TWO DISTINCT ALLOTYPIC DETERMINANTS ON THE ANTIGEN-SPECIFIC SUPPRESSOR AND ENHANCING T CELL FACTORS THAT ARE ENCODED BY GENES LINKED TO THE IMMUNOGLOBULIN HEAVY CHAIN LOCUS

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Thymus-derived (T) lymphocytes have been shown to display antigen specificity. The antigen-recognition units on T lymphocytes, in contrast to those on B lymphocytes, do not appear to be of the conventional immunoglobulin (Ig) type. Recent studies from various laboratories (1-5) have demonstrated that the antigen-specific T cell factors derived from helper or suppressor T cells possess the antigen-binding moiety and the products of genes in the I region of the H-2 complex, whereas no Ig constant determinant has been defined. Antibodies against the variable (V) region of the Ig heavy chain (Igh)\(^1\) or against idiotypes on the antibodies, however, have been shown to react with isolated T cell receptors for antigens as well as with antigen-specific helper and suppressor T cell factors (6-12). It is therefore suggested that the antigen-binding structure on T cell antigen-recognition units shares the V region markers with the conventional Ig on B cells. In this sense, the antigen-recognition units on T cells appear to possess a constant portion, as do Ig. However, the constant region determinants on T cell receptors or on antigen-specific T cell factors have so far not been defined.

If T cells utilize gene pools similar or identical to those coding for the V region of Igh (Igh-V), it is possible that the constant region determinants of the antigen-recognition units on T cells, distinct from those of B cell Igh (Igh-C), are also encoded by genes linked to the Igh locus. In this respect, the immunization of mice with T cells from Igh allotype congenic mice would be able to provide antibodies specific for new allotypic determinants on the constant region of the antigen-recognition units of T cells.

Based on these assumptions, Owen et al. (13-15) have, in fact, been successful in obtaining antisera reactive to the allotypic determinants on suppressor T cells of Igh\(^{1}\) mice (Tsu\(^d\)) encoded by genes adjacent to the Igh constant-region genes, Igh-C, on the side toward the prealbumin gene, Pre-1. The Tsu\(^d\) has been found to be expressed on Lyt-2\(^+\) suppressor T cells. Furthermore, the anti-Tsu\(^d\) antisera stimulate suppressor T cells of mice that have an appropriate Igh allotype and block the binding

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\(^1\) Abbreviations used in this paper: ARS, azobenzen arsonate; BPV, Bordetella pertussis vaccine; C, complement; Con A, concanavalin A; DNP, 2,4-dinitrophenyl; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; Igh, immunoglobulin heavy chain; Igh-C, constant region of the Igh; Igh-V, variable region of the Igh; KLH, keyhole limpet hemocyanin; NMS, normal BALB/c serum; PFC, plaque-forming cells; TaF, augmenting T cell factor; TsF, suppressor T cell factor; Tsu\(^d\), allotypic determinant on suppressor T cells of Igh\(^{1}\) mice; V, variable.

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of idiotyp-specific suppressor T cells to the idiotype-bearing immunoglobulins. Therefore, products recognized by the antisera seem to be the conserved determinants on, or adjacent to, the antigen-binding site of T cell receptors. No direct evidence has, however, demonstrated that the conserved allotypic determinants are in fact on the antigen-recognition units of T cells.

In this communication, we report that the alloantisem produced by Igh allotype congenic pairs defines the conserved allotypic determinants on the small population of T cells that belong to Thy-1 dull-stained Lyt-2+ or Lyt-2+ T cells of mice expressing Igh-1b allotype. The allotypic determinants recognized by the antisem are expressed on the antigen-binding moiety of the functionally different T cell factors (i.e., antigen-specific helper and suppressor T cell factors). Both of the determinants are considered to be encoded by genes located in the right side of the Igh-V locus on the 12th chromosome. Furthermore, the product on the antigen-specific suppressor T cell factor defined by the antisem is distinct from that on the antigen-specific helper (augmenting) T cell factor.

Materials and Methods

Animals. BALB/c CrSlc (H-2d, Igh-1b), C57BL/6 CrSlc (H-2b, Igh-1b), and C3H/HeSlc (H-2k, Igh-1j) mice were purchased from Shizuoka Experimental Animal Laboratory, Co. Ltd., Hamamatsu, Japan. SJL/J (H-2a, Igh-1b) and allotype congenic mice, CB-20 (H-2d, Igh-1b), BAB-14 (H-2d, Igh-1b), SJA/Hz (H-2a, Igh-1b), C3H.SW/Hz (CSW; H-2a, Igh-1b), and CWB/Hz (H-2a, Igh-1b) were kindly provided by Dr. K. Okumura, Department of Immunology, Faculty of Medicine, University of Tokyo, Japan.

Antigens. Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif. Dinitrophenylated KLH (DNP770-KLH) was prepared by coupling with 2,4-dinitrobenzenesulfonic acid under an alkaline condition. Bordetella pertussis vaccine (BPV) was purchased from the Chiba Serum Institute, Chiba, Japan.

