosome. In fact, previous reports have not formally excluded these possibilities. Further experiments are thus required to ascertain whether the *I-J*-controlled determinants on the antigen-specific suppressor T cell are identical to the determinants expressed on the antigen-specific suppressor T cell factors or on other immunocompetent cells with different functional activities.

It is generally known that *I*-region-encoded antigens are easily demonstrated on B lymphocytes but detected with difficulty on T lymphocytes (reviewed in 12). I-J

[1] *Abbreviations used in this paper:* C, rabbit complement; Con A, concanavalin A; DNP, 2,4-dinitrophenyl; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; HAT, hypoxanthine-aminopterin-thymidine; KLH, keyhole limpet hemocyanin; NS-1, P3-NSI/1-Ag4-1; P3U1, P3-X63-Ag8-U1; PEG, polyethylene glycol; PFC, plaque-forming cells.

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products on T cells are detected mainly by functional studies. For example, anti-I-J antisera are able to eliminate suppressor T lymphocytes but are not detectably cytotoxic on unfractionated spleen cells (1). Only a few attempts have been made to investigate the existence of I-J products in conventional cytotoxic assays on enriched splenic suppressor T cells (13, 14) and unfractionated lymph node cells (15, 16). However, direct demonstration of I-J products on T cells has not been formally reported. This may be due to the difficulty in obtaining high titers of anti-I-J antibodies and due to the limited expression of I-J products per cell.

Current techniques for producing monoclonal antibodies by cell hybridization provide helpful prospects for discriminating between the heterogenous I-J products and for defining new products expressed on functionally different immunocompetent cells, and also for preparing large quantities of monospecific antibodies. We report the establishment of B cell hybridomas producing monoclonal anti-I-J^b antibodies and describe two types of anti-I-J^b antibodies, one recognizing the private type of I-J^b determinant and the other recognizing the cross-reactive determinant between I-J^b and I-J^d products. Both of the determinants detected by the monoclonal antibodies are encoded for by a gene in the *I-J* subregion. We also demonstrate that the same I-J products are expressed on both antigen-specific suppressor T cells and their soluble factors. Furthermore, by using these monoclonal antibodies, I-J^b products are successfully demonstrated by membrane fluorescence techniques on the I-J^b-positive hybridoma and on the Con A-induced thymocyte blasts from C57BL/6 mice.

Materials and Methods

Animals. C3H/HeJ (*H-2*^{k}), C57BL/6 (*H-2*^{b}), DBA/2 (*H-2*^{d}), and BALB/c (*H-2*^{d}) mice were purchased from the Shizuoka Experimental Animal Co., Ltd., Shizuoka, Japan. C57BL/10 (*H-2*^{b}), A/WySn (*H-2*^{a}), CBA (*H-2*^{k}), AKR (*H-2*^{k}), B10.BR (*H-2*^{k}), B10.D2 (*H-2*^{d}), B10.S (*H-2*^{s}), B10.A (2R) (*H-2*^{h2}), B10.A(5R) (*H-2*^{h5}), and B10.A(3R) (*H-2*^{h3}) mice were raised in our animal facility. C3H.SW (*H-2*^{b}) mice were kindly provided by Dr. Okumura, Tokyo University, Tokyo, Japan.

Antigens. Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif. Dinitrophenylated KLH (DNP_{770}-KLH) was prepared by coupling with 2,4-dinitrobenzensulfonic acid under alkaline conditions by the method of Eisen et al. (17). *Bordetella pertussis* vaccine was obtained from the Chiba Serum Institute, Chiba, Japan.

Antibodies. The monoclonal anti-Thy-1.2 alloantibody (F7D5) was kindly supplied by Dr. P. Lake, Georgetown University, Bethesda, Md. Anti-I-J^b, B10.A(5R) anti-B10.A(3R), and anti-I-J^k, B10.A(3R) anti-B10.A(5R) were raised in our laboratory by the reciprocal immunization of the spleen and thymus cells. Fluorescein-conjugated rabbit anti-mouse IgG_{2a} antibodies were gratefully received from Dr. L. A. Herzenberg, Stanford University, Stanford, Calif.

Cell Lines. P3-X63-Ag8-U1 (P3U1) and P3-NSI/1-Ag4-1 (NS-1), derived from the BALB/c myeloma cell line MOPC-21, and AKR-derived thymoma BW5147 were obtained from Professor Takeshi Watanabe, Saga Medical School, Saga, Japan. I-J^b-positive T cell hybridoma lines (34S-18, 34S-704) with KLH-specific suppressor activity derived from a fusion of BW5147 and C57BL/6 KLH-specific suppressor T cells were established in our laboratory, as described elsewhere (8, 18, 19). The T cell lines express the product encoded by a gene in the *I-J* subregion of the major histocompatibility complex.

Immunization. B10.A(3R) spleen and thymus cells were treated with 100 μg/ml of mitomycin C (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) for 45 min at 37°C. B10.A(5R) mice were repeatedly immunized intravenously with 1×10^7 MMC-treated spleen and thymus cells of B10.A(3R) mice at weekly intervals for 7 wk.

Fusion and Cloning. 3 d after the last immunization, cell hybridization was carried out by the modified method of Oi et al. (20). In brief, 10^8 primed spleen cells of B10.A(5R) mice were

mixed with 1×10^7 NS-1 or P3U1 plasmacytoma in a 50-ml tube and pelleted by light centrifugation at 400 *g*. Cells were then resuspended in 1.0 ml prewarmed (37°C) 50% polyethylene glycol (PEG: 4,000 mol/wt; Sigma Chemical Co., St Louis, Mo.) by gentle shaking, followed by the dropwise addition of 1 ml of serum-free RPMI 1640 (Gibco Laboratories, Grand Island Biochemical Co., Grand Island, N. Y.). 1 ml of RPMI 1640 medium was successively added. The mixture was further diluted with another 7 ml of PRMI 1640. Cells were then centrifuged at 200 *g* for 5 min and were resuspended in warm hypoxanthine-aminopterin-thymidine (HAT) medium enriched with 10% fetal calf serum (FCS) (HAT medium: RPMI 1640 containing 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine; Wako Pure Chemical Industries, Ltd., Osaka, Japan). 100 μl of the cell suspensions was distributed to each well of a 96-well tissue culture plate (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) Hybrid cells were cultured in HAT medium for at least 2–4 wk. Culture supernates of hybrid cells grown in HAT medium were tested for their cytotoxic or their binding activity against the I-J^b-positive T cell hybridomas. Cells in the positive wells were cloned at least twice with single cell manipulation or limiting dilution.

