Changes in the Peripheral Blood T-Cell Receptor V β Repertoire In Vivo and In Vitro during Shigellosis

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A sequential activation of T cells in peripheral blood during shigellosis has been observed (D. Islam, P. K. Bardhan, A. A. Lindberg, and B. Christensson, *Infect. Immun.* 63:2941–2949, 1995). To further investigate the cellular response during the course of *Shigella* infection, changes in the T-cell receptor (TCR) repertoire in the peripheral blood were analyzed. The hypothesis for monitoring changes in the TCR V β repertoire of T-cell subsets in blood in patients during shigellosis was that *Shigella* antigens may modulate the function of T cells carrying TCRs capable of recognizing *Shigella*-specific epitopes or superantigens. Such a selective preference for T cells expressing certain TCR V β types could lead to the expansion or deletion of these T cells. In the present study of 27 adult male Bangladeshi patients with dysentery (14 cases caused by *Shigella dysenteriae* 1 and 13 cases caused by *Shigella flexneri*), the changes in the TCR V β repertoire of peripheral blood CD4⁺ and CD8⁺ T-cell subsets have been analyzed with a panel of nine anti-V β monoclonal antibodies by flow cytometry. Twenty healthy males from Bangladesh and 20 healthy males from Sweden served as controls. Compared with the Bangladeshi controls, the patients had an increased frequency of CD4⁺ T cells expressing V β 2, V β 3, and V β 17, with a maximum at day 7 after the onset of disease. The frequency of CD4⁺ T cells expressing V β 5.1 was increased only in patients with *S. flexneri* infection. Peripheral blood T cells from *Shigella*-infected patients also responded to in vitro stimulation in a TCR V β -specific manner. Stimulation with heat-killed *S. dysenteriae* 1 and Shiga toxin enhanced the frequency of cells expressing V β 2, V β 3, V β 5.1, V β 13.6, and V β 17, especially in samples obtained at day 7. The enhanced frequency of cells expressing V β 2, V β 3, V β 5.1, and V β 17 found both in vivo and in vitro could suggest that in shigellosis antigens or superantigens are presented to the immune system and preferentially activate certain TCR V β types in T-cell subsets. The kinetics of the change in the TCR V β repertoire in blood during shigellosis may indicate that following local activation, the antigen-activated T cells can be retrieved in the blood and restimulated in vitro. If confirmed by parallel analysis of T cells in the gut and blood by TCR sequence analysis, the possibility suggested by our findings would facilitate further analysis of the role of cell-mediated immune responses in the pathogenesis of and protection against *Shigella* infection.

Antigen-mediated activation of T-cell receptor (TCR) α/β -expressing T cells may be the result of several types of interactions between antigen-presenting cells and T cells. Both peptide and nonpeptide structures from infectious organisms may interact with T cells. The conventional major histocompatibility complex (MHC)-restricted trimolecular interaction of processed peptides bound to the groove of the MHC molecule and the variable part of the TCR may lead to clonal expansion of T cells carrying the appropriate TCR (13, 28). The polymorphism of the MHC system and the vast recombinatorial potential of the TCR may suggest that after exposure to infectious agents, a multitude of processed peptide antigens would lead to the expansion of sets of T-cell clones unique to each individual (16, 39). However, immunodominant epitopes in experimental systems of autoimmunity and infection as well as in humans have been described (15, 41, 46). Thus, a limited number of processed peptides may be identified that in certain

MHC haplotypes activate T cells expressing certain TCR α or β types. Those T cells could be responsible for a significant part of the specific T-cell response. In infected individuals, activation of T cells by immunodominant epitopes could then be reflected in a skewing of the TCR V β repertoire.

The cross-linking of the constant part of the TCR V β chain to MHC class II molecules on antigen-presenting cells by superantigens may lead to T-cell activation, anergy, or deletion, depending on the circumstances. Thus, superantigens may also selectively activate T cells expressing certain TCR V β gene products, leading to a skewing of the TCR V β repertoire (3, 4, 20, 26). The particular factor(s) determining the outcome of T-cell ligation by superantigens is not well understood. In certain bacterial and viral infections, caused by *Staphylococcus aureus* and human immunodeficiency virus, respectively, superantigen effects have been implicated. Superantigens have also been suggested to be involved in modulating T-cell function in chronic diseases in which skewing of the TCR V α or V β repertoire has been seen, such as rheumatoid arthritis (22, 34), sarcoidosis (17, 18), multiple sclerosis (7, 31, 33), AIDS (5, 9, 21), and Kawasaki disease (1, 2, 35).