Antisera. The BALB/c anti-CB-20 alloantisem was prepared by hyperimmunization of BALB/c mouse intravenously with 1 × 107 of CB-20 spleen cells precultivated with 5 #g/ml of concanavalin A (Con A; Sigma Chemical Co., St. Louis, Mo.) for 48 h at 37°C in a 5% CO2 incubator. The Con A-stimulated CB-20 spleen cells were treated with 100 #g/ml of mitomycin C (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) for 45 min at 37°C before immunization. The animals were immunized weekly, and beginning at the 11th wk, they were partially bled from a tail vein immediately before each immunization. The serum bled from each animal every week was pooled to make one lot and stored at -80°C before use. Only lot 2 of the antisem was used in the studies. Monoclonal mouse IgM anti-Thy-1.2 (F7D5) was kindly provided by Dr. P. Lake, Georgetown University, Wash., D. C. Monoclonal rat antibodies against Lyt-1 (53-7.3), Lyt-2 (53-6.7), Thy-1.2 (30-H-12), and affinity-purified mouse (SJL) anti-rat Ig were gifts from Dr. J. A. Ledbetter, Department of Genetics, Stanford University, Stanford, Calif.

Cytotoxic Test. The two-step cytotoxic assay was performed. 10 #l of 1 × 107/ml lymphoid cells from different tissues suspended in RPMI 1640 medium containing 1% fetal calf serum (FCS) and 0.1% NaN3 was incubated with 10 #l of diluted antisem for 30 min at 4°C. After one washing the cells were treated with 20 #l of a 1:8 diluted rabbit complement (C) for 30 min at 37°C. Live and dead cells were counted under a light microscope by the trypan blue dye exclusion method. Percent cytotoxicity was calculated by the following formula:

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

200 cells were counted per well in the dye exclusion cytotoxic assay.

Quantitative Absorption of BALB/c Anti-CB-20 Antisem with Cells. Quantitative absorption studies were carried out in a V-bottomed microtiter plate (Dynatech Laboratories, Inc.,
Dynatech Corp., Alexandria, Va.). 30 µl of the 1:10 diluted antiserum that showed the end point of maximum cytotoxicity was absorbed with graded numbers of lymphoid cells from various strains of mice for 1 h at 4°C. The supernatants were collected by centrifugation and then tested for their cytotoxic activities on C57BL/6 thymocytes.

Cytotoxic Treatment of Spleen Cells with a Monoclonal Anti-Thy-1.2. 10 million/ml C57BL/6 spleen cells were reacted with 10 µg of monoclonal mouse anti-Thy-1.2 (F7D5) antibodies for 30 min at room temperature. The cells were washed once and incubated with 1:10 diluted guinea pig C for 45 min at 37°C. The cells were again treated with anti-Thy-1.2 and C and then fractionated by Ficoll-Conley (Pharmacia Fine Chemicals, Uppsala, Sweden) to remove dead cells.

Purification of T Cells from Spleen Cells by a Nylon Wool Column. Spleen cells from C57BL/6 mice were fractionated with a nylon wool column (LP-1 Leukopack Leukocyte Filters; Fenwal Inc., Walter Kidde & Co. Inc., Ashland, Mass.) by the method of Julius et al. (16). A yield of lymphoid cells from the nylon wool column was ~15% unfraccionated spleen cells. Purified populations contained ~75% Thy-1-positive cells and <1% of surface Ig-positive cells by a membrane fluorescence technique.

Cell Surface Staining. 1 million lymphocytes in 100 µl RPMI 1640 medium containing 1% FCS and 0.1% NaN₃ were reacted with 1 µg of monoclonal rat antibody (anti-Lyt-1, anti-Lyt-2, or anti-Thy-1.2) for 30 min at 4°C. Cells were washed twice and then stained with fluorescein-conjugated mouse anti-rat Ig reagents for 30 min at 4°C. Stained cells were analyzed by a fluorescence-activated cell sorter (FACS-IV; B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, Calif.).

Preparation of the KLH-specific Suppressor T Cell Factor (TsF) and the KLH-specific Augmenting T Cell Factor (TaF). The methods for obtaining KLH-specific TsF and TaF have been described in detail elsewhere (1, 2). In brief, mice were immunized intraperitoneally twice with 200 µg of KLH at 2-wk intervals. 2 wk after the last immunization, thymocytes or spleen cells from C57BL/6 or BALB/c mice were obtained and subsequently disrupted by freezing and thawing. The cell-free extracts were obtained by ultracentrifugation at 40,000 g for 1 h. Monoclonal KLH-TsF was extracted by the same method as above (17) from suppressor T cell hybridoma (34S-704) made by a fusion of AKR thymoma cells (BW5147) and KLH-specific suppressor T cells from C57BL/6 mice.

Absorption and Elution of KLH-TsF and KLH-TaF with Immunoadsorbent Columns. 10 mg KLH or 1 ml gamma globulin fraction of antisera was coupled with 1 ml of Sepharose 4B (Pharmacia Fine Chemicals) by the method of Axén et al. (18). They were packed in a 1-ml disposable plastic syringe. The KLH-TsF or KLH-TaF was applied to the immunoadsorbent columns and incubated for 1 h at 4°C. Effluent was collected by passing through the columns. After extensive washing with RPMI 1640 medium, the active materials were eluted from the columns with 2 ml of 0.175 M glycine HC1 buffer, pH 3.2, at 4°C. The eluted materials were instantly neutralized with 1 M sodium bicarbonate and dialyzed with phosphate-buffered saline. They were then concentrated by vacuum pressure. Both effluent and eluate were tested for activity in the in vitro secondary antibody response as described below.

