

# REPERTOIRES OF T CELLS DIRECTED AGAINST A LARGE PROTEIN ANTIGEN, $\beta$ -GALACTOSIDASE

## II. Only Certain T Helper or T Suppressor Cells Are Relevant in Particular Regulatory Interactions

By URSZULA KRZYCH,\* AUDREE V. FOWLER,<sup>‡</sup> AND ELI E. SERCARZ\*

*From the Departments of \*Microbiology and <sup>‡</sup>Biological Chemistry, University of California at  
Los Angeles, Los Angeles, California 90024*

Suppressor cell interactions with their target cells have been studied in a variety of systems, often involving polypeptide antigens of unknown amino acid sequence such as keyhole limpet hemocyanin (KLH)<sup>1</sup> (1) and L-glutamic acid-L-alanine-L-tyrosine (GAT) (2), in which it is impossible to establish the relationship between epitopes recognized by the interacting T cell populations. Several antigen systems, however, offer the possibility of determining the specificity repertoires of T suppressor (Ts), T helper (Th), and other T regulatory cells, e.g., lysozyme (3), glycoporphin (4), myelin basic protein (5), and  $\beta$ -galactosidase (GZ) (6). In an effort to understand the molecular basis of the suppressive interactions involving Ts, Th, and B cells in the GZ system, we have initiated studies that we hope will provide a thorough description of the specificity repertoire of Ts directed against epitopes on this large protein (molecular weight of tetramer, 465,000). Little is known about the physical state of a large protein and its epitopes at the moment of confrontation by the variety of cells in the immune system. A large protein might be transformed into a smaller entity after *in vivo* macrophage processing, and then linked to surface macromolecules, or, conceivably, it could be recognized *in situ* in its native state by, for example, B cell receptors. GZ appeared to be suitable for exploring these topics because it is one of the few large proteins whose amino acid sequence is known (7), and because of the ready availability of its cyanogen bromide (CB)- and trypsin-cleaved peptides (70% of the whole GZ molecule was available in the form of purified peptides).

In this report, we assess the ability of Ts cells, raised against each of the identifiable Ts-inducing peptides of GZ, to affect Th cells, directed against the

---

This work was supported by grants AI-11183 and AI-04181 from the National Institutes of Health. U. Krzych was the recipient of an NIH postdoctoral fellowship and is presently at the Catholic University, Department of Biology, Washington D.C. 20064.

<sup>1</sup> *Abbreviations used in this paper:* Ars, arsonate; BD, B cell determinant; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; CB, cyanogen bromide; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GAMIg, goat anti-mouse Ig; GRBC, goat red blood cell; GZ,  $\beta$ -galactosidase; HBSS, Hanks' balanced salt solution; HD, helper determinant; IFA, incomplete Freund's adjuvant; KLH, keyhole limpet hemocyanin; LNC, lymph node cell; PBS, phosphate-buffered saline; PFC, plaque-forming cell; RCM, reduced, carboxymethylated; SD, suppressor determinant; Th, T helper; Ts, T suppressor; Tsi, T suppressor inducer.

two Th-inducing CB GZ peptides. In previous studies (6), it has been shown that only 2 of 11 CB peptides tested could induce a Th cell population able to augment the anti-fluorescein isothiocyanate (FITC) component of the response to GZ-FITC (6). Furthermore, in earlier work (8), it was evident that, with the GZ as well as the lysozyme (3), erythrocyte (4), and myelin basic protein (5) systems, suppression of the response induced by one peptide on the antigen seemed to affect the entire response (Th as well as B cell response, when tested) to any determinant on or attached to the same antigen.

At least three noteworthy new insights resulted from this work. First, not all Th cell targets could be suppressed, only those neighboring the Ts-inducing determinants. Second, a preferential hierarchy of induction occurred in the stimulation of Th cells by GZ and its peptides: not all potential Th were activated, and the GZ-induced Th differed from peptide-induced Th. Finally, the evidence compels us to picture the fragmentation of the very large GZ molecule into smaller overlapping peptides containing the combination of epitopes (Ts, Th, and B) required to detect intercellular regulatory effects.

### Materials and Methods

*Mice.* Female CBA/J mice at 10–12 wk of age were purchased from The Jackson Laboratory, Bar Harbor, ME, and were maintained in our animal facilities for at least 10 d before any experiments.

*Antigens.* *Escherichia coli*  $\beta$ -galactosidase (GZ) was prepared as described previously (9). The preparation of reduced carboxymethylated GZ (RCM-GZ), and CB-cleaved peptides has been described in detail elsewhere (10). KLH (Pacific Bio-Marine Laboratories Inc., Venice, CA) and bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) were used without further purification.

*Cell Culture Medium.* Modified Click medium is Hanks' balanced salt solution (HBSS) (Gibco Laboratories, Grand Island, NY) enriched with 2 $\times$  minimum essential medium (MEM) essential and 5 $\times$  nonessential amino acids, 25  $\mu$ g/ml each of adenosine, cytosine, guanosine, and uridine (Sigma Chemical Co.), 2 $\times$  MEM vitamins (Gibco Laboratories), 15 mM Hepes (Calbiochem-Behring Corp., San Diego, CA), and 863  $\mu$ g/ml sodium bicarbonate. It was further supplemented with 100  $\mu$ g/ml penicillin, 100  $\mu$ g/ml streptomycin, 4  $\mu$ M glutamine, 2.5  $\mu$ M sodium pyruvate, 50 mM 2-mercaptoethanol (Aldrich Chemical Co., Milwaukee, WI), and 5% fetal calf serum (FCS) just before use.

