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Cytotoxic activity of Vβ8$^+$ T cells in Crohn's disease: the role of bacterial superantigens

M. E. BACA-ESTRADA, D. K. H. WONG & K. CROITORU *Intestinal Disease Research Program, Department of Medicine, McMaster University, Hamilton, Ontario, Canada*

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SUMMARY

In Crohn's disease, disease-related stimuli could alter the T cell receptor (TCR) repertoire. To examine the possibility that changes in function may occur in T cell subsets without obvious changes in expression of TCR, we analysed the TCR repertoire of cytotoxic T lymphocytes in Crohn's disease peripheral blood. Furthermore, we examined the effect of bacterial superantigens, staphylococcal enterotoxin B (SEB) and E (SEE) on the cytotoxic function of T cell subsets bearing different TCR V genes using MoAbs specific for CD3 and TCR V gene products in a redirected cytotoxicity assay. There was no difference between patients and controls in the cytotoxicity measured in concanavalin A (Con A)-stimulated peripheral blood mononuclear cells (PBMC) with anti-CD3 or with six of seven anti-TCR V gene MoAbs. However, the cytotoxicity of Vβ8 T cells was decreased in Crohn's disease patients. This was not due to a decrease in total or CD8$^+$ T cells expressing Vβ8. Furthermore, in normal subjects, PBMC stimulation with SEE and SEB selectively expanded and increased the cytotoxicity of Vβ8 and Vβ12 T cells, respectively. In Crohn's disease, although SEB stimulation increased the number and cytolytic function of the Vβ12 subset, SEE stimulation failed to increase cytolytic activity of Vβ8$^+$ T cells in spite of the expansion of Vβ8$^+$ T cells. These results suggest that the changes in cytotoxic function observed in Vβ8 T cells in Crohn's patients may reflect previous exposure to a Vβ8-selective superantigen.

Keywords Crohn's disease superantigen T cell receptor cytotoxicity

INTRODUCTION

Crohn's disease (CD) is characterized by alterations in immune function; however, the relevance of these to disease pathogenesis or etiology remains unclear [1,2]. One of the difficulties in defining the role of the immune system in CD is the fact that an agent(s) or antigen(s) responsible for initiating and perpetuating the disease has not been identified. Several lines of evidence suggest that activated T cells are important in the pathogenesis of inflammatory bowel disease (IBD) [3,4], although most changes in T lymphocyte phenotype and function described in CD are not disease-specific [5,6]. Studies of antigen-specific cytotoxic T cell (CTL) activity in CD have been difficult. An indirect approach takes advantage of the fact that MoAbs specific for the T cell receptor (TCR)/CD3 complex can induce antigen-independent cytotoxicity of *in vivo* primed CTL [7]. Patients with IBD have an apparent increase in anti-CD3-triggered CTL in peripheral blood, suggesting a change in immune activity [8]; however, the nature of this activity in mucosal T cells of CD patients is not clear [9,10].

Recently, molecular probes and MoAbs to the variable (V) genes of the TCR complex and their products have been used to study the repertoire of T cell subsets in autoimmune and chronic inflammatory diseases [11,12]. Changes in the number of T cells expressing restricted TCR V gene products suggest that superantigens may be involved in the disease pathogenesis in such patients [13,14]. Superantigens activate T cell subsets through binding to a particular V region gene product, usually on the β-chain of the TCR [15,16]. In mice, *in vivo* stimulation with the staphylococcal enterotoxin superantigens can cause deletion or alterations in functional activity of T cells [17–19]. Recent studies have shown that a subgroup of CD patients have an increase in the number of Vβ8-expressing T cells in peripheral blood and mesenteric lymph nodes [20]. Although functional activity was not assessed, it is possible that superantigens may be involved in CD.

In this study, we examined whether functional changes could be demonstrated in peripheral blood T cells bearing particular TCR V gene products in CD, and whether such changes are related to a previous encounter with selected superantigens.

Correspondence: Kenneth Croitoru MDCM, Room 4H17, Intestinal Disease Research Program, McMaster University Medical Centre, 1200 Main St. W., Hamilton, Ontario, Canada L8N 3Z5.

PATIENTS AND METHODS

Subjects

Heparinized peripheral blood was obtained from 18 normal individuals and from 24 patients with CD. The diagnosis of CD was based on histologic, radiologic and clinical findings. Ten of the 24 patients with CD were receiving corticosteroid treatment, which did not interfere with the anti-CD3-triggered cytoxicity generated in peripheral blood mononuclear cells (PBMC) (data not shown).