Treatment of T Cell Lines with Neuraminidase. 10 million/ml I-J^b-positive T cell hybridoma and BW5147 thymoma cells, which are parental cells for the I-J^b-positive T cell hybridoma, were reacted with 20 μl of neuraminidase (Behringwerke AG, Marburg, West Germany) at a concentration of 10 U/ml at 37°C for 70 min in a CO_2 incubator. The treated cells were washed and used as target cells for the cytotoxic and cell binding assays.

Cytotoxic Assay. A two-step cytotoxic assay was performed. 40,000 target cells and 20 μl of the culture supernates were mixed and incubated for 30 min on ice in a V-bottomed microtiter plate (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, Va.). The cells were washed, pelleted, and resuspended in 20 μl of rabbit complement (C) at a 1:10 dilution, followed by further incubation for 30 min at 37°C. Dead and viable cells were counted under the light microscope by a trypan blue dye exclusion. The percent cytotoxicity was calculated according to the formula:

$$\text{percent cytotoxicity} = 100 \times \frac{\text{percent dead experimental} - \text{percent dead C control}}{100 - \text{percent dead C control}}.$$

Between 200 and 300 cells were counted per well of the dye exclusion assay.

Purification of the Monoclonal Antibodies. Ascites from the hybridoma-bearing mice were pooled, dialyzed against 0.01 M sodium phosphate buffer (NaPB), pH 8.0, and passed through a DE52 cellulose column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Uppsala, Sweden) with a continuous gradient buffer (0.01–0.2 M NaPB). Fractions containing monoclonal antibodies that gave a monoclonal spot by electrophoresis were collected. They were then dialyzed with 0.01 M phosphate-buffered saline, pH 7.2, containing 0.1% NaN_3. Myeloma proteins (CBPC 101:IgG_{2a}) were also purified by the same method as above.

Radioiodination. 100 μg of affinity-purified rabbit anti-mouse immunoglobulins was mixed with 200 μCi of ^{125}I Na in 100 μl of phosphate-buffered saline. They were then reacted with 2 μg of 1,3,4,6-tetrachloro-3α, 6α-diphenylglycoluril (IODO-GEN; Pierce Chemical Co., Rockford, Ill.) in a glass tube for 10 min on ice. The reaction mixture was then applied to 10 ml of the Bio-Gel P-6 column (Bio-Rad Laboratories, Richmond, Calif.) to remove free iodine.

Quantitative Absorption of Monoclonal Antibodies with Cells. 100 μl of the diluted culture supernate from the cloned hybridoma that gave the end point of the maximum cytotoxicity was absorbed with graded numbers of spleen cells from various strains of mice at 4°C for 60 min, with occasional shaking. They were then centrifuged, and the supernate was further tested for residual cytotoxic activity against neuraminidase-treated I-J^b-positive hybridoma cells (34S-18).

Enrichment of KLH-specific Suppressor T Cells. KLH-specific suppressor T cells from primed spleen cells of C57BL/6, BALB/c, or C3H mice were enriched by using KLH-coated petri dishes, as described previously (14). Briefly, spleen cells of C57BL/6, BALB/c, or C3H mice immunized twice with 200 μg of KLH at 2-wk intervals were applied to and incubated for 1 h on petri dishes coated with rabbit anti-mouse immunoglobulins at room temperature to remove B cells. Nonadherent cells that usually contain >95% T cells were further incubated with KLH-coated petri dishes under the same conditions as above. The cells that bound to KLH-coated dishes were recovered by the temperature shift (on ice) and pipetting with cold (4°C) medium.

The antigen-binding cells thus obtained were usually 0.1–0.5% of the unfractionated spleen cells.

Preparation of Immunoadsorbent Columns. Approximately 5 mg of monoclonal antibodies in 1 ml of ascites from the hybridoma-bearing mice was precipitated with ammonium sulfate at a concentration of 50%. They were extensively dialyzed with phosphate-buffered saline and were conjugated with 1 ml of Sepharose 4B (Pharmacia Fine Chemicals) by the method of Axen et al. (21).

Absorption and Elution of the Monoclonal I-J^b-bearing Suppressor Factor with Columns. The extracted materials equivalent to 3×10^5 I-J^b-positive hybridoma cells (34S-18 or 34S-704) were passed through immunoadsorbent columns. After extensive washing with RPMI 1640, the absorbed materials were eluted with 0.175 M glycine-HCl buffer, pH 3.2. The effluent and eluate were both tested for their activity in the in vitro secondary antibody response, as described elsewhere (22).

Fluorescent Stainings. 1 million of the neuraminidase-treated I-J^b-positive T cell hybridoma cells (34S-704) in 100 μl of Dulbecco's modified minimal essential medium containing 0.1% NaN_3 were reacted with 10 μg of purified monoclonal antibodies E10 (IgG_{2a}) at room temperature for 30 min. The cells were washed twice and then stained with fluorescein-conjugated rabbit anti-mouse IgG_{2a} at room temperature for 30 min. 10 μg of purified myeloma protein (CBPC 101: IgG_{2a}) were used as control for the stainings. The stained cells were then analyzed in a fluorescence-activated cell sorter (FACS IV; B-D FACS Systems, Beckton, Dickinson & Co., Sunnyvale, Calif.). Con A-induced thymocyte blasts were also used as target cells for a fluorescent-staining analysis. 10 million/ml thymocytes from C57BL/6 (*H-2*^{b}) and C3H (*H-2*^{k}) were cultured with 5 μg/ml of Con A (Sigma Chemical Co) for 48 h at 37°C in a CO_2 incubator. The cells were harvested and then extensively washed with medium. 1 million of the live cells fractionated by Ficoll-Conrey (Pharmacia Fine Chemicals) were reacted with 1 μg of purified monoclonal anti-I-J^b antibodies (E10 or H6: IgG_{2a}) or myeloma protein (CBPC 101: IgG_{2a}) followed by staining with fluorescein-conjugated rabbit anti-mouse IgG_{2a} antibodies. Approximately 20% of Con A-treated thymocytes, which are large blast cells, were analyzed by FACS-IV.