In some experiments, monoclonal KLH-TsF extracted from 1 × 10⁸ hybridoma cells (34S-704) was absorbed with the BALB/c anti-CB-20 column to cover the reactive sites of antibodies specific for TsF. After washing the column with 20 ml chilled medium (RPMI 1640), it was incubated with medium for 1 h at 0°C. Effluent from the column was then added to the culture of DNP-KLH spleen cells on day 0 or day 2, and then tested for activity to confirm whether monoclonal KLH-TsF was discharged from the column during the incubation. Spleen cell extract from KLH-primed C57BL/6 mice equivalent to 1 × 10⁷ cells was subsequently absorbed with the monoclonal KLH-TsF preabsorbed column of the anti-CB-20. Activity of KLH-TsF or KLH-TaF in the effluent or the eluate from the column was tested in the in vitro antibody response by addition of the materials to the culture on day 0 or day 2.

In Vitro Culture System. 4 million spleen cells from C57BL/6 or BALB/c mice primed 6 wk previously with 100 µg of DNP-KLH and 10⁸ BPV were cultured in the Mishell-Dutton system in the presence of 0.1 µg/ml of DNP-KLH at 37°C in 5% CO₂ in air for 5 d. For assaying the TaF or the TsF activity, the cell-free extract from 1 × 10⁸ KLH-primed spleen or thymus cells, or 5 × 10⁸ hybridoma cells were added to the culture on day 0 or day 2, respectively. 5 d later,
the numbers of anti-DNP IgG plaque-forming cells (PFC) were assayed by using DNP-coupled erythrocytes.

**Statistical Analysis.** For in vitro assays, results were analyzed with a two-tailed Student's t test. Probability values of > 10% were considered to be insignificant.

**Results**

**T Cell Reactivity of BALB/c Anti-CB-20 Antiserum.** The allotype congenic mice pairs, BALB/c (H-2\textsuperscript{d}, Igh-1\textsuperscript{a}) and CB-20 (H-2\textsuperscript{d}, Igh-1\textsuperscript{b}), were used to produce the antiserum used in this study. It seemed possible that the antiserum would contain antibodies against the allotypic determinants on T cell surfaces encoded by genes linked to the Igh genes, as previous studies have suggested that the antigen-recognition units on T cells carry the determinants shared with the Igh-\textit{V} gene products. To investigate the reactivity of the BALB/c anti-CB-20 antiserum to T cells, the antiserum was absorbed with graded numbers of unfractionated thymus or spleen cells of C57BL/6 (Igh-1\textsuperscript{b}) or BALB/c (Igh-1\textsuperscript{a}) mice, or anti-Thy-1-treated C57BL/6 spleen cells. The residual cytotoxic activity of the antiserum thus absorbed was then tested on C57BL/6 thymocytes as a target. Fig. 1 shows that spleen cells and thymocytes from C57BL/6 but not from BALB/c mice completely absorbed the cytotoxic activity of the antiserum. The reactivity of the antiserum to the C57BL/6 thymocytes was preserved, however, even when the antiserum was absorbed with $5 \times 10^6$ C57BL/6 spleen cells treated twice with anti-Thy-1 and C. The results clearly indicate that the BALB/c anti-CB-20 antiserum is specific for the products on T cells but not on B cells.

**Genetic Specificity of BALB/c Anti-CB-20 Antiserum.** To demonstrate the genetic specificity of the BALB/c anti-CB-20 antiserum, the reactivity was investigated by the dye exclusion cytotoxic assay on thymocytes derived from various strains of mice including allotype congenic mice: C57BL/6 (H-2\textsuperscript{b}, Igh-1\textsuperscript{b}), BALB/c (H-2\textsuperscript{d}, Igh-1\textsuperscript{a}), BAB-14 (H-2\textsuperscript{d}, Igh-1\textsuperscript{b}), SJL (H-2\textsuperscript{a}, Igh-1\textsuperscript{b}), SJ A (H-2\textsuperscript{a}, Igh-1\textsuperscript{b}), C3H (H-2\textsuperscript{k}, Igh-1\textsuperscript{a}), CSW (H-2\textsuperscript{b}, Igh-1\textsuperscript{b}), and CWB (H-2\textsuperscript{b}, Igh-1\textsuperscript{b}). As shown in Fig. 2a, the antiserum...
killed 15–20% of the thymocytes from C57BL/6, BAB-14, CWB, and SJL mice, whereas no cytotoxic activity was observed on those from BALB/c, C3H, CSW, and SJA mice. All mouse strains sensitive to the antiserum carry IgH-1b allotype with different genetic background. It is clear that the antiserum recognizes the products expressed on T cells from IgH-1b mice.