More recently, the activation of T cells expressing TCRs of α/β types by lipid antigens derived from mycobacteria has been shown to be restricted by the CD1 molecule and not by the

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MHC (6). In addition, other nonpeptide antigens, monoethyl phosphates from mycobacteria, may be involved in the activation of T cells expressing TCRs of the γ/δ type (44). The possible roles of such non-MHC-restricted T-cell activation mechanisms in human disease are still unknown.

The humoral immune response has been the focus of studies of defense mechanisms in shigellosis. Both local and systemic responses to lipopolysaccharide (LPS) and *Shigella* invasion plasmid-encoded protein antigens (Ipa) have been implicated in the protection against the infection. In contrast, little is known about the role of cell-mediated immune responses in shigellosis. Recently, we and others have shown evidence for systemic and local cellular activation, including activation of blood T cells and induction of cytokine production (10, 23, 37, 38). We have shown evidence for an in vivo activation of T cells in the blood in patients during shigellosis and a correlation between the degree of T-cell activation and disease severity (23). To understand the role of T cells in the pathogenesis of and protection against *Shigella* infection and to further the development of effective vaccines, knowledge about *Shigella*-derived molecules or epitopes that may induce T-cell-specific responses is required.

In the present study, an approach was made to analyze changes in the TCR V β repertoire in patients with shigellosis, both in vivo and after in vitro stimulation with *Shigella* antigens. The results suggest that both in vivo and in vitro *Shigella* spp. skew the TCR V β repertoire toward certain V β types in CD4⁺ and CD8⁺ T cells, and they provide new insight into T-cell activation in shigellosis. The apparently TCR V β -selective skewing would be compatible with a possibly oligoclonal activation of certain T-cell subsets, which could be related to the presence of either immunodominant peptides or superantigen-like molecules.

MATERIALS AND METHODS

Study populations. Twenty-seven male adult patients (20 to 45 years), seen at the International Centre for Diarrhoeal Disease Research, Bangladesh, with culture-confirmed *Shigella* infection were included in this study, which was approved by the Ethical Review Committee at the International Centre for Diarrhoeal Disease Research, Bangladesh. There were 14 *Shigella dysenteriae* 1-infected patients (SDIP) and 13 *Shigella flexneri*-infected patients (SFIP). The controls from Bangladesh (C-B), comprising 20 healthy subjects, were matched for sex, age, and socioeconomic status. In addition, 20 healthy male adult volunteers from Sweden (V-S) were included in the study. Neither controls nor patients had experienced bloody dysentery in the last 6 months prior to inclusion in the study or prior to the current infection, respectively. At onset of the illness, all patients exhibited typical clinical symptoms of bacillary dysentery, including fever, tenesmus, and blood and mucus in their feces. None of the patients had any other infection or additional major disease. All patients were given pivmecillinam chloride (200 mg four times daily) for at least 1 week from the day of admission (pivmecillinam is now considered to be the first-line drug for the treatment of shigellosis in areas where most *Shigella* isolates are resistant to other chemotherapeutic agents). Within 5 to 7 days all patients were clinically cured. Individuals in this study population were also included in studies relating to other aspects of the immune function in shigellosis (23, 25).

Samples. Blood was collected from each patient on the day of admission and 4, 7, 11, and 30 days thereafter. Single blood samples were collected from the C-B and V-S. The time course of the disease was categorized into five phases based on the sampling days.

Because of the limited amount of peripheral blood mononuclear cells (PBMC) available, samples from the same patients and controls could not be used for analysis of changes of the TCR V β repertoire in T-cell subsets during disease and for in vitro culture with antigens. PBMC from 22 patients (11 SDIP and 11 SFIP) and from 15 C-B and 15 V-S were analyzed for changes in the TCR V β repertoire in T-cell subsets. In five patients (three SDIP and two SFIP), sufficient amounts of PBMC were available in samples from days 1, 7, and 30 to allow TCR V β repertoire analysis after in vitro culture. As controls, PBMC from five C-B and five V-S were also cultured and analyzed for the expression of TCR V β types in T-cell subsets under the same conditions. The results were correlated to the disease course and infecting *Shigella* sp. (*S. dysenteriae* 1 or *S. flexneri*).

