

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

BY TAKESHI TOKUHISA AND MASARU TANIGUCHI

*From the Department of Immunology, School of Medicine, Chiba University, Chiba, Japan*

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)<sup>1</sup> 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<sup>d</sup>* mice (Tsu<sup>d</sup>) encoded by genes adjacent to the *Igh* constant-region genes, *Igh-C*, on the side toward the prealbumin gene, *Pre-1*. The Tsu<sup>d</sup> has been found to be expressed on Lyt-2<sup>+</sup> suppressor T cells. Furthermore, the anti-Tsu<sup>d</sup> antisera stimulate suppressor T cells of mice that have an appropriate *Igh* allotype and block the binding

<sup>1</sup> 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<sup>d</sup>, allotypic determinant on suppressor T cells of *Igh-1<sup>d</sup>* mice; V, variable.

of idiotype-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 alloantiserum 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<sup>-</sup> or Lyt-2<sup>+</sup> T cells of mice expressing *Igh-1<sup>b</sup>* allotype. The allotypic determinants recognized by the antiserum 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 antiserum is distinct from that on the antigen-specific helper (augmenting) T cell factor.

### Materials and Methods

**Animals.** BALB/c CrSlc (*H-2<sup>d</sup>*, *Igh-1<sup>a</sup>*), C57BL/6 CrSlc (*H-2<sup>b</sup>*, *Igh-1<sup>b</sup>*), and C3H/HeSlc (*H-2<sup>k</sup>*, *Igh-1<sup>j</sup>*) mice were purchased from Shizuoka Experimental Animal Laboratory, Co. Ltd., Hamamatsu, Japan. SJL/J (*H-2<sup>s</sup>*, *Igh-1<sup>b</sup>*) and allotype congenic mice, CB-20 (*H-2<sup>d</sup>*, *Igh-1<sup>b</sup>*), BAB-14 (*H-2<sup>d</sup>*, *Igh-1<sup>b</sup>*), SJA/Hz (*H-2<sup>s</sup>*, *Igh-1<sup>a</sup>*), C3H.SW/Hz (CSW; *H-2<sup>b</sup>*, *Igh-1<sup>j</sup>*), and CWB/Hz (*H-2<sup>b</sup>*, *Igh-1<sup>b</sup>*) 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 (DNP<sub>770</sub>-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 alloantiserum was prepared by hyperimmunization of BALB/c mouse intravenously with  $1 \times 10^7$  of CB-20 spleen cells precultivated with 5  $\mu\text{g}/\text{ml}$  of concanavalin A (Con A; Sigma Chemical Co., St. Louis, Mo.) for 48 h at 37°C in a 5% CO<sub>2</sub> incubator. The Con A-stimulated CB-20 spleen cells were treated with 100  $\mu\text{g}/\text{ml}$  of mitomycin C (Kyowa Hakkō 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 antiserum 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  $\mu\text{l}$  of  $1 \times 10^7/\text{ml}$  lymphoid cells from different tissues suspended in RPMI 1640 medium containing 1% fetal calf serum (FCS) and 0.1% NaN<sub>3</sub> was incubated with 10  $\mu\text{l}$  of diluted antiserum for 30 min at 4°C. After one washing the cells were treated with 20  $\mu\text{l}$  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:

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

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

**Quantitative Absorption of BALB/c Anti-CB-20 Antiserum with Cells.** Quantitative absorption studies were carried out in a V-bottomed microtiter plate (Dynatech Laboratories, Inc.,