The results shown in Fig. 2a were confirmed by quantitative absorption analysis. 30 μl of the 1:10 diluted antiserum was absorbed with graded numbers of thymocytes from various strains of mice. The residual cytotoxic activity was tested on C57BL/6 thymocytes. Fig. 2b shows cytotoxic curves of the antiserum after absorption with thymocytes from C57BL/6, BAB-14, BALB/c, CWB, CSW, SJL, or SJA mice. The cytotoxic activity of the antiserum was absorbed with thymocytes of C57BL/6, BAB-14, CWB, and SJL mice having IgH-1b allotype. No absorbing capacity was observed on BALB/c, CSW, or SJA thymocytes. Therefore, the absorption with IgH-1b thymocytes always removed antibody reactivity. The data in Fig. 2a and b suggest that the BALB/c anti-CB-20 antiserum contains antibodies directed against the allotypic determinants on T cells that are coded for by genes linked to the IgH genes. Thus, the determinants detected by the antiserum were shown to be encoded by genes located in the right side of the IgH-V gene locus, because the recombinant inbred strain, BAB-14, carries the IgH-C linked genes on the inserted chromosomal segment from C57BL/6 (Igh-1b) and the IgH-V linked genes from BALB/c (Igh-1a) mice (19).

Tissue Distribution of Cells Sensitive to BALB/c Anti-CB-20. To study the tissue distribution of cells sensitive to the antiserum, spleen, thymus, and lymph node cells from C57BL/6 mice were tested by a dye exclusion cytotoxic assay. The results shown in Fig. 3 demonstrate that the 1:10 diluted antiserum killed ~20% of the thymocytes (Thy-1+ >95%) or the lymph node cells (Thy-1+ 40%), and 5% of the spleen cells (Thy-1+ 30%). When spleen cells purified with the nylon wool column (splenic T, Thy-1+ 75%, Ig+ <1%) were treated with the 1:10 diluted antiserum and C, 25% were sensitive to the antiserum. These results indicate that some but not all peripheral T cells are also sensitive to the antiserum.

Subsets of T Cells Sensitive to BALB/c Anti-CB-20. Subpopulations of splenic T cells
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Fig. 3. Tissue distribution of the cells sensitive to BALB/c anti-CB-20 antiserum. Cytotoxicity of the antiserum was tested on nylon wool column-purified splenic T cells (□) and the unfractionated lymphoid cells from spleen (□), thymus (X), or lymph node (△) of C57BL/6 mice. The targets used contained >95% Thy-1+, <1% Ig+ cells in thymocytes, 30% Thy-1+, 50% Ig+ cells in spleen cells, 40% Thy-1+, 40% Ig+ cells in lymph node cells, and 75% Thy-1+, <1% Ig+ cells in nylon wool column-purified splenic T cells. Background was <5%.

FIG. 4. The cells sensitive to BALB/c anti-CB-20 in the subsets of splenic T cells with Lyt-2− or Lyt-2+ phenotypes. C57BL/6 splenic T cells purified with a nylon wool column were treated with the anti-CB-20 and C (△) or with NMS and C (▼). The cells thus treated were reacted with monoclonal rat anti-Thy-1.2, anti-Lyt-1, or anti-Lyt-2 antibodies, followed by staining with fluorescein-conjugated mouse (SJL) anti-rat Ig. They were subsequently analyzed by FACS-IV. The percentage of positively stained cells represents the analysis of 20,000 cells on FACS-IV.

Sensitive to the antiserum were investigated by membrane fluorescence technique and FACS analysis. C57BL/6 splenic T cells were treated with the 1:10 diluted antiserum and C. The remaining cells were treated with monoclonal rat antibodies (anti-Lyt-1, anti-Lyt-2, or anti-Thy-1.2) followed by staining with fluorescein-conjugated mouse anti-rat Ig. Splenic T cells treated with normal BALB/c serum (NMS) and C were used as controls. The treated cells were analyzed by FACS IV. The results in Fig. 4 demonstrate that 16–17% of Thy-1+ or Lyt-1+ population in the splenic T cells were eliminated by the treatment with the BALB/c anti-CB-20 and C. Similarly, ~5% Lyt-2+ T cells were shown to be sensitive to the antiserum. Thus, the antiserum contains antibodies reactive to some T cells belonging to both Lyt-2− and Lyt-2+ T cells. The results in Fig. 5 show the fluorescence profiles of the Thy-1+ splenic T cells treated with BALB/c anti-CB-20 or NMS and C. The antiserum selectively eliminated the Thy-1 dull-stained population in the splenic T cells.

Absorption of KLH-TsF with the Column of BALB/c Anti-CB-20. The experiments
Fig. 5. Fluorescence profiles of Thy-1+ splenic T cells with or without treatment of BALB/c anti-CB-20. Splenic T cells (75% Thy-1+ cells) purified with a nylon wool column were treated with the anti-CB-20 and C (—) or with NMS and C (—). The cells were subsequently reacted with a monoclonal rat anti-Thy-1.2 antibodies followed by staining with fluorescein-conjugated mouse (SJL) anti-rat Ig. Background (cells stained with fluorescein-conjugated mouse anti-rat Ig) is shown as a dotted line. Fluorescence distributions were analyzed by FACS-IV fitted with a logarithmic amplifier.