MAbs. Anti-TCR V β 5.1-, V β 5.3-, V β 6.7-, V β 8-, and V β 12-specific monoclonal antibodies (MAbs) were obtained from T-Cell Diagnostics (Woburn, Mass.);

V β 2-, V β 3-, V β 13.6-, and V β 17-specific MAbs were obtained from Immunotech (Marseille Cedex, France). All MAbs were conjugated with fluorescein isothiocyanate. The following antibodies were obtained from Becton Dickinson (San Jose, Calif.): phycoerythrin-conjugated anti-Leu-2a (CD8) and anti-CD69; peridinin chlorophyll-A protein-conjugated anti-Leu-3a (CD4) and anti-Leu-4 (CD3); and fluorescein isothiocyanate-, phycoerythrin-, and peridinin chlorophyll-A protein-conjugated isotype controls.

Cell preparation and staining. Cell preparation and staining were exactly as described previously (23, 24). PBMC were isolated from heparinized peripheral blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, frozen in RPMI medium containing 20% fetal bovine serum and 10% dimethyl sulfoxide, and stored at -196°C . After thawing and washing, the viability was $>95\%$ by trypan blue exclusion. A total of 10^6 cells in 100 μl of phosphate-buffered saline (pH 7.4) (PBS) containing 2% heat-inactivated pooled human serum (AB sera) were stained with triple combinations of MAbs for 30 min at 4°C . After being washed, the cells were resuspended in PBS containing 1% paraformaldehyde (except for cells to be stained with propidium iodide for viability counts) and analyzed within 4 h. Negative controls were included in each experiment.

Antigen, mitogen, and superantigen. Heat-killed (HK) *S. dysenteriae* 1 bacteria and polysaccharide (PS) (from phenol-water extracted LPS), HK *S. flexneri* Y (the basic common structure for types 1a-5b) bacteria and PS, *Escherichia coli* CVD-172 PS, Ipa, and Shiga toxin were used as antigens. The mitogen phytohemagglutinin A (M form; GIBCO BRL Life Technologies, Grand Island, N.Y.) and the superantigen toxic shock syndrome toxin 1 (TSST-1) (Toxin Technology, Madison, Wis.) were also used. The preparation of antigens was as described previously (12, 25). All of the PSs were provided by Inger C. Fält (Division of Clinical Bacteriology, Karolinska Institute, Huddinge Hospital, Sweden). All of the PSs and protein antigens were checked by chromogenic *Limulus* amoebocyte lysate assay (Chromogenix AB, Mölndal, Sweden) for the presence of endotoxin (lipid A). Protein antigens and medium alone (RPMI 1640 culture medium [GIBCO] supplemented with 20% inactivated pooled human serum, 2 mM L-glutamine, 1 mM Na-pyruvate, 100 U of penicillin per ml, and 100 μg of streptomycin per ml) contained between 0.01 and 0.02 ng of endotoxin per ml, whereas PSs contained between 0.1 and 5.0 ng/ml.

Stimulation of PBMC. Optimal concentrations of antigens, mitogen, and superantigen as well as culture conditions were based on previously published methods and confirmed by titration and kinetic studies (19, 40). PBMC were cultured in supplemented RPMI 1640 culture medium (described above) in 24-well Costar plates (A/S Nunc, Roskilde, Denmark) for subsequent cell surface marker analysis and in parallel in U-bottomed microtiter plates (10^5 cells per well, in triplicate) for thymidine incorporation studies. PBMC were incubated for 4 h at 37°C in the absence of stimulant or in the presence of PS (20 $\mu\text{g}/\text{ml}$), Ipa (20 $\mu\text{g}/\text{ml}$), Shiga toxin (0.2 $\mu\text{g}/\text{ml}$), phytohemagglutinin (10 $\mu\text{g}/\text{ml}$), TSST-1 (1 ng/ml), or HK bacteria ($10^6/\text{ml}$, as determined by CFU counts of serially diluted bacterial suspensions before heat killing). After the 4-h incubation, the cells were washed twice and cultured for 72 h at 37°C . After addition of recombinant human interleukin-2 (10 U/ml) (Lymphocult; Biotest AG, Landsteinerstrasse, Germany), an additional 24-h incubation was performed to allow reexpression of potentially modulated TCR (26, 40). For cell replication assays, cells were pulsed with [^3H]thymidine (Amersham; specific activity, 5 mCi/mmol; 0.5 μCi per well) 18 h before harvest. Labeled cells were collected on filter paper by using a cell harvester, and the [^3H]thymidine incorporation was measured as counts per minute with a beta counter (LKB, Wallac, Pharmacia). Results were expressed as mean stimulation indices (counts per minute of stimulated cells/counts per minute of unstimulated cells) for triplicate determinations.