Dynatech Corp., Alexandria, Va.). 30  $\mu$ l 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  $\mu$ g 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% unfractionated 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  $\mu$ l RPMI 1640 medium containing 1% FCS and 0.1%  $\text{NaN}_3$  were reacted with 1  $\mu$ g 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  $\mu$ g 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 Immunoabsorbent 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 immunoabsorbent 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 HCl 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  $1 \times 10^9$  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  $1 \times 10^7$  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  $\mu$ g of DNP-KLH and  $10^9$  BPV were cultured in the Mishell-Dutton system in the presence of 0.1  $\mu$ g/ml of DNP-KLH at 37°C in 5%  $\text{CO}_2$  in air for 5 d. For assaying the TsF or the TaF activity, the cell-free extract from  $1 \times 10^7$  KLH-primed spleen or thymus cells, or  $5 \times 10^6$  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 ( $H-2^d$ ,  $Igh-1^a$ ) and CB-20 ( $H-2^d$ ,  $Igh-1^b$ ), 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-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 ( $Igh-1^b$ ) or BALB/c ( $Igh-1^a$ ) 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 ( $H-2^b$ ,  $Igh-1^b$ ), BALB/c ( $H-2^d$ ,  $Igh-1^a$ ), BAB-14 ( $H-2^d$ ,  $Igh-1^b$ ), SJL ( $H-2^s$ ,  $Igh-1^b$ ), SJA ( $H-2^s$ ,  $Igh-1^a$ ), C3H ( $H-2^k$ ,  $Igh-1^j$ ), CSW ( $H-2^b$ ,  $Igh-1^j$ ), and CWB ( $H-2^b$ ,  $Igh-1^b$ ). As shown in Fig. 2 a, the antiserum

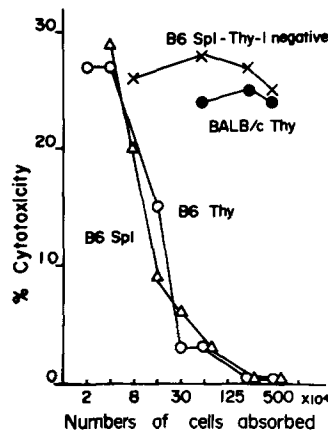


FIG. 1. Reactivity of BALB/c anti-CB-20 antiserum against T cells. Quantitative absorption analyses of the antiserum with lymphocytes were carried out. 30  $\mu$ l of the 1:10 diluted antiserum was absorbed with graded numbers of unfractionated thymus or spleen cells from C57BL/6 ( $Igh-1^b$ ) or BALB/c ( $Igh-1^a$ ) mice or C57BL/6 spleen cells treated with anti-Thy-1 and C. The residual cytotoxic activity of the antiserum thus absorbed was tested on C57BL/6 thymocytes (see Materials and Methods). The background in this experiment was <5%.

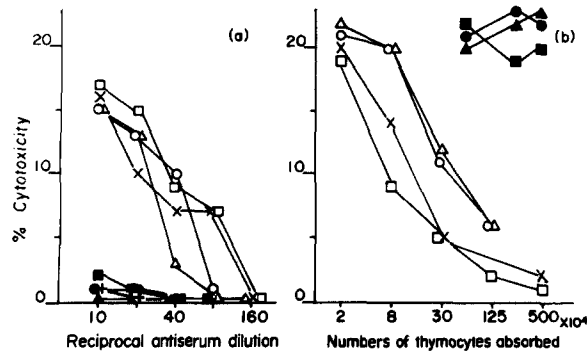


FIG. 2. Genetic specificity of BALB/c anti-CB-20 antiserum. (a) Cytotoxic activity of the antiserum on thymocytes from various strains of mice, i.e., C57BL/6 (*Igh-1<sup>b</sup>*) (x), BALB/c (*Igh-1<sup>a</sup>*) (■), BAB-14 (*Igh-1<sup>b</sup>*) (□), CWB (*Igh-1<sup>b</sup>*) (Δ), C3H (*Igh-1<sup>l</sup>*) (+), CSW (*Igh-1<sup>l</sup>*) (▲), SJL (*Igh-1<sup>b</sup>*) (○), and SJA (*Igh-1<sup>a</sup>*) (●). (b) Quantitative absorption of 1:10 diluted anti-CB-20 antiserum with graded numbers of thymocytes from various strains of mice. The residual cytotoxic activity of the absorbed antiserum was tested on C57BL/6 thymocytes. Symbols are the same as those in a. Background was <5%.