Table I
Successful Absorption of the KLH-TsF from C57BL/6 (Igh-1b) but Not from BALB/c (Igh-1a) Mice with the Column of BALB/c Anti-CB-20

| KLH-TsF absorbed with columns* | Materials added      | Anti-DNP IgG PFC/culture |
|-------------------------------|----------------------|--------------------------|
|                               |                      | C57BL/6 §§                 |
|                               |                      |  P value  | BALB/c §§ |  P value  |
| Unabsorbed TsF                | —                    | 4,020 ± 180 | —         | 16,600 ± 1,700 | —         |
| —                              | Effluent             | 120 ± 50 | <0.001   | 8,100 ± 900 | <0.001   |
| BALB/c anti-CB-20             | —                    | 3,180 ± 360 | NS        | 8,300 ± 200 | <0.001   |
| BALB/c anti-CB-20             | Eluate               | 550 ± 390 | <0.001   | 15,200 ± 1,600 | NS       |
| KLH                            | Effluent             | 3,500 ± 260 | NS        | 15,800 ± 1,500 | NS       |
| KLH                            | Eluate               | 950 ± 170 | <0.001   | 6,800 ± 500 | <0.001   |

* The extract of spleen cells from KLH-primed mice equivalent to 1 × 10⁷ cells was absorbed to and eluted from immunoadsorbent columns. The effluent or the eluate of the C57BL/6 or BALB/c TsF from the columns was added to the culture of DNP-KLH-primed spleen cells on day 0.
‡ DNP-KLH-primed spleen cells from C57BL/6 mice were cultured with the effluent or the eluate of C57BL/6 KLH-TsF from the columns.
§ DNP-KLH-primed spleen cells from BALB/c mice were cultured with the effluent or the eluate of BALB/c KLH-TsF from the columns.

Arithmetic means and standard deviations of four cultures.

The results were designed to examine whether the determinants recognized by the BALB/c anti-CB-20 were related to the antigen-recognition units on T cells, because the factor derived from suppressor T cells was found to bear the antigen-binding moiety (1). The KLH-TsF obtained from C57BL/6 (Igh-1b) or BALB/c (Igh-1a) mice was absorbed with the immunoadsorbent columns of KLH or the BALB/c anti-CB-20. The absorbed materials were also eluted from the columns with glycine-HCl buffer. Both effluent and eluate were added to the in vitro secondary response of DNP-KLH-primed C57BL/6 or BALB/c spleen cells on day 0 and tested for their suppressor activities. As shown in Table I, the suppressor activity of the KLH-TsF from C57BL/6 mice was absorbed to and eluted from the column of the anti-CB-20 antiserum, whereas the BALB/c derived KLH-TsF did not react to the same column. Furthermore, the factors from C57BL/6 and BALB/c mice were antigen specific, since the activities were absorbed to and eluted from the KLH column. The results
Successful Absorption of the KLH-TaF from C57BL/6 (Igh-1b) but Not from BALB/c (Igh-1a) Mice with the Column of BALB/c Anti-CB-20

| KLH-TaF absorbed with columns* | Materials added | Anti-DNP IgG PFC/culture |
|--------------------------------|-----------------|--------------------------|
|                                |                 | C57BL/6/§ P value | BALB/c§ P value |
| Unabsorbed TaF                 |                 | 5,290 ± 830 | 4,330 ± 1,130 |
| BALB/c anti-CB-20 Effluent     |                 | 11,835 ± 835 | <0.001 | 10,400 ± 500 | <0.001 |
| BALB/c anti-CB-20 Eluate       |                 | 3,680 ± 1,080 | NS | 11,550 ± 1,560 | NS |
| KLH Effluent                   |                 | 12,870 ± 795 | <0.001 | 4,225 ± 835 | NS |
| KLH Eluate                     |                 | 3,705 ± 600 | NS | 4,800 ± 1,040 | NS |

* The extract of thymocytes from KLH-primed mice equivalent to 1 × 10^7 cells was absorbed to and eluted from immunoadsorbent columns. The effluent or the eluate of the C57BL/6 or BALB/c TaF from the columns was added to the culture of DNP-KLH-primed spleen cells on day 2.
† DNP-KLH-primed spleen cells from C57BL/6 mice were cultured with the effluent or the eluate of C57BL/6 KLH-TaF from the columns.
§ DNP-KLH-primed spleen cells from BALB/c mice were cultured with the effluent or the eluate of BALB/c KLH-TaF from the columns.
¶ Arithmetic means and standard deviations of four cultures.

Presence of Two Distinct Antibodies in BALB/c Anti-CB-20, Each of Which Defines the KLH-TsF or the KLH-TaF

| Anti-CB-20 column | Materials added | Anti-DNP IgG PFC/culture |
|-------------------|-----------------|--------------------------|
| Absorbed with     | Subsequently    | Monoclonal TaF added on  |
|                   | absorbed with*  | C57BL/6-extract added on |
|                   |                 | day 0 | day 0 | day 2 |
|                  |                 | 2,050 ± 250 | 7,320 ± 195 | 5,990 ± 195 |
|                  |                 | 500 ± 390 | 830 ± 60 | 10,710 ± 683 |
| Monoclonal TaF§   | Effluent        | 1,580 ± 190 | ND | ND |
| Monoclonal TaF§   | Eluate          | 170 ± 150 | ND | ND |
| Monoclonal TaF§   | Medium Effluent | ND | 7,210 ± 580 | 4,540 ± 670 |
| Monoclonal TaF§   | C57BL/6 factor Effluent | ND | 650 ± 670 | 5,380 ± 310 |
| Monoclonal TaF§   | C57BL/6 factor Eluate | ND | ND | 10,430 ± 120 |