Flow cytometric analysis. Three-color fluorescence analysis was performed according to previously described procedures (23, 24) on a FACSort flow cytometer (Becton Dickinson). Data acquisition was done with Lysys II software (Becton Dickinson). For each sample data for 3,000 lymphocytes were acquired by using log-amplified fluorescence and linearly amplified side and forward scatter signals. The data were analyzed with the Paint-A-Gate⁺ software (Becton Dickinson). All samples were analyzed by setting appropriate forward and side scatter gates around the lymphocyte population, using back gating on CD45⁺ CD14⁻ cells. The proportion of nonviable leukocytes was analyzed by staining with propidium iodide and peridinin chlorophyll-A protein-conjugated CD45. Multiple samples from individual patients were always stained and run at the same time.

To analyze the effect of in vivo and in vitro activation on the distribution of cells expressing particular TCR V β types within the CD4⁺ and CD8⁺ T-cell subsets, ratios were constructed. For analysis of in vivo activation, the frequency of cells expressing a particular TCR V β type in samples obtained at days 0, 7, and 30 was divided by the corresponding mean value in samples from controls. For analysis of in vitro activation, the frequency of cells expressing a particular TCR V β type in a culture stimulated with an antigen or a superantigen was divided by the corresponding value in the unstimulated control culture from that individual.

Statistical analysis. Data were analyzed by analysis of variance with Bonferroni-Dunn statistics at the 5% significance level with the StatView statistical software (Abacus Concepts, Inc., Berkeley, Calif.). Group comparisons were made by the Student *t* test.

TABLE 1. Frequency of peripheral blood T-cell subsets expressing the nine TCR V β types in C-B and V-S

TCR V β type	Expression (%) ^a by:			
	CD4 ⁺ T cells		CD8 ⁺ T cells	
	V-S	C-B	V-S	C-B
2	6.80 $\pm$ 1.20	6.19 $\pm$ 0.87	3.19 $\pm$ 0.79	3.08 $\pm$ 1.41
3	3.04 $\pm$ 0.92	2.44 $\pm$ 0.97	6.59 $\pm$ 1.12*	2.52 $\pm$ 0.96
5.1	2.83 $\pm$ 0.76*	4.85 $\pm$ 1.14	2.02 $\pm$ 0.45	1.49 $\pm$ 0.25
5.3	1.80 $\pm$ 0.65	2.54 $\pm$ 1.11	1.33 $\pm$ 0.46*	0.57 $\pm$ 0.34
6.7	3.99 $\pm$ 1.21	3.62 $\pm$ 0.63	2.71 $\pm$ 1.10	1.17 $\pm$ 0.30
8	4.35 $\pm$ 1.14	4.64 $\pm$ 1.13	3.83 $\pm$ 1.40	3.02 $\pm$ 1.01
12	2.57 $\pm$ 1.60	2.31 $\pm$ 0.35	2.08 $\pm$ 0.94	1.64 $\pm$ 0.50
13.6	1.92 $\pm$ 0.53	1.63 $\pm$ 0.61	1.13 $\pm$ 0.51	1.33 $\pm$ 0.45
17	4.06 $\pm$ 0.67	4.81 $\pm$ 0.72	3.76 $\pm$ 1.30	4.89 $\pm$ 1.20

^a Expressed as mean $\pm$ standard deviation. *, significantly different from the corresponding C-B value.

RESULTS

Lymphocyte subset distribution. The distributions of lymphocytes in peripheral blood from the patients with shigellosis, the healthy controls, and the volunteers studied here have been previously reported (23). In short, there was no significant change in the proportion of CD3⁺ cells in patients compared with controls. The proportion of CD4⁺ T cells in patients was similar to that in C-B, whereas the proportion of CD4⁺ T cells was significantly higher in V-S (58.5%) than in C-B (44.5%). The proportion of CD8⁺ as well as CD3⁺4⁺8⁺ T cells was significantly higher in C-B than in V-S. In SDIP, the proportion of CD8⁺ T cells was significantly lower throughout the disease course than it was in C-B (23).

Expression of TCR V β repertoire. The TCR V β gene segment expression in PBMC from SDIP and SFIP at different times after the onset of the disease and in the C-B and V-S groups was analyzed.