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-1<sup>b</sup>* allotype with different genetic background. It is clear that the antiserum recognizes the products expressed on T cells from *Igh-1<sup>b</sup>* mice.

The results shown in Fig. 2a were confirmed by quantitative absorption analysis. 30  $\mu$ l 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-1<sup>b</sup>* allotype. No absorbing capacity was observed on BALB/c, CSW, or SJA thymocytes. Therefore, the absorption with *Igh-1<sup>b</sup>* 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-1<sup>b</sup>*) and the *Igh-V* linked genes from BALB/c (*Igh-1<sup>a</sup>*) 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<sup>+</sup> >95%) or the lymph node cells (Thy-1<sup>+</sup> 40%), and 5% of the spleen cells (Thy-1<sup>+</sup> 30%). When spleen cells purified with the nylon wool column (splenic T, Thy-1<sup>+</sup> 75%, Ig<sup>+</sup> <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

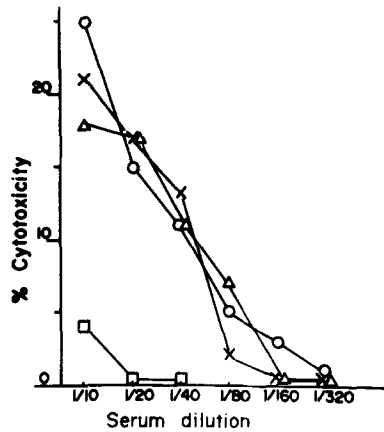


FIG. 3. Tissue distribution of the cells sensitive to BALB/c anti-CB-20 antiserum. Cytotoxicity of the antiserum was tested on nylon wool column-purified splenic T cells (○) and the unfractionated lymphoid cells from spleen (□), thymus (×), or lymph node (△) of C57BL/6 mice. The targets used contained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

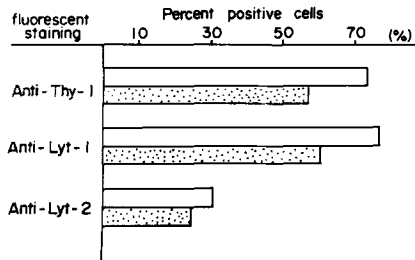


FIG. 4. The cells sensitive to BALB/c anti-CB-20 in the subsets of splenic T cells with Lyt-2<sup>-</sup> or Lyt-2<sup>+</sup> phenotypes. C57BL/6 splenic T cells purified with a nylon wool column were treated with the anti-CB-20 and C (▨) or with NMS and C (▩). The cells thus treated were reacted with 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<sup>+</sup> or Lyt-1<sup>+</sup> population in the splenic T cells were eliminated by the treatment with the BALB/c anti-CB-20 and C. Similarly, ~5% Lyt-2<sup>+</sup> 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<sup>-</sup> and Lyt-2<sup>+</sup> T cells. The results in Fig. 5 show the fluorescence profiles of the Thy-1<sup>+</sup> 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

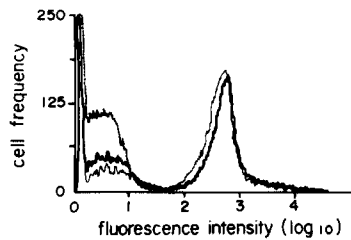


FIG. 5. Fluorescence profiles of Thy-1<sup>+</sup> splenic T cells with or without treatment of BALB/c anti-CB-20. Splenic T cells (75% Thy-1<sup>+</sup> cells) purified with a nylon wool column were treated with the anti-CB-20 and C (—) or with NMS and C (---). The cells were subsequently reacted with 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-1<sup>b</sup>*) but Not from BALB/c (*Igh-1<sup>a</sup>*) Mice with the Column of BALB/c Anti-CB-20

KLH-TsF absorbed with columns*	Materials added	Anti-DNP IgG PFC/culture			
		C57BL/6‡	<i>P</i> value	BALB/c§	<i>P</i> value
—	—	4,020 ± 180	—	16,600 ± 1,700	—
—	Unabsorbed TsF	120 ± 50	<0.001	8,100 ± 900	<0.001
BALB/c anti-CB-20	Effluent	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  $1 \times 10^7$  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.