*Fluoresceination of Proteins.* GZ, KLH, and BSA were fluoresceinated according to Goldman (11). Briefly, to 10 mg protein in 1 ml of 0.1 M phosphate-buffered saline (PBS), pH 7.1, was added 0.1 ml of 0.5 M, pH 9.5 sodium carbonate containing 500  $\mu$ g FITC (Baltimore Biological Lab, Baltimore, MD). The reaction was carried on for 4 h at room temperature; then the mixture was dialyzed in the cold against 0.1 M phosphate buffer, pH 7.1, to remove any free FITC. The protein concentration and the number of FITC molecules per 10<sup>5</sup> of each protein were determined according to the formula: FITC per molecule protein =  $(1.9 \times \text{OD}_{496} \times \text{molecular weight of protein}) / \text{protein concentration}$ . Different average levels of fluoresceination resulted (FITC<sub>25</sub>GZ, FITC<sub>17</sub>GZ, FITC<sub>6</sub>GZ, FITC<sub>30</sub>KLH, and FITC<sub>50</sub>BSA). FITC<sub>17</sub>GZ through FITC<sub>25</sub>GZ were used, because it was found that FITC<sub>6</sub>GZ was suboptimally derived.

*Immunizations.* (a) Th cell priming. 10–12 d before culture, mice were immunized in each hind footpad and at the base of the tail with 50  $\mu$ g/mouse of GZ, RCM-GZ, or KLH in 50  $\mu$ l of PBS, emulsified with an equal volume of complete Freund's adjuvant (CFA) (Difco Laboratories, Inc., Detroit, MI). Immunizations with peptides were at a molar equivalent to 50  $\mu$ g GZ, i.e., 2.5  $\mu$ g CB-2 or 1.4  $\mu$ g CB-10 peptide per mouse in CFA. Mice primed with PBS-CFA emulsion served as controls where indicated. Popliteal and periaortic lymph node cells (LNC) were used as sources of Th cells.

(b) Ts cell priming. CB peptides were emulsified with an equal volume of incomplete

Freund's adjuvant (IFA) (Difco Laboratories, Inc.) and used for Ts cell priming at a dose the molar equivalent of 100  $\mu\text{g}$  GZ. Immunizations were intraperitoneal with 0.2 ml, and 7–21 d later, spleens were used as a source of Ts cells. Mice primed with PBS-IFA (0.2 ml) served as controls.

(c) B cell priming. Mice were immunized with 25  $\mu\text{g}$  FITC<sub>50</sub>BSA i.p. in CFA (Gibco Laboratories) and, 4–6 wk later, challenged with 5–10  $\mu\text{g}$  i.p. soluble BSA-FITC. Splenic B cells were prepared from mice challenged 10 d previously.

*Cell Preparations.* (a) Th cell preparation. Populations of primed, pooled popliteal and periaortic LNC served as a source of T cells, after passage on goat anti-mouse immunoglobulin (GAMIg)-coated plates, according to Mage et al. (12). Briefly, after the preparation of a single-cell suspension and two washes in cold BSS,  $5 \times 10^7$  cells in culture medium containing 10% FCS were incubated for 1 h at 4°C on 100-mm plastic petri dishes (Lab Tek Products, Naperville, IL) that had been previously coated with 100  $\mu\text{g}/\text{ml}$  purified GAMIg. Nonadherent cells were recovered by gentle washing with cold HBSS. Routinely, 35–45% of input cells were recovered and <5% of these were Ig-positive as determined by fluorescein staining with anti-mouse Ig. The recovered cells were >95% viable by trypan blue exclusion.

(b) Ts cell preparation. Splenic lymphocytes served as a source of T suppressor cells after enrichment on GAMIg-coated plates as described above, with an additional step involving lysis of red cells with 0.85% ammonium chloride in Tris buffer, 2% FCS (before the lymphocytes were passed on the plates).

(c) B cell preparation. B cells, obtained from three to four spleens treated with rat monoclonal anti-Thy-1.2 (kindly provided by I. Trowbridge, Salk Institute, San Diego, CA) at a final dilution of 1:40 at 4°C for 30 min, were washed and then incubated with agarose-absorbed rabbit complement at a 1:8 final dilution at 37°C for 30 min. The splenic cells were then washed three times in HBSS and adjusted to a proper concentration for culture. ~50% of the splenic cells were recovered.

*Cell Culture System for Th Cell Activity.* In vitro cultures were set up using a "miniculture" system (96-well, flat-bottom Linbro plates). As described previously (6), the culture medium was modified Click medium containing 5% FCS. Briefly, each culture contained  $3.6 \times 10^5$  B cells in 0.2 ml and titrated Th cells to a maximum of  $4 \times 10^4$  Th cells (10% Th). Nine 0.2-ml wells were set up for each titration point, each containing 1  $\mu\text{g}/\text{ml}$  of either GZ-FITC, KLH-FITC, or medium alone. The cell mixtures were cultured for 4 d at 37°C in a humidified atmosphere containing 2% CO<sub>2</sub>. For plaque-forming cell (PFC) determinations, three wells were pooled and considered as one culture.

*Ts Cell Cultures.* To measure suppression, in addition to the above, the culture contained graded numbers of GAMIg plate-passed T cells from a specific Ts population primed for suppressor activity. The number of Ts cells added was  $0.2\text{--}3.2 \times 10^4$  (0.5–8% of total cells); the Ts were always admixed into helper cultures containing  $4 \times 10^4$  T cells and  $3.6 \times 10^5$  B cells. After 4 d of culture at 37°C in a humidified atmosphere, the cultures were analyzed for anti-FITC PFC as described below.