The frequency of CD4⁺ T cells expressing the nine TCR V β

types in V-S was similar to that in C-B, except for the expression of V β 5.1, which was higher in C-B than in V-S (Table 1). The frequency of CD8⁺ T cells expressing V β 3 and V β 5.3 was significantly higher in V-S than in C-B. In general, the TCR V β types analyzed were expressed more frequently on CD4⁺ T cells than on CD8⁺ T cells (Table 1).

Figure 1 shows the frequency of CD4⁺ T cells expressing the nine TCR V β types analyzed in controls, SFIP, and SDIP at onset (day 0). Even at onset the frequency of V β 2⁺ CD4⁺ T cells was significantly higher in SDIP than in C-B, whereas the frequency of CD4⁺ T cells expressing V β 5.1, V β 5.3, or V β 6.7 was significantly lower in SDIP than in C-B.

Throughout the 30-day observation period, the frequency of V β 2⁺ CD4⁺ T cells was significantly higher in SDIP than in C-B. The frequency of V β 2⁺ CD4⁺ T cells in SFIP was significantly higher than that in C-B only at days 4 and 7 (Fig. 2A). The frequency of V β 2⁺ CD4⁺ T cells was significantly higher in SDIP than in SFIP at days 11 and 30.

The frequency of V β 3⁺ CD4⁺ T cells was significantly higher in SDIP at days 7 and 11 than in C-B. In SFIP a significantly increased frequency was seen only at day 7. At day 11, the frequency of V β 3⁺ CD4⁺ T cells was significantly higher in SDIP than in SFIP (Fig. 2B).

A selective expansion of V β 5.1⁺ CD4⁺ T cells was seen only in SFIP; it peaked at day 7 (a nearly fourfold increase) (Fig. 2C).

Compared with V β 2⁺, V β 3⁺, and V β 5.1⁺ CD4⁺ T cells, the frequency of V β 13.6⁺ CD4⁺ T cells was low in all individuals. Only in SFIP was the frequency of V β 13.6⁺ CD4⁺ T cells increased (Fig. 2D). In SDIP the frequency of V β 13.6⁺ CD4⁺ T cells decreased during the disease course.

The peak expansion of V β 17⁺ CD4⁺ T cells appeared earlier in SFIP than in SDIP. At day 11, the frequency of V β 17⁺ CD4⁺ T cells was significantly higher in SDIP than in SFIP (Fig. 2E).

By comparing patients with controls, in vivo changes in the TCR V β repertoire over time were further analyzed. Figure 3 shows the ratios obtained by dividing the mean frequency of T

FIG. 1. Frequency (%) of peripheral blood CD4⁺ T cells expressing individual TCR V β types in SDIP (○) and SFIP (●) at onset of the disease and in C-B (×).

FIG. 2. Frequency (%) of peripheral blood $CD4^+$ T cells expressing individual TCR $V\beta$ types in SDIP and SFIP at different time points during the disease course and in controls. The frequency of positive cells was determined with the antibodies to TCR $V\beta 2$ (A); $V\beta 3$ (B); $V\beta 5.1$ (C); $V\beta 13.6$ (D), and $V\beta 17$ (E). Means and 95% confidence intervals are shown. Dashed lines connect mean for consecutive sampling days (days 0, 4, 7, 11, and 30).

cells expressing individual TCR $V\beta$ types in patient samples obtained at days 0, 7, and 30 by the mean frequency for the corresponding TCR $V\beta$ types in C-B. The frequencies of $V\beta 2^+$ $CD4^+$ T cells and $V\beta 3^+$ $CD4^+$ T cells peaked at day 7 both in SDIP and SFIP. At all time points, the frequency of $CD4^+$ T cells expressing $V\beta 5.1$, $V\beta 5.3$, and $V\beta 6.7$ was lower in SDIP than in C-B, whereas in SFIP only $V\beta 5.3$ had a frequency lower than that in C-B (Fig. 3A and B). At all time points the frequency of $V\beta 13.6^+$ $CD4^+$ T cells, as well as that of $CD8^+$ T cells, was increased in SFIP (Fig. 3B and D). In contrast, the increase in $V\beta 5.1^+$ T cells (seen in both $CD4^+$ and $CD8^+$ T cells) was most pronounced at day 7.

The skewing over time of the TCR $V\beta$ repertoire in vivo could be due to recirculation of activated *Shigella*-specific T cells carrying certain TCR $V\beta$ types. To further analyze the role of *Shigella* (super)antigen in the skewing of the TCR $V\beta$ repertoire, PBMC from 5 patients and 10 controls (5 C-B and 5 V-S) were stimulated in vitro.