Cell Culture. 4 million spleen cells of C57BL/6 mice immunized 4 wk previously with 100 μg of DNP-KLH and *Bordetella pertussis* vaccine were cultured in the presence of 0.1 μg DNP-KLH in the Mishell-Dutton system. The materials from the immunoadsorbent columns were added to the culture at the start of cultivation. 5 d later, anti-DNP plaque-forming cells (PFC) were enumerated by using DNP-coupled sheep erythrocytes.

Results

Production of Anti-I-J^b Monoclonal Antibodies. The monoclonal antibodies D7, E10, F4, D4, and H6 were selected from the products of the fusion of mouse myeloma cells (P3U1 or NS-1) with spleen cells of B10.A(5R) mice hyperimmunized with mitomycin C-treated spleen and thymus cells of B10.A(3R) mice. Fused cells were plated in 0.2-ml tissue culture wells and cultured in HAT medium for at least 2–4 wk. The supernatant fluids were then harvested and assayed for their antibody activities against the products encoded by genes in the *I-J*^{b} subregion of the *H-2* complex by using a cell-binding assay with radioiodinated rabbit anti-mouse immunoglobulins. For screening the anti-I-J^b-producing hybridomas, several T cell hybridomas (lines 34S-18, 34S-704) with antigen-specific suppressor activities were used as target cells. AKR-derived T lymphoma cells, BW5147, which are parental cells of T cell hybridomas, were utilized as control target cells. The T cell hybridomas used express the products of genes in the *I-J* subregion, as described elsewhere (8, 18, 19). All positive supernatant fluids were further examined by a C-dependent cytotoxic assay.

The D7 and D4 antibodies were shown to be serologically IgM, and the E10, F4, and H6 antibodies were IgG_{2a}, IgG_{2b}, and IgG_{2a}, respectively. Fig. 1 shows cytotoxic patterns of the conventional anti-I-J^b antiserum [B10.A(5R) anti-B10.A(3R)], the

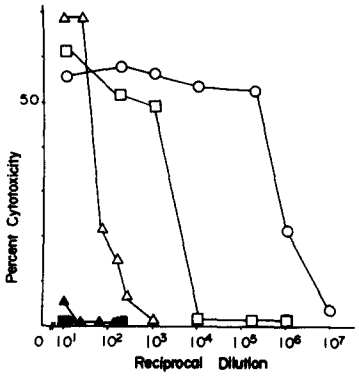

FIG. 1. Cytotoxic curves of anti-I-J^b antibodies on the I-J^b-positive T cell hybridoma (34S-18) or on the parental T lymphoma cell line, BW5147, of the T cell hybrids. The cytotoxicity of B10.A(5R) anti-B10.A(3R) (△, ▲), culture supernates (E10) (□, ■), and ascitic fluid from the hybridoma (E10)-bearing mice (○, ●) was investigated by dye exclusion cytotoxic assays. Target cells were pretreated with neuraminidase (see Materials and Methods). Other monoclonal antibodies (D7, F4, H6, and D4) gave results similar to the E10 antibody. The open and closed symbols indicate the cytotoxic titers on the *I-J*^{b}-positive hybridoma (△, □, ○) and on BW5147 (▲, ■, ●), respectively. The background in these experiments was <10%.

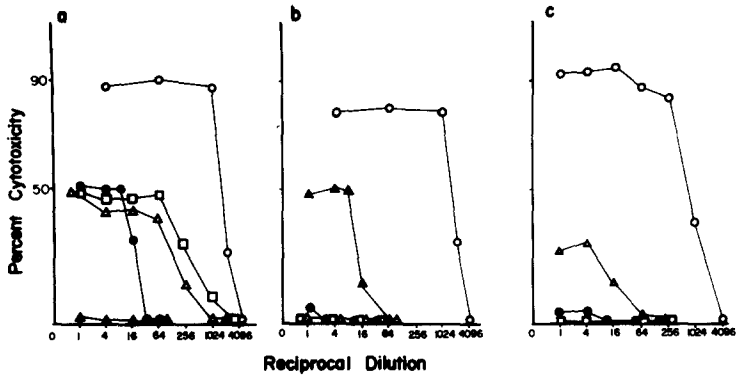

FIG. 2. Cytotoxic activities of the monoclonal antibodies E10 and H6 on the KLH-specific suppressor T cells from C57BL/6 (*H-2*^{b}) (a), C3H (*H-2*^{k}) (b), and BALB/c (*H-2*^{d}) (c) mice enriched with KLH-coated petri dishes. 4 × 10^4 enriched cells were treated with monoclonal anti-Thy-1.2 (○), B10.A(5R) anti-B10.A(3R) (●), B10.A(3R) anti-B10.A(5R) (▲), or monoclonal antibodies E10 (□) and H6 (△), as described in Materials and Methods. The background in these experiments was <5%.

culture supernate, and the ascitic fluid from the mice bearing the hybridoma (E10) on the I-J^b-positive suppressor T cell hybridoma (34S-18). Conventional anti-I-J^b antiserum showed low titers of cytotoxic activity on the I-J^b-positive T cell hybridoma. However, high titers of cytotoxic activity were obtained by the ascitic fluid and the culture supernate. No cytotoxic activity was observed on the BW5147 cells. Similar results were obtained by other monoclonal antibodies (D4, D7, F4, and H6).