*PFC Assay.* Triplicate cultures were harvested and tested for direct PFC, according to the Cunningham and Szenberg technique (13), against FITC-derived goat red blood cells (GRBC-FITC) or GRBC alone. The GRBC were derived with FITC according to Wolf et al. (14): 0.1 mg/ml FITC in 0.15 M sodium bicarbonate buffer, pH 9.5, 0.15 M NaCl were mixed with 0.25 ml packed GRBC and the reaction carried out at 4°C for 1 h, after which the GRBC-FITC were washed extensively in HBSS. Results are expressed as the mean PFC  $\pm$  SD of nine wells assayed in triplicate.

*Anti-T Cell Antibodies.* Various monoclonal antibodies were used to characterize T cells involved in suppression. Non-allele-specific anti-Thy-1.2 treatment was as described above for B cell preparation. Anti-Lyt-1 and anti-Lyt-2 were gifts from Dr. L. A. Herzenberg, Stanford University, and anti-arsenate (anti-Ars) monoclonal antibody was a gift from Dr. J. Goodman, University of California, San Francisco. Since, the anti-Lyt-1 and -Lyt-2 were not cytotoxic, both antibodies were derived with Ars to permit cytotoxicity after application of affinity-purified anti-Ars (0.1  $\mu\text{g}/\text{ml}$ ). T cells enriched on GAMIg plates were adjusted to  $1 \times 10^7$  cells/ml and incubated with either anti-Lyt-1-Ars at a

final dilution of 1:40 or anti-Lyt-2-Ars at 1:25, and were incubated at 4°C for 45 min. Affinity-purified anti-Ars (0.01 µg/ml) was added for an additional 45 min at 4°C. At the end of incubation, the cells were pelleted and then resuspended at the original cell density for complement treatment at 1:8 final dilution for 45 min at 37°C. Finally, cells were washed three times in HBSS and used in culture at the indicated concentrations.

## Results

*System Used to Study Suppression of GZ-specific Th Cells.* The question of the Ts cell repertoire was addressed by evaluating the ability of Ts raised by CB peptides of GZ to suppress specifically primed Th cells directed against a specific peptide of GZ. These two T cell populations interacted for the first time with each other and FITC-primed B cells upon in vitro challenge using GZ-FITC as antigen. Regarding the proliferative and Th cell repertoires specific for GZ, priming CBA/J mice with CB peptides of GZ revealed a broad spectrum of T cell reactivity. Cells from mice primed with almost any peptide could recognize the native or reduced RCM-GZ molecule during subsequent in vitro challenge, whereas only T cells specific for CB-2 (amino acid residues 3–92) and CB-10 (residues 378–418) could engage in collaborative interaction with FITC-specific B cells in the presence of GZ-FITC (7). Since the apparent Th repertoire was so limited despite many potential T cell-recognizing epitopes on the large GZ molecule (1,023 residues per monomer), we asked whether the specificity repertoire of Ts cells that arises after priming with CB peptides of GZ is similarly restricted.

Regarding the Ts-inducing activity of the CB-2 fragment of GZ, prior evidence (8) indicated that CB-2-primed splenic T cells suppressed GZ-primed splenic helper cells, resulting in a reduced antihapten response to GZ hapten. To evaluate this (8) and other evidence (15) showing that, soon after priming, popliteal LNC populations lacked Ts cell activity in the lysozyme system, we began using 9–12-d-primed LNC as a source of less complex Th targets for the putative Ts cells. In Fig. 1, composite data are shown from several experiments in which splenic T cells from mice were primed 7–14 d earlier with different CB peptides in IFA.  $1.6 \times 10^4$  primed Ts cells were added to cultures containing  $4 \times 10^4$  GZ-primed Th cells and  $3.6 \times 10^5$  FITC-primed B cells. After 4 d culture in the presence of GZ-FITC, anti-FITC PFC were enumerated.

*Limited Ts Cell Repertoire Directed Against the Large Protein Antigen GZ.* The composite data in Fig. 1 show that only two CB peptides, CB-2 and CB-3 (residues 93–187), activated Ts cells that can specifically suppress the anti-FITC PFC in response to stimulation with GZ-FITC. The remaining fragments had marginal or no suppressive activity. These results are reminiscent of the mapping studies (7) aimed at uncovering helper determinants, in which the helper determinants were also restricted to two CB peptides, CB-2 and CB-10.

*Characteristics of the Peptide-specific Ts.* The specificity of CB-2 -and CB-3-induced Ts was tested using KLH-primed LNC as the source of Th, in the presence of FITC-primed B cells and KLH-FITC. The results (Fig. 2) demonstrate that both CB-2- and CB-3-induced Ts can be titrated into GZ-primed Th stimulated with GZ-FITC. The KLH-specific anti-FITC response remained unaltered, despite the presence of GZ-specific Ts cells. Maximum suppression of the GZ-specific anti-FITC PFC response was observed with as few as 4% ( $1.6 \times$

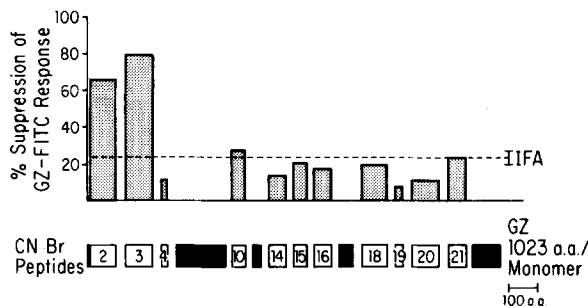


FIGURE 1. T<sub>s</sub> cell repertoire against GZ is restricted. The available CB peptides of GZ were tested 7–14 d after injection for their ability to induce splenic T<sub>s</sub> in CBA/J mice. Peptides in molar equivalent amounts, corresponding to 100 μg of GZ, were emulsified 1:1 with IFA and used for priming. Using 4 × 10<sup>4</sup> Th cells from GZ-primed lymph nodes as the targets for suppression, peptide-primed splenic T cells were added that had been negatively selected on GAM1g plates, up to a level of 0.5–4% of the total cell number. Each culture in the 96-well plate also contained 3.6 × 10<sup>5</sup> FITC-primed B cells, and was stimulated with 1 μg/ml GZ-FITC. On day 4 of culture, anti-FITC PFC were enumerated using an anti-FITC PFC assay.