Proliferative response of PBMC to in vitro stimulation. The proliferative response to phytohemagglutinin, used as a positive control, showed a stimulation index of 50, which was fivefold higher than that of the HK *S. dysenteriae* 1. Peak stimulation was seen in samples obtained at day 7, irrespective of the stimulant, as shown in Fig. 4. HK *S. dysenteriae* 1 gave a higher response than Ipa, Shiga toxin, or TSST-1. The responses to HK *S. flexneri* Y and *S. flexneri* Y PS were slightly lower than those to HK *S. dysenteriae* 1 and *S. dysenteriae* 1 PS, respectively.

Viability count with propidium iodide and peridinin chlorophyll-A protein-conjugated CD45. After 4 h of stimulation and after 96 h of culture, the leukocyte viability was analyzed

by exclusion of propidium iodide staining in CD45-positive cells. After 4 h, the viability was 95% in all cultures. After 96 h of culture, the percentage of viable cells in control cultures from C-B and V-S was 75%, whereas in control cultures from patients it was 70%. In cultures initially exposed to stimulants, the viability was approximately 60% in patient samples and 70% in samples from control individuals. The decreased forward and side scatter characteristics of nonviable cells allowed their separation from viable cells. The percentage of nonviable cells within the lymphocyte scatter gate was 2 to 3% for control samples and 5 to 8% for patient samples.

Frequency of T cells and T-cell subsets in stimulated PBMC.

When stimulated and unstimulated cell cultures were compared, no significance difference in the frequency of $CD3^+$ cells or the $CD4/CD8$ ratio was seen. In day 7 samples stimulated with HK *S. dysenteriae* 1, the proportion of $CD3^+$ cells (91.33%) was higher than that in unstimulated cells (84.3%).

CD69 expression on T cells by in vitro *Shigella* antigen stimulation. The frequency of $CD4^+$ T cells and $CD8^+$ T cells expressing the early T-cell activation marker CD69 was analyzed after 4 h of stimulation with antigens, as well as after 96 h of culture of cells. After 4 h of stimulation with HK *S. dysenteriae* 1 and HK *S. flexneri*, the frequency of $CD69^+$ $CD4^+$ T cells was more than twofold higher than that in unstimulated controls. Stimulation with TSST-1 gave a 1.5-fold increase. The frequency of $CD69^+$ $CD8^+$ T cells was increased slightly less than that of $CD4^+$ T cells. Analysis of CD69 expression in individual TCR $V\beta$ -expressing T-cell subsets showed no significant changes compared with controls. After 96 h of culture, the frequencies of $CD69^+$ cells in stimulated cultures and control cultures were similar (data not shown).

FIG. 3. Relative changes in the frequencies of peripheral blood CD4⁺ T cells and CD8⁺ T cells expressing nine TCR V β types, expressed as the ratios of the frequencies of positive cells in SDIP or SFIP at days 0 (□), 7 (▤), and 30 (▥) to those in C-B.

TCR V β repertoire of in vitro-stimulated T cells. For CD4⁺ and CD8⁺ T cells, the frequency of cells positive for the nine TCR V β types was assessed. The changes were expressed as the ratio between the frequencies of positive cells in stimulated

and unstimulated cultures. The mean ratios for cultures stimulated with HK *S. dysenteriae* 1, Shiga toxin, and TSST-1 are shown in Fig. 5 and 6 for CD4⁺ T cells and CD8⁺ T cells, respectively. With a ratio of 2 or higher considered to be a definite increase, stimulation with TSST-1 gave an increase in the frequency of V β 2⁺ CD4⁺ T cells in V-S, C-B, and patient samples obtained at day 7 (Fig. 5A, B, and D). For CD8⁺ T cells TSST-1 induced increases in V β 2-positive cells in C-B and in patient samples from day 0 (Fig. 6B and C).

The V β 17⁺ CD4⁺ T-cell ratio increased in response to HK *S. dysenteriae* 1 and Shiga toxin in patients at all time points as well as in controls. In addition, the V β 2⁺ CD4⁺ T-cell ratio increased in controls and in patient samples from day 7. In patient samples the higher responses in CD4⁺ T cells were observed at day 7. In contrast, for CD8⁺ T cells the higher responses were seen at day 0. Within both the CD4⁺ and CD8⁺ T-cell subsets, cells expressing TCR V β 5.1 and V β 13.6 responded more to HK *S. dysenteriae* 1 and Shiga toxin.