Cytotoxic Activity on the Antigen-specific Suppressor T Cells. The reactivity of the monoclonal antibodies D7, E10, F4, D4, and H6 was investigated by dye exclusion cytotoxic assay on KLH-primed splenic suppressor T cells of C57BL/6 (*H-2*^{b}), C3H (*H-2*^{k}), and BALB/c (*H-2*^{d}) mice enriched with KLH-coated petri dishes. The methods to enrich I-J-positive splenic suppressor T cells were described elsewhere (14).

The suppressor activity of the enriched cells thus obtained has usually been shown to be >100-fold as much as that of unfractionated spleen cells (14). As shown in Fig. 2 a–c, ~80% of the cells enriched from C57BL/6, C3H, or BALB/c mice were Thy-1-positive T cells. In addition, ~50% of the cells from C57BL/6 mice were susceptible to conventional anti-I-J^b but not to anti-I-J^k antisera. Similarly, anti-I-J^k antisera killed ~50% of C3H but not C57BL/6-enriched T cells. Therefore, enriched cell populations from each strain of mice contain ~50% I-J-positive cells. Thus, we used these I-J-positive suppressor T cells as target cells to determine the reactivity of the monoclonal antibodies.

All of the monoclonal antibodies tested killed ~50% of the enriched suppressor T cells from C57BL/6, although no cytotoxic activity was observed on those from C3H mice. It is clear that the monoclonal antibodies recognize antigen-specific suppressor T cells expressing I-J^b products. However, Fig. 2 also demonstrates that some of the monoclonal antibodies revealed cross-reactive cytotoxic activity on the *H-2*^{d} suppressor T cells because ~30% of BALB/c-derived enriched T cells were lysed by the monoclonal H6 but not by the E10 antibodies. The cross-reactivity was also observed by the D4 antibody but was not detected by the conventional anti-I-J^b antisera [B10.A(5R) anti-B10.A(3R)] nor by other monoclonal antibodies (D7 and F4) (some data not shown).

Genetic Specificity of Monoclonal Antibodies. To demonstrate the genetic specificity of monoclonal antibodies D7, F4, E10, H6, and D4, 100 μl of the diluted culture supernates of the clones that gave the end point of the maximum cytotoxicity was absorbed with graded numbers of spleen cells from various strains of mice. The residual cytotoxic activity was tested on the I-J^b-positive hybridoma (34S-18). Fig. 3 a and b shows the cytotoxic curves of the monoclonal antibodies E10 (Fig. 3a) and H6 (Fig. 3b) before or after absorption with spleen cells from B10.BR (*H-2*^{k}), B10.S (*H-2*^{s}), B10.D2 (*H-2*^{d}), C57BL/10 (*H-2*^{b}), C57BL/6 (*H-2*^{b}), C3H (*H-2*^{k}), or C3H.SW

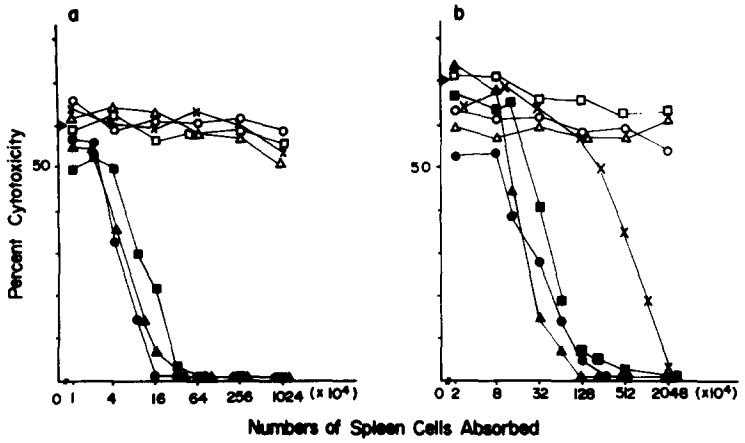

FIG. 3. Quantitative absorption analysis of the monoclonal antibodies E10 and H6. 100 μl of culture supernates from the E10 clone (a) at a 1:2,000 dilution and the same amount from the H6 clone (b) at a 1:3,000 dilution were absorbed with twofold dilution of the following spleen cells: C57BL/6 (*H-2*^{b}) (●), C57BL/10 (*H-2*^{b}) (▲), C3H.SW (*H-2*^{b}) (■), B10.S (*H-2*^{s}) (○), C3H (*H-2*^{k}) (□), B10.D2 (*H-2*^{d}) (×), and B10.BR (*H-2*^{k}) (△). The residual cytotoxic activity was assayed on the neuraminidase-treated I-J^b-positive T cell hybridoma (34S-18). The background was <20% (see Materials and Methods).

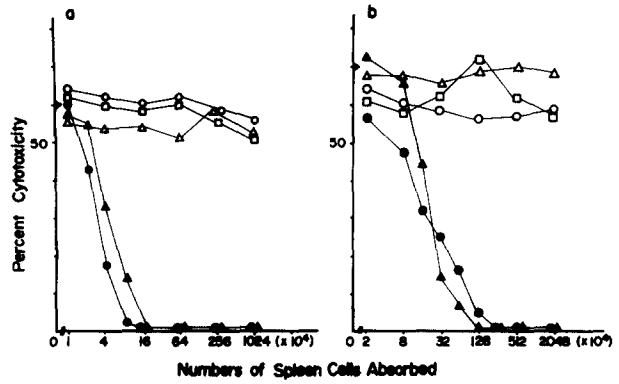

FIG. 4. Quantitative absorption analysis of the monoclonal antibodies E10 and H6. 100 μl of culture supernates from the E10 clone (a) at a 1:2,000 dilution and the same amount from the H6 clone (b) at a 1:3,000 dilution were absorbed with twofold dilution of the following spleen cells: C57BL/10 ($H\text{-}2^b$) (▲), B10.A(3R) ($H\text{-}2^{i3}$) (●), B10.A(2R) ($H\text{-}2^{h2}$) (△), B10.A(5R) ($H\text{-}2^{i5}$) (○), and A/WySn ($H\text{-}2^a$) (□). The residual cytotoxic activity was assayed as in Fig. 3. The background was <20%.