The width of each bar in the figure reflects the number of amino acid residues in the CB peptides; filled rectangles indicate peptides not available for testing. The results represent composite data from several experiments. The responses from each experiment have been normalized to the percent of the response achieved after GZ or RCM-GZ priming. (These response range from 150 to 300 PFC/culture).

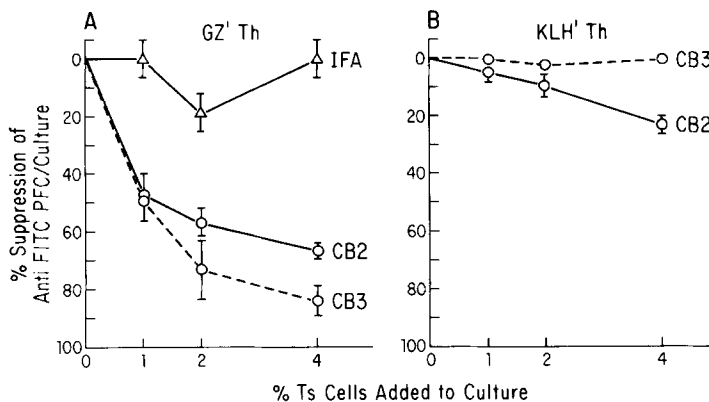


FIGURE 2. CB-2 and CB-3 peptides of GZ induce T<sub>s</sub> cells specific for GZ-FITC. (A) Spleen cells from CBA/J mice, primed intraperitoneally 7–14 d before culture with 5 μg CB-2 or CB-3 peptide emulsified in an equal volume of IFA, were enriched on GAM1g plates and then titrated into a standard helper culture containing 0.4 × 10<sup>5</sup> GZ-primed Th cells and 3.6 × 10<sup>5</sup> FITC-primed B cells, and stimulated with 1 μg/ml GZ-FITC. Spleen cells from CBA/J mice primed with IFA alone served as controls in a parallel culture. The cultures were maintained for 4 d and anti-FITC PFC were enumerated using GRBC-FITC as indicator cells. (B) The same CB-2- and CB-3-primed splenic T cells were also titrated into Th cultures containing 0.4 × 10<sup>5</sup> KLH-primed Th and 3.6 × 10<sup>5</sup> FITC-primed B cells, and maintained for 4 d in the presence of 1 μg/ml KLH-FITC. Anti-FITC PFC were enumerated as in A.

10<sup>4</sup>) CB-2- or CB-3-primed T<sub>s</sub> cells. In other experiments, approximately the same degree of GZ-specific suppression persisted when additional T<sub>s</sub> were added. Kinetics data (not shown) indicate that 7-d- or 21-d-primed splenic T cells serve equally well as sources of T<sub>s</sub> cells.

Surface markers on the T<sub>s</sub> cells, raised as described above and enriched by

TABLE I  
*CB-2-induced, GZ-specific Suppression Is Mediated by Lyt-2<sup>+</sup>, I-J<sup>k</sup> Cells*

Cell mixtures in cultures ( $\times 10^{-5}$ )			Antiserum + C treatment of CB- primed cells	Direct PFC per culture
FITC- primed B	GZ-primed Th	CB-2- primed Ts		
4	—	—	—	15 $\pm$ 0
—	4	—	—	5 $\pm$ 0
3.6	0.4	—	—	165 $\pm$ 14
3.6	0.4	0.4	—	43 $\pm$ 18
3.6	0.4	0.4	Anti-Thy-1	125 $\pm$ 7
3.6	0.4	0.4	Anti-Lyt-1	33 $\pm$ 12
3.6	0.4	0.4	Anti-Lyt-2	137 $\pm$ 3
3.6	0.4	0.4	Anti-I-J <sup>k</sup>	133 $\pm$ 25
3.6	0.4	0.4	Anti-I-J <sup>b</sup>	42 $\pm$ 18

Cultures identical to those described in Figs. 1 and 2 were established, to which CB-2 splenic T cells were added.  $4 \times 10^3$  CB-2-primed T cells from 28-d primed spleens ("Ts") were treated at  $1 \times 10^7$  cells/ml with one of the following antisera (and C'): anti-Thy-1, anti-Lyt-1, anti-Lyt-2, anti-I-J<sup>k</sup>, or anti-I-J<sup>b</sup>. Ts were then washed and admixed with  $4 \times 10^4$  10-d GZ-primed Th from the popliteal lymph node and  $3.6 \times 10^5$  BSA-FITC-primed B cells.

removal of B cells over GAMIg plates, were characterized by treatment with cytotoxic antisera against Thy-1, Lyt antigens, and I-J<sup>k</sup> (Table I). Suppression was caused by Lyt-2<sup>+</sup>, I-J<sup>k</sup>-bearing T cells, although it isn't known whether Lyt-2 and I-J<sup>k</sup> were present on the same cell. Treatment with anti-Lyt-1 monoclonal antibody plus complement was without effect, as was anti-I-J<sup>b</sup> plus complement.