DISCUSSION

In the present study, using three-color flow cytometry, we have shown changes in the peripheral blood TCR V β repertoire during shigellosis. To further analyze the relationship of the skewed repertoire to *Shigella*-derived molecules, PBMC were stimulated in vitro. The *Shigella* antigen-induced in vitro response showed a TCR V β perturbation similar to that in vivo, with a maximum response at days 7 to 11.

FIG. 4. *Shigella* antigen- and TSST-1-induced proliferative responses in PBMC obtained at days 0, 7, and 30 from patients with shigellosis and from controls. □, HK *S. dysenteriae* 1; ▤, *S. dysenteriae* 1 PS; ■, Shiga toxin; ▥, Ipa; ▦, TSST-1.

FIG. 5. Effect of in vitro stimulation on the frequency of peripheral blood $CD4^+$ T cells expressing nine TCR $V\beta$ types. PBMC obtained at days 0, 7, and 30 were stimulated for 4 h with HK *S. dysenteriae* 1, Shiga toxin, or TSST-1 and cultured for 96 h. The change in the frequency of $V\beta^+ CD4^+$ T cells was expressed as the ratio between positive cells in stimulated and control cultures. The mean ratios are shown for V-S (A), C-B (B), and patient samples from days 0 (C), 7 (D), and 30 (E).

Our observations are not likely to fully reflect the full spectrum of TCR $V\beta$ changes in shigellosis. First, only 9 of over 20 recognized TCR $V\beta$ types could be assessed because of limitations in antibody availability and sample volume. Second, since shigellosis is a local gut infection, the PBMC would not be expected to fully reflect the extent of the local T-cell activation. The MABs were selected on the basis of their differential expression in various diseases in previous studies (5, 18, 35). In noncultured cells from Swedish and Bangladeshi controls, this panel identified 33% of $CD4^+$ T cells and 20% of $CD8^+$ T cells. When the V-S and C-B control populations were compared, minor differences in the TCR $V\beta$ repertoire were seen, mainly corroborating findings in previous studies comparing the TCR $V\beta$ repertoires in Scandinavian and Asian populations (35, 36).

By analysis of the TCR $V\beta$ repertoire in samples from days 0 to 30, a consistent pattern was seen, with a peak increase of the individual $V\beta$ types in samples obtained at days 7 to 11. This finding is consistent with our previous observations on the kinetics of peripheral blood T-cell activation in shigellosis (23) and is compatible with the notion that antigen-specific T cells locally activated in the gut may be sampled in the periphery at certain time points. Within the total recirculating pool of T cells, subsets with specific tissue-homing properties, such as gut-homing T cells, seem to exist (42, 43). A subset of the locally activated T cells will subsequently leave the site of antigen stimulation and after recirculation will again home

back to their tissue compartment (45). Thus, by sampling peripheral blood lymphocytes at different time points after their local activation, recirculating antigen-activated T cells leaving the inflamed tissue may be analyzed for their TCR $V\beta$ repertoire. Similar to the findings in vivo, in vitro stimulation of PBMC was associated with a skewing of the TCR $V\beta$ repertoire. The induction of this response pattern in vitro seemed to follow the same kinetics as seen in vivo, with peak increases of particular $V\beta$ types in samples obtained from days 7 to 11. The similarities seen in the $V\beta$ response patterns in samples from different days, but with differences in the magnitude, may indicate that the frequency of antigen-specific T cells appearing in the peripheral blood increases up to about day 11 and then subsequently decreases, possibly because of rehomeing into the gut-associated lymphoid tissues or, alternatively, because of apoptotic cell death. Interestingly, the peak response seemed to appear earlier in $CD8^+$ than in $CD4^+$ T cells.

The most prominent changes were seen in $V\beta 2$, $V\beta 3$, and $V\beta 17$ in both SDIP and SFIP, whereas $V\beta 5.1$ and $V\beta 13.6$ was increased only in SFIP. Moreover, the particular $V\beta$ types that were increased in $CD8^+$ T cells largely overlapped those that were increased in $CD4^+$ T cells. Of particular interest was the prominent peak in the $V\beta 5.1$ subset of $CD4^+$ T cells seen around day 7. This notable response, seen only in SFIP, would be compatible with a superantigen-like stimulation. We have previously seen a particular expansion of NK and NK-like cells in SFIP but not in SDIP (23). It has previously been suggested