* The anti-CB-20 column, which had been absorbed with a large quantity of the monoclonal KLH-TaF, was extensively washed and was further incubated with medium (RPMI 1640) for 1 h at 0°C. The effluent from the column was then added to the culture on day 0 or day 2, and was tested for the activity to confirm whether the active materials were discharged from the column. Spleen cell extract from KLH-primed C57BL/6 mice equivalent to 1 × 10^7 cells was subsequently absorbed with the monoclonal TaF preabsorbed anti-CB-20 column. The effluent or the eluate from the column was added to the culture of DNP-KLH-primed C57BL/6 spleen cells on day 0 or day 2 in order to test suppressor or enhancing activity.
† Arithmetic means and standard deviations of four cultures.
§ Monoclonal KLH-TaF from the T cell hybridoma (34S-704) equivalent to 5 × 10^6 cells was absorbed to the anti-CB-20 column. The absorbed materials were eluted from the column with acid. The eluate or the effluent was tested for its suppressor activity in the in vitro secondary response of DNP-KLH-primed C57BL/6 spleen cells.
¶ Not done.

strongly suggest that the antiserum reacts to the allelotype determinants on the antigen-specific suppressor T cell factor.

Absorption of KLH-TaF with the Column of BALB/c Anti-CB-20. It is known that the soluble extract obtained from KLH-primed spleen cells or thymocytes augments antibody responses in an antigen-specific fashion when the factor was added to culture on day 2 (2). Furthermore, antigen-specific TaF was also found to bear the antigen-
binding moiety and the products encoded by genes in the {\textit{I-A}} subregion of the {\textit{H-2}} complex (2). As a result, experiments were carried out to establish whether the KLH-TaF could have determinants detected by the BALB/c anti-CB-20.

The KLH-TaF from primed spleen cells of C57BL/6 or BALB/c mice was absorbed with the immunoabsorbent columns of KLH or the antiserum. Augmenting activities in the effluent or the eluate from the columns were tested by addition of the materials to the in vitro secondary culture on day 2. Table II shows results demonstrating that KLH-TaF from both C57BL/6 and BALB/c mice were absorbed to and eluted from the KLH column. The column composed of the anti-CB-20 antiserum, however, absorbed KLH-TaF from C57BL/6 but not from BALB/c mice. These results indicate that the anti-CB-20 antiserum also detects allotypic determinants on the antigen-specific TaF.

**BALB/c Anti-CB-20 Defines Two Distinct Allotypic Determinants on the Antigen-recognition Units on T Cells, Each of Which Is Expressed on the KLH-TaF or the KLH-TsF.** In our previous experiments, monoclonal KLH-TsF derived from T cell hybridoma (34S-704) made by the fusion of a thymoma cell line, BW5147, and C57BL/6 (Igh-1\textsuperscript{b}) KLH-specific suppressor T cells, was shown to have the same immunochemical and physicochemical properties as that of conventional KLH-TsF (17, 20). In fact, the results shown in the upper part of Table III demonstrate that the monoclonal KLH-TsF, like the C57BL/6-derived KLH-TsF, carried the determinants recognized by the anti-CB-20 antiserum, because activity of the monoclonal KLH-TsF was absorbed to and eluted from the anti-CB-20 column.

Based on these results, experiments were carried out to investigate whether the determinants on the KLH-TsF detected by the anti-CB-20 antiserum are distinct from those on the KLH-TaF. The anti-CB-20 column was incubated with monoclonal KLH-TsF in extracted materials equivalent to {\textit{1 × 10\textsuperscript{6}}} hybridoma cells at 0°C for 1 h, and then extensively washed with the medium. 500 \mu l RPMI 1640 was further incubated with the column at 0°C for 1 h. The effluent from the column was added to the culture on day 0 or day 2 to test whether the active materials were discharged from the column during the 1-h incubation. As shown in Table III, the effluent from the anti-CB-20 column absorbed with a large amount of the monoclonal KLH-TsF had no detectable suppressor and enhancing activities, indicating that active materials were not discharged from the column during incubation with medium. Therefore, KLH-primed spleen cell extract of C57BL/6 mice equivalent to {\textit{1 × 10\textsuperscript{7}}} cells was subsequently absorbed with the anti-CB-20 column preabsorbed with a large amount of monoclonal KLH-TsF. Suppressor or enhancing activity in the effluent or the eluate from the column was tested in the in vitro secondary response by addition of materials to culture on day 0 or day 2. As shown in the lower part of Table III, enhancing activity was absorbed to and eluted from the column, whereas there was no longer any suppressor activity absorbed. These results indicate that the monoclonal KLH-TsF preabsorbed column preserves the capacity to absorb KLH-TaF but not KLH-TsF.