*Each Ts Cannot Suppress All Th Targets.* The fact that each of the peptide-induced Ts populations was able to suppress the GZ-specific anti-FITC PFC response  $\sim 75$ – $90\%$  suggested that all target Th cells were susceptible to the activity of any one Ts. To test this notion, we performed experiments similar to those in Figs. 1 and 2, with each of the target Th populations that we had enumerated in previous work (7), i.e., Th primed with GZ, RCM-GZ, CB-2, or CB-10. Figs. 3 and 4 show results obtained by titration of CB-2- and CB-3-induced Ts into each of the four target Th populations.

Both GZ- and RCM-GZ-primed Th were fully susceptible to each Ts population. CB-3-induced Ts were, generally, more suppressive for GZ-primed Th, and RCM-GZ Th were slightly less suppressible than GZ Th.

Most striking were the results showing that not all Th were susceptible to suppression by each Ts. CB-10-induced Th were totally unaffected by either Ts cell. CB-2-induced Th activity was resistant to Ts cells generated by CB-3, although sensitive to CB-2 Ts. The inability of CB-3-induced Ts to suppress the collaboration between CB-2-specific Th and FITC-specific B cells, compared with the sensitivity of GZ-primed Th, indicates that the Th cells triggered by GZ injection were not identical to those induced by CB-2.

One possible explanation of the comparative suppressibility of GZ- and CB-2-specific Th cells is that GZ-primed Th were highly focused and are directed against a determinant topologically closer to CB-3-induced Ts, perhaps between CB-2 and CB-3. Therefore, the uncleaved fragment CB-2-3 (residues 3–187) was used to induce Th cells. In Table II, it can be seen that CB-2-3-induced, but

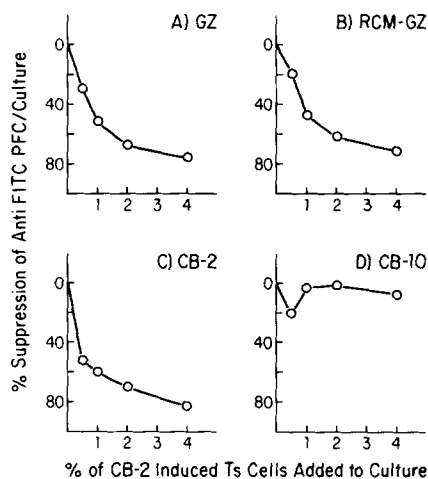


FIGURE 3. Interaction between Th and Ts cells is limited: CB-2-primed Ts. Popliteal lymph node Th cells, induced with either GZ (50–100  $\mu\text{g}$ ), RCM-GZ (50–100  $\mu\text{g}$ ), CB-2 (5  $\mu\text{g}$ ), or CB-10 (2.5  $\mu\text{g}$ ) were used as target cells for splenic Ts cells specific for CB-2 in a 0.2 ml microculture system containing  $3.6 \times 10^5$  FITC-primed B cells (anti-Thy-1.2 and C'-treated spleen cells) and  $0.4 \times 10^5$  Th cells (RAMIg plate-passed) with the indicated number of Ts cells, expressed as a percent of total culture. The culture was stimulated with 1  $\mu\text{g}/\text{ml}$  GZ-FITC and maintained for 4 d. Direct anti-FITC PFC were enumerated and the responses expressed as percent suppression, compared with cultures without added Ts.

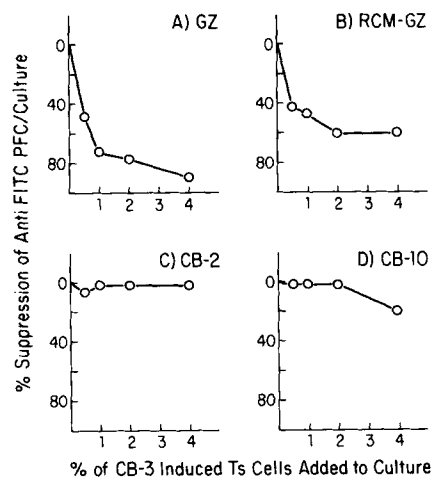


FIGURE 4. Interaction between Th and Ts cells is limited: CB-3-primed Ts. Same as in Fig. 3 except CB-3-primed Ts cells were added to each of the four target Th cell populations.

not CB-2-induced Th, were susceptible to CB-3-induced Ts. This suggests that the major Th induced after native GZ priming may be specific for an overlapping area between CB-2 and CB-3.

Another possible explanation of the failure of Ts cells induced by CB-3 to suppress Th cells induced by CB-2 is that the target population includes Th cells but lacks the appropriate T suppressor inducer (Tsi) cells to interact with CB-3-specific Ts. A preliminary experiment was performed to test this, mixing CB-3-

TABLE II  
*Immunodominant Th Cell Epitope May Reside on CB-2-3*

Cell mixture in culture ( $\times 10^{-5}$ )			Anti-FITC PFC culture
B 3.6	Th 0.4	Ts 0.1	
primed with:			
FITC	GZ	—	433 $\pm$ 72
FITC	GZ	CB-2	196 $\pm$ 21
FITC	GZ	CB-3	145 $\pm$ 47
FITC	CB-2	—	262 $\pm$ 32
FITC	CB-2	CB-2	122 $\pm$ 25
FITC	CB-2	CB-3	218 $\pm$ 38
FITC	CB-2-3	—	408 $\pm$ 88
FITC	CB-2-3	CB-2	44 $\pm$ 6
FITC	CB-2-3	CB-3	133 $\pm$ 39

Details of experiment are identical to those in legend to Fig. 3.  $1 \times 10^4$  Ts cells were added to the cultures where indicated.

induced Ts with popliteal LNC from CB-3 footpad-primed mice, which should provide the required Tsi but are already known not to be a source of Th (6). Even with the addition of CB-3-primed popliteal LNC to the CB-2-primed LNC targets, the CB-3-specific Ts were unable to suppress the CB-2-primed Th. On balance, the evidence seems to favor an explanation based on the topology of relevant epitopes.