($H\text{-}2^b$). The cytotoxic activity of the E10 and H6 antibodies was absorbed with spleen cells of C57BL/6, C57BL/10, and C3H.SW having $H\text{-}2^b$ haplotype. The reactivity of the monoclonal antibodies was preserved, however, even when the antibodies were absorbed with 2×10^7 spleen cells of B10.BR, B10.S, and C3H mice. Therefore, absorption with $H\text{-}2^b$ spleen cells always removed the antibody activity. However, when the antibodies were absorbed with 2×10^6 to 20×10^6 of B10.D_2 D2 ($H\text{-}2^d$) spleen cells, the reactivity of the H6 but not E10 antibody was abrogated. The same results on the cross-reactivity between $H\text{-}2^b$ and $H\text{-}2^d$ spleen cells were obtained with the D4 but not with the F4 and D7 antibodies. These data in Fig. 3 a and b suggested that the monoclonal antibodies are directed against the $H\text{-}2^b$ gene products and that the H6 and D4 antibodies possessed the cross-reactivity to the $H\text{-}2^d$ products.

The I-J specificity of monoclonal antibodies was further characterized. The recombinants, B10.A(5R) and B10.A(3R), which were raised during back-crosses between C57BL/10 and A/WySn, might have the unequal chromosomal segment from A/WySn on the 17th chromosome of C57BL/10 mice. The immunization of B10.A(5R) with B10.A(3R) is possible to make antibodies to the $I\text{-}J^b$ products as well as those against the unknown products encoded by genes located somewhere in the right side end of the extra-*H-2* on the chromosomal segment from C57BL/10 or A/WySn. To exclude the reactivity against the unknown products, the antibodies were absorbed with spleen cells of C57BL/10, B10.A(3R), B10.A(5R), B10.A(2R), or A/WySn. As shown in Fig. 4 a and b, the cytotoxicity of the monoclonal antibodies with cross-reactive (H6) or noncross-reactive activities (E10) were abrogated by absorption with C57BL/10 and B10.A(3R) but not with B10.A(5R), B10.A(2R), and A/WySn. The same results were obtained by other monoclonal antibodies (D4, F4, and D7). It is therefore concluded that the monoclonal antibodies are specific for the $I\text{-}J^b$ subregion gene products.

Cross-reactivity of the H6 and D4 Antibodies. To confirm the previous results showing the cross-reactivity between $I\text{-}J^b$ and $I\text{-}J^d$ products detected by the H6 and D4 antibodies, quantitative absorption analysis of the cross-reactive antibody (H6) was

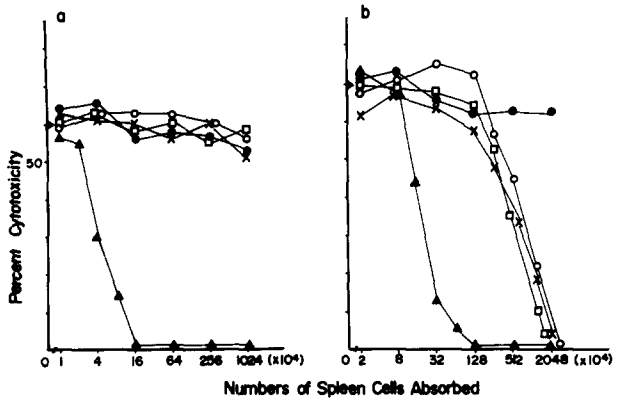

FIG. 5. Quantitative absorption analysis of the monoclonal antibodies E10 and H6. 100 μl of culture supernates from the E10 clone (a) at a 1:2,000 dilution and the same amount from the H6 clone (b) at a 1:3,000 dilution were absorbed with twofold dilution of the following spleen cells: C57BL/10 (*H-2*^{b}) (▲), BALB/c (*H-2*^{d}) (Δ), DBA/2 (*H-2*^{d}) (○), B10.D2 (*H-2*^{d}) (×), and B10.BR (*H-2*^{k}) (●). The residual cytotoxic activity was assayed as in Fig. 3. The background was <20%.

TABLE I

Cytotoxicity of Monoclonal Antibodies after Absorption with Spleen Cells from Various Strains of Mice

Absorption with*	H-2 complex								Percent cytotoxicity of the absorbed monoclonal antibodies				
	K	A	B	J	E	C	S	D	E10	F4	D7	H6	D4
None					—				63	59	65	71	63
C57BL/10	b	b	b	b	b	b	b	b	<5	<5	<5	<5	<5
C57BL/6	b	b	b	b	b	b	b	b	<5	—‡	—	<5	<5
B10.S	s	s	s	s	s	s	s	s	58	49	59	54	69
B10.BR	k	k	k	k	k	k	k	k	52	51	68	63	69
CBA	k	k	k	k	k	k	k	k	—	—	—	—	68
AKR	k	k	k	k	k	k	k	k	66	53	65	59	67
C3H	k	k	k	k	k	k	k	k	55	55	—	63	65
C3H.SW	b	b	b	b	b	b	b	b	<5	—	—	<5	<5
B10.A(3R)	b	b	b	b	k	d	d	d	<5	<5	<5	<5	<5
B10.A(5R)	b	b	b	k	k	d	d	d	56	54	68	59	61
A/WySn	k	k	k	k	k	d	d	d	57	60	60	58	54
B10.A(2R)	k	k	k	k	k	d	d	b	53	62	65	67	62
BALB/c	d	d	d	d	d	d	d	d	59	58	—	<5	<5
DBA/2	d	d	d	d	d	d	d	d	56	61	60	<5	<5
B10.D2	d	d	d	d	d	d	d	d	53	—	63	<5	<5

* 100 μl of culture supernates from the E10 clone at a 1:2,000 dilution, from the F4 clone at a 1:2,000 dilution, from the D7 clone at a 1:500 dilution, from the H6 clone at a 1:3,000 dilution, and from the D4 clone at a 1:800 dilution were absorbed with 2×10^7 spleen cells of various strains of mice. The residual cytotoxic activities of the absorbed antibodies were tested on the neuraminidase-treated I-J^b-positive hybridoma cells (34S-18).