**Discussion**

It is known that two distinct types of T cell factors, with different functions, are found in materials extracted from spleen or thymus cells of mice hyperimmunized with KLH (1, 2). These factors, KLH-TsF and -TaF, can suppress or enhance the
antihapten IgG PFC responses to DNP-KLH (1, 2, 21). Furthermore, we have clearly
shown that both KLH-TsF and KLH-TaF bear the antigen-binding moiety and the
products coded for by genes in the I subregion of the H-2 complex (1, 2). Subregion
specificities of the I region products on the KLH-specific TsF and TaF were serologically
determined to be the I-J and I-A subregion of the H-2 complex, respectively (2, 21).

Imunochemical analyses of KLH-TsF derived from T cell hybridomas have also
shown that the suppressor factor is composed of two distinct polypeptide chains, i.e.,
the antigen-binding and the I-J-encoded chains in covalent or noncovalent association,
whereas no Ig constant region determinant has been demonstrated (1, 17, 20, 22).
Recent reports from various laboratories (6-12) have, however, indicated that the
antigen-specific helper and suppressor factors carry a structure analogous or identical
to the V region of Ig. As a result, it is conceivable that at least two genes are involved
in coding for the structural entity of antigen-specific T cell factors: one located in the
I subregion of the H-2 complex, and the other linked to the Ig region. If the variable
region of the antigen-binding moiety on the T cell factors is encoded by genes in the
Igh linkage group, the constant region determinants of the antigen-recognition units
on T cells or T cell factors should be encoded by genes on the same chromosome as
those coding for the antigen-binding structure. No report, however, has formally
demonstrated the presence of the constant region determinants on the antigen-
recognition units on T cells.

In this respect, the combination of BALB/c and CB-20 mice is ideal for producing
antibodies against the products encoded by genes in the Igh linkage group. The Igh
allotype congenic mice, CB-20, are derived from a cross between BALB/c (Igh-1a)
and C57BL/6 (Igh-1b), and carry BALB/c background genes with the chromosomal
segment of C57BL/6 origin that extends to somewhere around the Igh locus. Thus,
the genetic difference between BALB/c and CB-20 mice is the Igh-linked
genes on the 12th chromosome.

We have in fact been successful in obtaining antibodies reactive to the allotypic
determinants expressed on T cells of Igh-1b mice by repeated immunization of BALB/c
mice with Con A-stimulated CB-20 spleen cells. The BALB/c anti-CB-20 antiserum
killed 5-20% Thy-1+ T cells in the lymphoid organs including thymus, spleen, and
lymph node (Figs. 3 and 4). The reactivity of the antiserum to the T cell population
was confirmed by the quantitative absorption analysis with lymphoid cells. As shown
in Fig. 1, the antibody activity was completely absorbed with thymus and spleen
cells, whereas spleen cells treated twice with anti-Thy-1 and C (Thy-1 negative
population) did not absorb the reactivity to thymocytes at all. The results indicate
that BALB/c anti-CB-20 antiserum definitely contains antibodies against the alloan-
tigenic determinant on T cells but not on B cells. The products detected by antiserum
were found to be expressed on splenic T cells far more than on thymocytes, because
2 × 10^8 spleen cells containing 30% T cells (in which only 5% were sensitive to the
antiserum) (Fig. 3) had the same capacity to absorb activity as 2 × 10^6 thymocytes
(Fig. 1). Moreover, the antiserum selectively killed the Thy-1 dull-stained population
in splenic T cells (Fig. 5). In general, T cells lose Thy-1 antigens on their surface with
the progression of maturation (23). Thus, mature T cells are more sensitive to the
antiserum than thymocytes. This is in agreement with the data reported by Owen et
al. (13).
The genetic specificity of the anti-CB-20 antiserum was determined in two ways. First, the antibody activity was tested by the dye exclusion cytotoxic assay on thymocytes from various strains of mice with different Igh allotypes. Second, quantitative absorption studies with thymocytes from various Igh allotype mice were carried out. The absorbed antiserum was then tested for cytotoxic activity on thymocytes of Igh-1<sup>b</sup> mice. From these experiments, antibodies were shown to be reactive with thymocytes of Igh-1<sup>b</sup> mice but not of Igh-1<sup>a</sup> and Igh-1<sup>c</sup> mice. It is clear then that the antiserum recognizes the allotypic determinants expressed only on T cells of the Igh-1<sup>b</sup> mice, and that they are distinct from B cell Igh allotype.

The antiserum reacted more specifically to BAB-14 thymocytes. The recombinant inbred mice, BAB-14, are derived from a cross between BALB/c (Igh-1<sup>a</sup>) and C57BL/6 (Igh-1<sup>b</sup>) mice. They carry the Igh-<i>V</i><sub>Dex</sub>, the anti-dextran idiotype locus, from BALB/c, and the Igh-<i>C</i> genes from C57BL/6 mice (19). Therefore, the inserted chromosomal segment of C57BL/6 origin on the 12th chromosome of BAB-14 mice seems to be extended from the cross-over point between the Igh-<i>V</i> genes and the Igh-<i>C</i> to somewhere in the right side of the Igh-<i>C</i>. As shown in Fig. 2b, antibody activity was completely absorbed with BAB-14 thymocytes. The results show conclusively that the antiserum has specificity to products of genes located in the right of the Igh-<i>V</i> genes.