### Discussion

In the absence of detailed evidence, there has been speculation that large proteins such as KLH and GZ (with 1,023 amino acid residues per monomer) are decorated with a large variety of determinants, all available for the functions expressed by different lymphoid subpopulations. The present work indicates that very few determinants induce functional T cells after administration of a large antigen such as GZ. Whether Th cells are expressed depends on whether there exist Ts cells directed against determinants in close proximity to the Th. Finally, some Th that can be raised by peptide priming are not engaged by immunization with the native protein antigen.

#### *In One Interaction, Ts- and Th-reactive Epitopes Must Be Close*

The lack of sensitivity of CB-10-induced as well as CB-2-induced Th cells to CB-3-specific Ts cells strongly implies a regional effect in the interaction between Ts and Th. We assume (a) that Ts and Th cells relate through recognition of determinants lying on a single usable structure; and (b) that an optimal suppressor determinant-helper determinant (SD-HD) distance must exist on the antigen molecule. This distance is clearly smaller than peptide 3-187 [CB-2-3], and may be as short as 20-40 amino acid residues. This estimate derives from the likelihood that a variety of fragments are created from GZ by the antigen-processing machinery. For Ts cells to effectively bridge and suppress target Th,



the only relevant fragments of antigen for cell interaction would be those containing an SD and a functional HD. Since CB-3-specific Ts never suppress CB-2-specific Th, we assume that these determinants generally separated onto distinct fragments via antigen processing. Presumably, at an earlier stage of processing, SD are still connected to other HD and Th directed against connected HD are then susceptible to suppression.

Our earlier work (6) suggested that the HD had to be relatively close to B cell epitopes to be detectable. Based on this idea, although Ts-Th interaction fragments containing only an SD and HD should be sufficient to allow the suppressive Ts-Th interaction, the SD would only become evident when the proximal HD lies near a B cell determinant. (The SD and B cell determinant [BD] may lie on overlapping fragments that result from antigen processing with a single HD in the area of overlap; thus, the SD and HD could lie on one fragment and the same HD and a BD on an overlapping fragment). Whatever the case, any limitation in the number of BD, as when low levels of hapten derivatization exist, should obscure *both* potential HD and SD. A fragment with an SD and an HD, but lacking a BD, would be functionally silent. Consequently, the SD-HD-BD triad seems inextricably linked, as suggested in a preliminary communication (16) and elegantly shown in a recent report (17).

That the Ts may act as an antigen-processing cell can be inferred from work in the lactic dehydrogenase system (18) and is supported to some extent by findings that Ts can display Ia molecules (19–21), similar to the B cell (22). The symmetry of Ts-Th and B-Th interactions, in the context of antigen-processing by Ts or B cells, may be a model to help explain the constraints in the GZ system. After recognition and uptake of antigen through receptor interaction at the SD, Ts cells may process proximal HD for display (in conjunction with Ia) more effectively than distal HD. Such a relationship would mean that the proximity requirements for Th- and Ts-directed determinants are similar to those suggested for Th- and B cell-directed determinants in T-B collaboration (23, 24).

#### *A Limited and Dominantly Expressed Portion of the Anti-GZ Th Repertoire Is Engaged After GZ Priming*

*The dominant Th activated by GZ.* The data in Figs. 3 and 4 strongly suggest that, in the context of a large antigen such as GZ, and probably also for small protein antigens, the interactions between Th and Ts are highly ordered, involving T cells with carefully matched specificities. Surprisingly, while CB-3-specific Ts were ineffective in suppressing either CB-2- or CB-10-induced Th activities, they efficiently suppressed Th induced by GZ. Which Th cells, then, are triggered by GZ? The clear implication of these results is that a Th cell of previously undefined specificity is induced after GZ priming and is suppressible by CB-2- and CB-3-specific Ts cells. This may be the major and dominant Th after GZ stimulation. It is likely that the CB cleavage at the methionine which separates CB-2 and CB-3 destroys this dominant Th-inducing epitope. As one test of this notion, we used an incomplete CB digestion fragment, CB-2-3 (residues 3–187), to immunize popliteal lymph nodes to see whether the immunodominant Th or the CB-2-type Th would be generated. As shown in Table II,

the helper activity induced by CB-2-3 was like that induced by GZ and was suppressible by either CB-2- or CB-3-induced Ts.

*GZ-induced Th are different from peptide-induced Th.* We did not expect that the immunodominant Th arising after GZ stimulation would be different from Th arising after priming with any of the CB peptides. The mechanism of this dominance is unknown. Let us consider the example of the CB-10-specific Th cell, which is one that seems not to be activated after GZ priming. If this Th cell had been primed, a large proportion of the GZ-induced helper activity would have been resistant to CB-2- or CB-3-induced Ts cells. Despite their immunodominance, the Th that emerge after native GZ stimulation are largely suppressible by either CB-2- or CB-3-specific Ts. It is possible that a private Ts close to CB-10 is raised after GZ immunization and interferes with the induction of CB-10-specific help. Alternatively, a hierarchy of Th engagement may occur, based on antigen presentation, with CB-10 quite low on the list. Nevertheless, the residual unsuppressible activity seen after CB-2- and CB-3-induced suppression of GZ-primed Th cells may represent a small degree of CB-10-specific T cell help.