‡ Not done.

carried out by using various *H-2*^{d} spleen cells (BALB/c, B10.D2, and DBA/2) in comparison with the noncross-reactive antibodies (E10). The results are shown in Fig. 5 a and b. The spleen cells from BALB/c, B10.D2, and DBA/2 mice show the capacity to absorb the cytotoxic activity of the H6 but not E10 antibodies, although no

TABLE II

Absorption of KLH-specific Hybridoma-derived Suppressor Factor with Monoclonal Anti-I-J^b Antibodies

TsF absorbed with*	Materials added	Anti-DNP IgG PFC/culture‡
None	None	4,650 ± 660
None	Unabsorbed TsF	1,490 ± 320
NMS	Effluent	1,650 ± 550
	Eluate	5,280 ± 670
F4	Effluent	4,240 ± 470
	Eluate	1,340 ± 430
E10	Effluent	4,300 ± 510
	Eluate	1,820 ± 310
D7	Effluent	4,080 ± 420
	Eluate	1,610 ± 380
H6	Effluent	6,360 ± 750
	Eluate	1,960 ± 750
D4	Effluent	4,250 ± 190
	Eluate	1,230 ± 390

* The extract of the KLH-specific suppressor T cell hybridoma (34S-18) equivalent to 5×10^5 cells was absorbed to and eluted from immunoadsorbent columns composed of monoclonal antibodies (F4, E10, D7, H6, and D4) or normal mouse serum (NMS). The effluent and the eluate from the columns were tested for their activity in the in vitro secondary anti-DNP IgG PFC response (see Materials and Methods). TsF is T suppressor factor.

‡ The arithmetic means of PFC numbers of three cultures ± standard deviations.

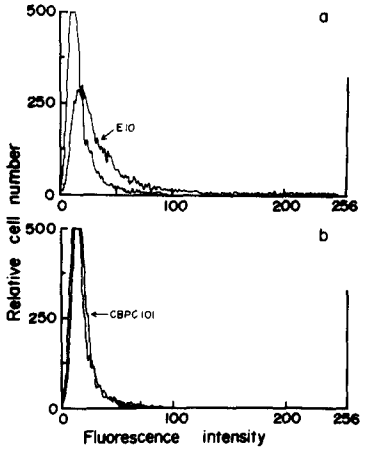

FIG. 6. Fluorescence profiles of the I-J^b-positive T cell hybridoma (34S-18) with the monoclonal anti-I-J^b antibody E10. The T hybridoma cells pretreated with neuraminidase were reacted with (a) 10 μg of the purified E10 antibody (IgG_{2a}) or with (b) 10 μg of the purified myeloma protein (CBPC 101:IgG_{2a}) followed by staining with fluorescein-conjugated rabbit anti-mouse IgG_{2a} antibodies. They were compared with T cell hybridomas stained with fluorescein-conjugated rabbit anti-mouse IgG_{2a} antibodies. Cells thus treated were analyzed by FACS IV with a linear amplifier.

absorption was observed with B10.BR (*H-2^k*) spleen cells. However, 10×10^6 to 20×10^6 *H-2^d* spleen cells (10–20 times as many as *H-2^b* spleen cells) were required to absorb the total antibody activity. The D4 antibody gave the same results as the H6

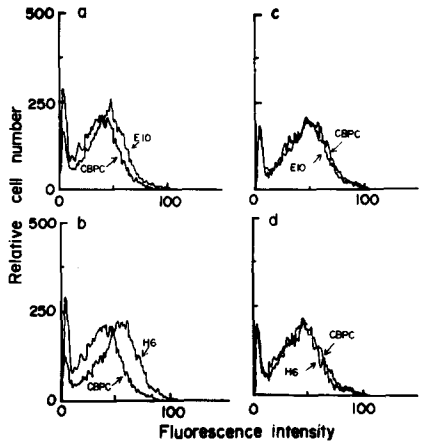

FIG. 7. FACS analysis of the monoclonal anti-I-J^b antibodies E10 and H6 on Con A-induced thymocyte blasts from C57BL/6 and C3H mice. Con A-induced thymocyte blasts from C57BL/6 mice treated with 1 μg of the purified E10 (IgG_{2a}) (a) or H6 (IgG_{2a}) (b) antibodies were compared with those reacted with 1 μg of the purified CBPC 101 myeloma protein (IgG_{2a}) as a control. Similarly, Con A-induced C3H thymocyte blasts reacted with 1 μg of the purified E10 (c) and H6 (d) antibodies were compared with those treated with 1 μg of the purified CBPC 101 myeloma protein. All cells treated with monoclonal antibodies and myeloma protein were stained with fluorescein-conjugated rabbit anti-mouse IgG_{2a}. Large blast cells, which are ~20% of Con A-stimulated thymocytes, were analyzed by FACS IV with a logarithmic amplifier. Note that Con A blasts of C57BL/6 (a, b) but not C3H (c, d) were significantly stained by the monoclonal anti-I-J^b antibodies E10 (a) and H6 (b).

antibody (data not shown). Table I summarizes the results of the absorption of the monoclonal antibodies with spleen cells from various mouse strains, demonstrating that the cytotoxicity of the E10, F4, and D7 antibodies was absorbed only with *H-2*^{b} spleen cells, whereas that of the H6 and D4 antibodies was absorbed by the *H-2*^{d} as well as *H-2*^{b} spleen cells. From the results in Figs. 3, 4, and 5, it is concluded that the D4 and H6 antibodies were able to react with I-J^b and I-J^d products, whereas the E10, F4, and D7 antibodies reacted only with I-J^b products.