¶ Not significant.

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-1<sup>b</sup>*) or BALB/c (*Igh-1<sup>a</sup>*) 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

TABLE II  
*Successful Absorption of the KLH-TaF from C57BL/6 (Igh-1<sup>b</sup>) but Not from BALB/c (Igh-1<sup>a</sup>) 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
—	—	5,280 ± 830	—	4,330 ± 1,130	—
—	Unabsorbed TaF	11,835 ± 835	<0.001	10,400 ± 500	<0.001
BALB/c anti-CB-20	Effluent	5,680 ± 1,080	NS¶	11,550 ± 1,560	<0.01
BALB/c anti-CB-20	Eluate	12,870 ± 795	<0.001	4,225 ± 835	NS
KLH	Effluent	5,705 ± 600	NS	4,800 ± 1,040	NS
KLH	Eluate	11,385 ± 1,185	<0.001	10,100 ± 695	<0.01

\* The extract of thymocytes from KLH-primed mice equivalent to  $1 \times 10^7$  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.

¶ Not significant.

TABLE III  
*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 absorbed with*		Monoclonal TsF added on	C57BL/6-extract added on	
			day 0	day 0	day 2
—	—	—	2,050 ± 250‡	7,730 ± 195	5,090 ± 195
—	—	Unabsorbed factor	500 ± 390	830 ± 60	10,710 ± 685
Monoclonal TsF§	—	Effluent	1,520 ± 190	ND	ND
Monoclonal TsF§	—	Eluate	170 ± 150	ND	ND
Monoclonal TsF¶	Medium	Effluent	ND	7,210 ± 580	4,540 ± 670
Monoclonal TsF¶	C57BL/6 factor	Effluent	ND	980 ± 670	5,380 ± 710
Monoclonal TsF¶	C57BL/6 factor	Eluate	ND	ND	10,430 ± 720

\* The anti-CB-20 column, which had been absorbed with a large quantity of the monoclonal KLH-TsF, 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  $1 \times 10^7$  cells was subsequently absorbed with the monoclonal TsF-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-TsF from the T cell hybridoma (34S-704) equivalent to  $5 \times 10^5$  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.

¶ Monoclonal KLH-TsF in hybridoma extract equivalent to  $1 \times 10^9$  cells was applied to the anti-CB-20 column to cover the binding site of antibodies specific for TsF.

strongly suggest that the antiserum reacts to the allotypic 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 *I-A* subregion of the *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<sup>b</sup>*) 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  $1 \times 10^9$  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  $1 \times 10^7$  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).

Immunochemical 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 Igh. 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 *Igh* genes. 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-1<sup>a</sup>*) and C57BL/6 (*Igh-1<sup>b</sup>*), 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-1<sup>b</sup>* 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<sup>+</sup> 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 alloantigenic 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  $2 \times 10^6$  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  $2 \times 10^6$  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>j</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-V Dex*, the anti-dextran idiotype locus, from BALB/c, and the *Igh-C* 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-V* and the *Igh-C* to somewhere in the right side of the *Igh-C*. 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-V* 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>d</sup>) of Lyt-2<sup>+</sup> suppressor T cells. The Tsu<sup>d</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>d</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>d</sup> antigen is associated with the T cell receptor. However, they have not formally demonstrated direct evidence that the anti-Tsu<sup>d</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-V*, 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-V*.

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  $Igh-1^b$  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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