Similar observations have been reported by Owen et al. (13-15), demonstrating that the immunization of BALB/c mice with Con A-stimulated azobenzen arsonate (ARS)-primed CAL-20 splenic T cells produced antibodies recognizing the surface products (Tsu<sup>a</sup>) of Lyt-2<sup>+</sup> suppressor T cells. The Tsu<sup>a</sup> has been demonstrated to be controlled by a gene linked to the right side of the Igh gene cluster. Furthermore, the anti-Tsu<sup>a</sup> antiserum can stimulate Lyt-2<sup>+</sup> suppressor T cells that suppress the antibody responses to the unrelated antigens (sheep erythrocytes, DNP-KLH, and ARS-KLH) (14). From these results, they have postulated that the Tsu<sup>a</sup> antigen is associated with the T cell receptor. However, they have not formally demonstrated direct evidence that the anti-Tsu<sup>a</sup> antiserum recognizes the determinants on the T cell receptor.

Our results, illustrated in Tables I and II, provided explicit evidence that the anti-CB-20 antiserum recognized the determinants on the antigen-binding units of the KLH-specific suppressor and enhancing T cell factors, since the BALB/c anti-CB-20 antiserum should not contain antibodies against the I region gene products that are parts of the KLH-TsF and TaF. Furthermore, we have recently confirmed that the anti-CB-20 antiserum definitely reacts to the antigen-binding molecule on the KLH-TsF (T. Tokuhisa and M. Taniguchi, manuscript in preparation).

As the anti-CB-20 antiserum was shown to be reactive to the products controlled by genes in the right side, but not within the side of Igh-<i>V</i>, it is likely that the antiserum reacts to the allotypic determinants of the constant region of the T cell factors. However, we cannot exclude the possibility that the antiserum recognizes the allotypic determinants on the V region of the antigen-binding molecule of T cell factors if there are sets of T cell V gene clusters in the right side of the B cell Igh-<i>V</i>.

A striking difference between Owen’s (13-15) and our antisera is that our anti-CB-20 antiserum contains at least two distinct antibodies with different specificities: the one specific for antigen-specific TsF, and the other for the antigen specific TaF. Owen’s antiserum reacts only to the Lyt-2<sup>+</sup> suppressor T cells. As shown in Table III, the immunoadsorbent column of the anti-CB-20 preabsorbed with a large quantity of monoclonal KLH-TsF could preserve the absorbing capacity of KLH-TaF from Igh-
$I^b$ mice, while conventional KLH-TsF was no longer absorbed with the same column. These results were supported by the evidence that T cells sensitive to anti-CB-20 belonged to both Lyt-2$^-$ and Lyt-2$^+$ populations (Fig. 4), each of which produces antigen-specific TaF and TsF. Consequently, it is apparent that antigen-specific TaF and TsF carry the distinct allotypic determinants on the antigen-recognition units, both of which are encoded by genes located in the right side of $Igh-V$ genes.

The allotypic determinants detected by the anti-CB-20 antiserum seem to be commonly shared within each functional activity of the respective T cell factors, as preabsorption of the anti-CB-20 column with the monoclonal KLH-TsF completely eliminated antibody activity against the conventional KLH-TsF (Table III). As the antiserum was produced by immunizations with unprimed Con A-stimulated CB-20 spleen cells, it should not contain antibodies against idiotype-like determinants on the T cell antigen-receptors. Thus, it is conceivable that allotypic markers defined by antiserum represent determinants on the antigen-binding molecules of antigen-specific T cell factors outside of the antigen-binding site (i.e., constant region determinants). If so, every T cell factor with a particular function may express a structurally related determinant like the $Igh-C$ allotype. In this sense, it is reasonable to consider that the cluster of genes coding for different allotypic markers on the constant region of functional T cell factors or receptors is located somewhere around the $Igh$-genes on the 12th chromosome.

Summary

The alloantiserum was raised in BALB/c ($H-2^d$, $Igh-1^a$) mice hyperimmunized with spleen cells of $Igh$ allotype congenic mice, CB-20 ($H-2^d$, $Igh-1^b$). It was found to define the new allotypic determinants (distinct from B cell $Igh$ constant region determinants: $Igh$ allotype) expressed only on a small population of T cells belonging to the Thy-1 dull-stained Lyt-2$^-$ or Lyt-2$^+$ population of $Igh-I^b$ mice. Genes coding for the determinants were shown to be accommodated somewhere in the right side of the $Igh$ variable region gene ($Igh-V$) cluster, as the antibody activity was completely absorbed with BAB-14 thymocytes. It was also demonstrated that the products detected by the antiserum represent the allotypic determinants (probably constant region determinants) on the antigen-binding moiety of the antigen-specific augmenting (TaF) and suppressor (TsF) T cell factors. Moreover, determinants on TsF were found to be distinct from those on TaF. Therefore, it can be suggested that the two genes coding for the T cell allodeterminants (distinct from those of the B cell Igh) are located in the right side of the B cell $Igh-V$ on the 12th chromosome, and that both encode the antigen-recognition units of the functionally distinct T cell factors.

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