Absorption of an Antigen-specific Suppressor Factor with Columns Composed of Monoclonal Antibodies. To determine whether the monoclonal antibodies have the capacity to absorb the antigen-specific suppressor T cell factor, a gamma globulin fraction of ascites from the hybridoma-bearing mice was coupled to the Sepharose 4B. The KLH-specific monoclonal suppressor factor bearing I-J^b products was absorbed with these columns. The absorbed materials to the columns were also eluted with 0.175 M glycine HCl buffer, pH 3.2. The effluent or the eluate from the columns was tested for its suppressor activity in the in vitro secondary antibody response. The results in Table II demonstrate that the hybridoma-derived I-J^b-bearing factor significantly suppressed the antibody response. The suppressor activity was absorbed to and eluted from the columns of monoclonal antibodies E10, F4, D7, D4, and H6. It is suggested that the determinants on antigen-specific suppressor T cells recognized by the monoclonal antibodies are also expressed on their soluble antigen-specific suppressor factors.

Immunofluorescent Stainings. The I-J^b-positive T cell hybridoma (34S-704) cells and Con A-induced thymocyte blast cells were reacted with purified monoclonal antibodies E10 (IgG_{2a}) and H6 (IgG_{2a}) followed by staining with fluorescein-conjugated

rabbit anti-mouse IgG_{2a}. The same amounts of CBPC 101 myeloma protein (IgG_{2a}) were used as a control for immunofluorescent stainings. They were then analyzed by FACS IV. The staining profiles of the monoclonal E10 antibody on the I-J^b-positive hybridoma are shown in Fig. 6a with the control stainings shown for comparison. The E10 antibody weakly but significantly stained the I-J^b-positive hybridoma cells, whereas no staining was observed with CBPC 101 myeloma protein (IgG_{2a}) having the same immunoglobulin subclass as the E10 antibody (Fig. 6b). The same results were also obtained in Fig. 7 by the stainings of Con A-induced thymocyte blasts with the monoclonal reagents (E10 and H6). The monoclonal E10 or H6 antibodies stained Con A-induced blast cells only from C57BL/6 (Fig. 7 a and b) but not from C3H mice (Fig. 7 c and d). No significant staining was obtained on either normal C57BL/6 or C3H thymocytes with these antibodies (data not shown).

Discussion

We described the monoclonal antibodies recognizing the product of a gene in the *I-J* subregion of the *H-2* major histocompatibility complex. The monoclonal antibodies were produced by hybridomas of NS-1 or P3U1 myeloma cells lacking the enzyme hypoxanthine guanine phosphoribosyl transferase, and spleen cells from B10.A(5R) mice repeatedly immunized with mitomycin C-treated B10.A(3R) lymphoid cells. The combination of B10.A(5R) and B10.A(3R) mice is ideal for making antibodies specific for the *I-J* subregion gene product because the only genetic difference between B10.A(5R) and B10.A(3R) is supposed to be the *I-J* subregion. Furthermore, an I-J^b-positive hybridoma with specific suppressor function was available as a useful target cell for selecting monoclonal anti-I-J^b-producing hybridomas. Two groups of hybridomas producing monoclonal anti-I-J^b antibodies with different specificities have been established: one recognizing the private type of the I-J^b determinant, the other recognizing the public or cross-reactive determinant on the I-J^b, which is at least shared by I-J^d products.

The specificity of monoclonal antibodies was determined in three different ways. First, the antibody activity was tested on the suppressor T cells of various strains of mice by a cytotoxic assay. It has been demonstrated that antigen-specific suppressor T cells enriched by antigen-coated dishes or columns bear the I-J products (13, 14). By using the enriched suppressor T cells, it is clearly demonstrated in Fig. 2 that the monoclonal antibodies have the ability to lyse I-J-bearing suppressor T cells of C57BL/6 but not those of C3H mice.

Secondly, quantitative absorption analysis with unprimed spleen cells from various strains of mice including independent haplotype strains, *H-2* recombinant, and *H-2* congenic mice was carried out to define the genetic specificity of monoclonal antibodies. From these experiments, the monoclonal antibodies were shown to be reactive with spleen cells having $I\text{-}J^b$ haplotype but not those with $I\text{-}J^k$ and $I\text{-}J^s$ haplotypes. Especially, all monoclonal antibodies tested were not reactive with C3H mice, whereas C3H.SW having $H\text{-}2^b$ haplotype with the same background as C3H ($H\text{-}2^k$) mice absorbed the antibody activity. Similarly, the antibody reactivity was always removed by absorption of C57BL/10 ($H\text{-}2^b$) spleen cells but not that of B10.BR ($H\text{-}2^k$) and B10.S ($H\text{-}2^s$) with C57BL/10 background, suggesting that the antibodies are directed against $H\text{-}2^b$ products. Moreover, the antibodies could be absorbed by spleen cells of

B10.A(3R) but not B10.A(5R) mice. Therefore, it seems clear that the monoclonal antibodies established are specific for the I-J^b products.

The *I-J* subregion is defined as a chromosomal segment encompassing the *Ia-4* locus with the cross-over positions in strains B10.A(5R) and B10.A(3R) forming regional boundaries. The recombinants are derived from a cross between A/WySn and C57BL/10 (reviewed in 23) and carry the chromosomal segment of A/WySn origin, which is extended from the intra-*H-2* recombinant points to somewhere to the right of the *H-2D*. The extra-*H-2* (right) end of this inserted chromosomal segment is totally unknown, and the crossover points at the right end in B10.A(3R) and B10.A(5R) are probably not identical. It is thus possible that the immunization in this combination gives rise to antibodies not only against the products of genes within the *I-J* subregion but also against those of unknown genes on the right side of the chromosomal segment, which can be either A/WySn or C57BL/10 type. To exclude this possibility, the antibodies were tested for their reactivity on spleen cells of B10.A(2R) and A/WySn. Recombinant B10.A(2R), which arose in the same series of the back-crossing that produced B10.A(5R) and B10.A(3R), should have the inherited chromosomal segement to the right of the *H-2D* from the parental C57BL/10 mice and the inserted chromosome to the left of the *H-2S* from A/WySn. B10.A(2R) and A/WySn strains are *k* type at the *I-J* subregion, and the right side end of the 17th chromosome in these mice should represent the respective parental type. Therefore, antibodies to unknown products of the right side of the extra-*H-2* genes of B10.A(3R) mice should react with one of B10.A(2R) and A/WySn, whereas anti-I-J^b antibodies should not. As shown in Table I and Fig. 4 a and b, none of the monoclonal antibodies, including cross-reactive (H6) and noncross-reactive (E10), was absorbed by B10.A(2R) or A/WySn spleen cells. These results show conclusively that these monoclonal antibodies are directed to the products of genes within the *I-J* subregion of the *H-2* complex.