*Consequences of the existence of epitope hierarchies.* (a) T repertoire definition. In addition to the lack of expression of T cells directed against certain peptides, the epitopes used to induce Th also differed when the immunogen was the native molecule rather than a peptide derived from it. Therefore, to define the complete T cell repertoire available to an antigen, component overlapping peptides, as well as the whole molecule, must be used for induction. This difficulty in defining repertoires may also extend to Ts cells; in studies of Ts induced by peptides, it may lead to ambiguous identification of the Ts actually engaged by the native molecule.

(b) Complete suppression caused by single Ts. Priming with a single suppressor cell-inducing peptide has often completely suppressed the subsequent response to the native protein (3, 8, 25). It had been reasoned that Ts cells directed against a single site could suppress Th cells directed against all other determinants on the molecule. The present results suggest a less global alternative. Since there seems to be a hierarchical use of determinants, the only Th cell that would need to be suppressed to achieve nearly complete lack of response would be the dominant Th. Therefore, in cases of *Ir*-controlled nonresponsiveness, some Th would not be expressed because they are subdominant; other(s), the dominant one(s), would be suppressed.

(c) Limited use of epitopes on multideterminant antigens. The repeated finding of hierarchical dominance reveals a general rule of limited use of epitopes on a native antigen. When a macromolecule is processed by the immune system, a hierarchy is established in which a dominant epitope is used to the exclusion of other potential epitopes (despite the demonstrated ability to respond to the subdominant epitope). Such a situation is also found in the lysozyme system, where activation by the C-terminal CB peptide does not occur among B10.A proliferative T cells when native hen egg-white lysozyme is used as immunogen (26, 27), although the T cells exist in the available repertoire of the haplotype. The restriction may result from competition between antigenic determinants at the level of antigen processing/presentation, or may conceivably be attributed

to regulatory encounters. As suggested by Miller (28), it may be that, during processing by antigen-presenting cells, the T cell recognizing the most "available" Ia-antigen complex slows down or stops the further processing of the antigen, preempting any other T cell activation. Possibly, a zipperlike unfurling of the molecule occurs, accounting for the seemingly preferred status of the amino-terminal end of the molecule (CB-2, CB-3). If an end-to-middle polarity exists in the processing of a protein by the immune machinery, there may be little opportunity for the internal regions of a large protein to influence the response specificity. The actual mechanism(s) underlying hierarchical preference is being examined.

### Summary

11 cyanogen bromide (CB) peptides, comprising 70% of the large protein, *Escherichia coli*  $\beta$ -galactosidase (GZ), were studied for their ability to induce T suppressor (Ts) cells capable of strongly suppressing the in vitro anti-fluorescein (FITC) response to GZ-FITC. Only CB-2 (amino acid residues 3-92) and CB-3 (residues 93-187) were found to bear such Ts-inducing epitopes.

In examining the specificity of T helper cell (Th) targets susceptible to CB-2 and CB-3-specific Ts, it appeared that only nearby Th targets could be suppressed. Thus, CB-10-primed Th were not suppressed by either Ts; even CB-3-primed Ts did not suppress CB-2-specific Th, although CB-2-specific Ts were effective.

Furthermore, analysis of the suppression pattern revealed a hierarchical use of potential epitopes on native GZ in triggering functional regulatory T cells. A dominant Th epitope near the amino terminus of GZ tops a hierarchy of potential Th, most of which are never engaged. The dominant determinant seems to exist on the peptide CB-2-3 (residues 3-187), and presumably is destroyed by its cleavage at Met 92; the Th cells that it induces are suppressible by each of the Ts-inducing peptides.

In the GZ system, where the native antigen is quite large, the interactions between Th and Ts are highly circumscribed. This may be attributable to the topology of antigen fragments produced during processing; any relevant fragment must bear at least a Ts- and Th-reactive determinant to permit intercellular regulation. A final implication of these results is that, not only does the existence of a Th-inducing determinant depend on its being an appropriate distance from a B cell epitope, but the existence of Ts-inducing determinants likewise depends on the existence of a neighboring Th-B cell association.

We thank Alex Miller for helpful discussions, Margaret Kowalczyk for preparing the figures, and Patricia Walters for preparation of the manuscript.

*Received for publication 5 March 1985 and in revised form 15 April 1985.*

### References

1. Tada, T., and T. Takemori. 1974. Selective roles of thymus-derived lymphocytes in the antibody response. I. Differential suppressive effect of carrier-primed T cells on hapten-specific IgM and IgG antibody responses. *J. Exp. Med.* 140:239.