FIG. 6. Effect of in vitro stimulation on the frequency of peripheral blood CD8⁺ T cells expressing nine TCR V β types. PBMC obtained at days 0, 7, and 30 were stimulated for 4 h with HK *S. dysenteriae* 1, Shiga toxin, or TSST-1 and cultured for 96 h. The change in the frequency of V β ⁺ CD8⁺ T cells was expressed as the ratio between positive cells in stimulated and control cultures. The mean ratios individual are shown for V-S (A), C-B (B), and patient samples from days 0 (C), 7 (D), and 30 (E).

that superantigen stimulation, in addition to V β -specific T-cell activation, may also influence NK cell activity (11). In several animal models and also in AIDS patients, it has been shown that superantigen-mediated T-cell activation is followed by the elimination or down regulation of the activated T cells (14, 27, 30). Such a mechanism could also explain the transitory appearance of the expanded V β types (especially V β 5.1) during shigellosis. The possible role of superantigens in the inflammatory response in shigellosis has not been well studied. In favor of a superantigen effect would be the similarity in response pattern seen in several individuals, resulting in increased mean ratios in particular for V β 5.1, V β 13.6, and V β 17. However, antigen-driven expansions also might be more predominant within particular V β subsets even in HLA-unrelated individuals (8, 18, 31). Recently it has been shown that a high proportion of TCR α/β cells may express more than one functional TCR type, involving different α and β chains (17). Moreover, recently subsets of T cells which seem to respond to nonpeptide antigens presented by nonpolymorphic HLA-like molecules such as CD1b have been identified (6). The possible role of such unconventional antigen presentation pathways in the defense against infections is as yet unclear, however.

The increased stimulatory response seen in samples from day 7 compared with controls suggests that the T cells had been antigenically primed in vivo. The stimulation protocol was

based on antigen pulsing of short duration (19, 47): the pulsing was long enough to allow antigen uptake by antigen-presenting cells but short enough to avoid any potential long-term toxic effects of the antigen preparations, especially Shiga toxin (32). We also excluded interleukin-2 from the early culture period in order to avoid expansion of non-antigen-activated T cells (29). The bonus of this system is the potentially lower "noise level" and the expansion of only cells stimulated in vitro. The trade-off is a lower level of oligoclonal amplification (26). Increased CD69 expression was already evident 4 h after stimulation, indicating that an early T-cell activation was taking place. However, analysis of the frequency of CD69-expressing cells within individual TCR V β T-cell subsets did not reveal any TCR V β preference in the early activation. The reason for this is not clear. It may be that CD69 expression is not a valid marker for the full activation of T cells. CD69 expression may be induced by microenvironmental changes also on "bystander" T cells which will not go on to replicate in response to antigen or superantigen.

Interestingly, stimulation with HK *S. dysenteriae* 1 and Shiga toxin gave very similar effects on the skewing of the TCR V β repertoire. Several explanations could be envisaged. One would be that those preparations may have one or several stimulants in common, either processed antigenic peptides and/or superantigen-like molecules. The possibility of an LPS-

mediated response due to contamination of these protein antigens is unlikely, since the preparations were negative in endotoxin tests and an LPS response would not be expected to involve only certain V β types. The PS-mediated response observed by thymidine incorporation was of a low magnitude (Fig. 4) and could be due in part to endotoxin contamination of these preparations (0.1 to 5.0 ng/ml). Both patients and controls (C-B), being from an area where shigellosis is endemic, may have been previously exposed to several *Shigella* spp., resulting in a T-cell memory for these and other *Shigella* antigens. The current results, however, seem to indicate that further studies specifically designed to map immunodominant epitopes in *Shigella* spp. would be of importance in *Shigella* vaccine trials.

Our data thus indicate that in shigellosis, both in vivo and after in vitro stimulation, particular TCR V β types are expanded in response to molecules known to induce a humoral immune response, suggesting that a T-helper response of type 2 may be induced. Whether activated T cells carrying V β specific receptors also are involved in a T-cell-mediated cytotoxic immune response to *Shigella* infection remains to be determined. Our findings also seem to support the hypothesis that following local activation, antigen-reactive T cells recirculate and can be sampled in the blood, which indicates that the effect of antigens or vaccines on the T-cell system may be monitored in the peripheral blood during dysenteric diseases. If this notion is confirmed by comparative analysis of TCR sequences in the blood and gut, it would certainly facilitate further analysis of the role of T cells in the immune defense against invasive enterobacterial disease.

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