Thirdly, our previous studies (3, 8, 22, 24) have demonstrated that various alloantibodies directed against I-J products have the capacity to absorb the KLH-specific suppressor factors from primed splenic T cells and KLH-specific suppressor hybridomas. Similar observations have been reported by several other investigators with different systems (reviewed in 2). Table II shows that the monoclonal suppressor factor was absorbed to and eluted from the columns of the monoclonal antibodies. Thus, it is strongly suggested that the monoclonal antibodies described here are specific for the product of a gene in the *I-J* subregion of the *H-2* complex.

Some cross-reactivities between I-J^k and I-J^s products and between I-J^d and I-J^k products have been reported by Murphy et al. (1) and Frelinger et al. (10), respectively. The results shown in Figs. 2c and 5b and Table I clearly demonstrate that the monoclonal D4 and H6 antibodies could react with $H\text{-}2^d$ as well as $H\text{-}2^b$ spleen cells. That the D4 and H6 antibodies were shown to be specific for the I-J^b products (see Fig. 4 and Table I) and that they also killed suppressor T cells enriched from KLH-primed BALB/c ($H\text{-}2^d$) spleen cells (Fig. 2c), indicates that the I-J^b determinant detected by the monoclonal antibodies H6 and D4 is the cross-reactive determinant shared by the I-J^d products. It is therefore likely that I-J products have both the private and public type determinants, like other Ia molecules controlled by the *I-A* or *I-E/C* subregion expressed on B cells (reviewed in 12). However, quantitative absorption studies showed that 2×10^7 spleen cells from $H\text{-}2^d$ mice, which are 10–20 times as much as the $H\text{-}2^b$ spleen cells, are necessary for the complete absorption of the H6

and D4 monoclonal antibodies. This is probably due to the lower affinity of the D4 and H6 monoclonal antibodies for the I-J^d products than that for the I-J^b products.

No direct demonstration of the *I-J*-encoded products has so far been reported. By using the monoclonal anti-I-J^b antibodies, the results in Fig. 7 a and b clearly demonstrate that the I-J products were detected on the cell surface of the Con A-induced thymocyte blasts from C57BL/6 but not C3H mice. However, no significant staining pattern was observed with normal thymocytes from either C57BL/6 or C3H mice (data not show). The findings suggest that the stimulation with Con A induces a proliferation of I-J-positive cells or the expression of I-J products on the cell surface.

The antigen-specific suppressor T cells and their factors have been found to bear the determinants detected by conventional anti-I-J alloantisera. There is, however, no evidence that I-J determinants on the suppressor T cells and their factors are encoded by the same gene. The monoclonal reagents made this problem clear. The monoclonal anti-I-J^b antibodies we described detected the determinants on the suppressor T cell hybridoma and on the enriched suppressor T cells by membrane stainings and/or cytotoxic assays. The antibodies also stained Con A-stimulated thymocytes, supporting the previous findings reported by Frelinger et al. (10) that Con A-reactive T cells bear I-J products. Moreover, the same monoclonal antibody has the capacity to absorb the antigen-specific suppressor T cell factor. From these results (Table II; Figs. 2 and 6) it is clear that the product encoded by a gene in the *I-J* subregion is expressed on both suppressor T cells and their factors.

Murphy et al. (1) originally determined that the *Ia-4* locus controls the determinants expressed on the allotype-specific suppressor T lymphocytes. However, the presence of the *Ia-4*-encoded product on the antigen-specific suppressor T cells and their factors has not been formally investigated. The results presented here also have not determined whether the monoclonal anti-I-J antibodies recognize the *Ia-4* product.

Cumulative evidence has suggested that two or more genes are accommodated in the *I-J* subregion of the *H-2* complex (9).[2] In this respect, monoclonal antibodies against I-J products with different specificities would be useful in discriminating between the heterogenous I-J products expressed on functionally different lymphocyte subsets. Further research, currently in progress, is required to answer these questions.

Summary

The B cell hybridomas producing monoclonal antibodies (E10, D7, F4, H6, and D4) were established by the fusion of P3U1 or NS-1 murine myeloma cell lines and spleen cells of B10.A(5R) mice hyperimmunized with mitomycin C-treated B10.A(3R) spleen and thymus cells. Two types of monoclonal antibodies specific for the products controlled by a gene in the *I-J*^{b} subregion of the *H-2* complex were characterized: one specific for the private type of I-J^b determinant, the other recognizing the cross-reactive determinant between the I-J^b and I-J^d products. By using these monoclonal reagents, the *I-J*-encoded product on the antigen-specific suppressor T cells was found to be expressed on their soluble suppressor factors. Furthermore, the I-J^b products were successfully detected not only on the T cell hybridoma with suppressor activity specific for keyhole limpet hemocyanin (KLH), but also on KLH-primed suppressor T cells enriched by antigen-coated petri dishes and concanavalin A-induced thymo-

[2] Takei, I., T. Saito, K. Hiramatsu, M. Kanno, and M. Taniguchi. Heterogeneity of I-J subregion gene products expressed on functionally different T cell hybridomas. Manuscript in preparation.

cyte blasts of C57BL/6 mice by complement-dependent cytotoxic assays and membrane fluorescence techniques.

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