2. Kapp, J. A., C. W. Pierce, S. Schlossman, and B. Benacerraf. 1974. Genetic control of immune response in vitro. V. Stimulation of suppressor T cells in nonresponder mice by the terpolymer L-glutamic acid<sup>60</sup>-L-alanine<sup>30</sup>-L-tyrosine<sup>10</sup> (GAT). *J. Exp. Med.* 140:648.
3. Adorini, L., M. A. Harvey, A. Miller, and E. E. Sercarz. 1979. Fine specificity of regulatory T cells. II. Suppressor and helper T cells are induced by different regions of hen eggwhite lysozyme in a genetically nonresponder mouse strain. *J. Exp. Med.* 150:293.
4. Fresno, M., G. Nabel, L. McVay Boudreau, H. Furthmayer, and H. Cantor. 1981. Antigen-specific T lymphocyte clones. I. Characterization of a T lymphocyte clone expressing antigen-specific suppressive activity. *J. Exp. Med.* 153:1246.
5. Swanborg, R. H. 1975. Antigen-induced inhibition of experimental allergic encephalomyelitis. III. Localization of an inhibitory site distinct from the major encephalitogenic determinant of myelin basic protein. *J. Immunol.* 114:191.
6. Krzych, U., A. V. Fowler, A. Miller, and E. E. Sercarz. 1982. Repertoires of T cells directed against a large protein antigen,  $\beta$ -galactosidase. I. Helper cells have a more restricted specificity repertoire than proliferative cells. *J. Immunol.* 128:1529.
7. Fowler, A. V., and I. Zabin. 1978. Amino acid sequence of  $\beta$ -galactosidase. XI. Peptide ordering procedures and the complete sequence. *Proc. Natl Acad. Sci. USA.* 253:588.
8. Turkin, D., and E. E. Sercarz. 1977. Key antigenic determinants in regulation of the immune response. *Proc. Natl. Acad. Sci. USA.* 74:3984.
9. Fowler, A. V. 1972. High level production of  $\beta$ -galactosidase by *Escherichia coli* merodiploids. *J. Bacteriol.* 112:656.
10. Fowler, A. V. 1978. Amino acid sequence of  $\beta$ -galactosidase. VII. Isolation of the 24 cyanogen bromide peptides. *J. Biol. Chem.* 253:5499.
11. Goldmann, M. 1968. *Fluorescent Antibody Methods*. Academic Press, Inc., New York.
12. Mage, M. G., L. L. McHugh, and T. Rothstein. 1977. Mouse lymphocytes with and without surface immunoglobulin: preparative scale separation in polystyrene tissue culture dishes coated with specifically purified anti-immunoglobulin. *J. Immunol. Methods.* 15:47.
13. Cunningham, A. J., and A. Szenberg. 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. *Immunology.* 14:599.
14. Wolf, B., C. A. Janeway, R. R. A. Coombs, D. Catty, P. G. H. Gell, and A. S. Kelus. 1971. Immunoglobulin determinants on the lymphocytes of normal rabbits. III. As4 and As6 determinants on individual lymphocytes and the concept of allelic exclusion. *Immunology.* 20:931.
15. Araneo, B. A., R. L. Yowell, and E. E. Sercarz. 1979. Ir gene defects may reflect a regulatory imbalance. I. Helper T cell activity revealed in a strain whose lack of response is controlled by suppression. *J. Immunol.* 123:961.
16. Krzych, U., A. Fowler, and E. E. Sercarz. 1983. Antigen structures used by regulatory T cells in the interaction among T suppressor, T helper and B cells. *In* Protein Conformation as an Immunological System. F. Celada, V. W. Schumaker, and E. E. Sercarz, editors. Plenum Publishing Corp., New York. 395-408.
17. Asano, Y., and R. J. Hodes. 1984. T cell regulation of B cell activation: an antigen-mediated tripartite interaction of Ts cells, Th cells, and B cells is required for suppression. *J. Immunol.* 133:2864.
18. Baxevanis, C. N., Z. A. Nagy, and J. Klein. 1983. The nature of the interaction between suppressor and helper T cells in the response to LDH<sub>B</sub>. *J. Immunol.* 131:628.
19. Koch, N., B. Arnold, G. J. Hammerling, J. Heuer, and E. Kolsch. 1983. Structural

- comparison of I-A antigens produced by a cloned murine T suppressor cell line with B cell-derived I-A. *Immunogenetics*. 17:497.
20. Trial, J. A., and J. A. Kapp. 1983. Expression of cell surface antigens by suppressor T cell hybridomas. I. Comparison of phenotype and function. *J. Immunol.* 130:565.
  21. Araneo, B. A., and R. L. Yowell. 1985. MHC-linked immune response suppression mediated by T cells bearing I-A encoded determinants. *J. Immunol.* In press.
  22. Chesnut, R. W., and H. M. Grey. 1981. Studies on the capacity of B cells to serve as antigen-presenting cells. *J. Immunol.* 126:1075.
  23. Berzofsky, J. A. 1983. T-B reciprocity: an Ia-restricted epitope-specific circuit regulating T cell-B cell interaction and antibody specificity. *Surv. Immunol. Res.* 2:223.
  24. Celada, F., A. Kunkl, F. Manca, D. Fenoglio, A. Fowler, U. Krzych and E. E. Sercarz. 1984. Preferential pairings in T-B encounters utilizing Th cells directed against discrete portions of  $\beta$ -galactosidase and B cells primed with the native enzyme or a hapten epitope. In *Regulation of the Immune System*. H. Cantor, L. Chess, E. E. Sercarz, editors. Alan Liss, Inc. New York. p. 637.
  25. Schwartz, M., C. Waltenbaugh, M. Dorf, R. Cesla, M. Sela, and B. Benacerraf. 1976. Determinants of antigenic molecules responsible for genetically controlled regulation of immune responses. *Proc. Natl. Acad. Sci. USA.* 73:2862.
  26. Maizels, R. M., J. A. Clarke, M. A. Harvey, A. Miller, and E. E. Sercarz. 1980. Epitope specificity of the T cell proliferative response to lysozyme: proliferative T cells react predominantly to different determinants from those recognized by B cells. *Eur. J. Immunol.* 10:509.
  27. Katz, M. E., R. M. Maizels, L. Wicker, A. Miller, and E. E. Sercarz. 1982. Immunological focusing by the mouse MHC: mouse strains confronted with distantly related lysozymes confine their attention to very few epitopes. *Eur. J. Immunol.* 12:535.
  28. Miller, A. 1978. A model implicating altered macrophage function in H-2-linked nonresponsiveness to hen lysozyme. *Adv. Exp. Med. Biol.* 98:131.