Isolation and culture of peripheral blood lymphocytes

PBMC were isolated from heparinized blood by centrifugation over Ficoll–Paque (Pharmacia, Uppsala, Sweden), washed three times and resuspended at $0{\cdot}5 \times 10^6$/ml in culture media containing RPMI 1640 (GIBCO, Grand Island, NY) supplemented with penicillin (100 U/ml) (GIBCO), 100 μg/ml streptomycin (GIBCO), 2 mM glutamine (GIBCO), 10% heat-inactivated fetal calf serum (FCS; GIBCO), 50 μM 2-mercaptoethanol (Sigma) and 10 mM HEPES (GIBCO). To generate CTL, cells were cultured in 24-well plates (Falcon, Oxnard, CA) at a concentration of 10^6 cells/well in the presence of 10 μg/ml concanavalin A (Con A) (Pharmacia). After 2 days in culture, 5 U/ml of human recombinant IL-2 (Genzyme, Cambridge, MA) were added. After 7 days these cultures contained ⩾ 95% $CD3^+$ T cells with >98% viability.

To examine the effect of bacterial enterotoxins on T cell subsets, PBMC were cultured for 3 days with 5 μg/ml Con A or optimal concentrations of bacterial superantigens: 1 μg/ml staphylococcal enterotoxins B or E (SEB or SEE; Toxin Technologies, Madison, WI). Cultured cells were then washed in RPMI containing 1% FCS and incubated in the presence of 5 U/ml recombinant IL-2 (Genzyme) for an additional 24 h to allow for re-expression of any T cell receptor down-regulated by the enterotoxin [15].

Cytotoxicity assay

The Fc receptor-expressing murine mastocytoma cell line P815 was used as the target cell for the redirected cytotoxicity assay. P815 were labelled with $Na^{51}CrO_4$ (New England Nuclear, Boston, MA), then washed four times, counted and resuspended in supplemented media at $1{\cdot}5 \times 10^6$/ml. ^{51}Cr-labelled P815 cells (100 μl) were then transferred to separate tubes and incubated with 200 ng of one of the following MoAbs: anti-CD3 (OKT3; Ortho Diagnostics, Raritan NJ), anti-CD8 (T8; Coulter, Hialeah, FL), Vβ5a (or anti-TCR Vβ5.2 and 5.3; clone 1C1), Vβ5c (anti-TCR Vβ5.1; clone LC4), Vβ6a (anti-TCR Vβ6.7; clone OT145), Vβ8a (anti-TCR Vβ8 subfamily; clone 16G8), Vβ12a (anti-TCR Vβ12.1; clone S511) and Vα2a (anti-TCR Vα subfamily 2.3 clone; clone F1) (T-Cell Diagnostics, Cambridge, MA). Five thousand antibody-coated target cells were then added to each well of a round-bottomed microtitre plate (Corning Inc., Corning, NY) with 100 μl of effector cells at $2{\cdot}5 \times 10^6$/ml to give an effector : target (E : T) ratio of 50 : 1. Spontaneous and maximum ^{51}Cr release was determined by incubating labelled cells with media alone or 1 N hydrochloric acid (HCl), respectively. All assays were done in triplicates. After 4 h incubation, ^{51}Cr release in 100 μl of supernatant from each well was measured in a gamma counter (LKB, Turku, Finland). Specific cytotoxicity was calculated as follows: (measured ct/min – spontaneous ct/min)/(maximum release ct/min – spontaneous ct/min) × 100. Spontaneous release as well as cytotoxicity in the presence of an irrelevant antibody was <20% of total release. Given the multiple number of antibodies used in testing the cytotoxicity of the T cell subsets and the further requirement for sufficient cells to stain for V region expression, a range of E : T ratios could not be examined for each patient. Since differences in cytotoxicity could reflect differences in number of killer cells, we compared the proportion of T cells expressing the given TCR between control and CD patients.

In experiments examining the effect of soluble superantigens on the cytotoxic activity of T cell subsets expressing different TCR V gene products, the absolute increase in cytotoxicity was calculated as (cytotoxicity after toxin with anti-TCR antibody – with irrelevant antibody) – (cytotoxicity after Con A with anti-TCR antibody – with irrelevant antibody).

Immunofluorescence analysis

Cultured PBMC were washed and stained with FITC-conjugated MoAbs to CD4 (T4; Coulter) or anti-TCR V regions (see above; T-Cell Diagnostics) and PE-conjugated MoAb to CD8 (Leu-2a; Becton Dickinson, San Jose, CA) or PE-conjugated anti-CD3 (OKT3; Coulter). Briefly, 1×10^6 cells were incubated in 100 μl PBS containing 0·1% sodium azide and 0·2% bovine serum albumin (BSA; Boehringer, Mannheim, Germany) containing appropriate dilution of conjugated antibodies for 30 min at 4°C. Cells were washed and fixed in fresh 1% paraformaldehyde and analysed on an EPICS 541 (Coulter) or FACScan (Becton Dickinson) flow cytometer. In experiments examining the effect of soluble superantigens on TCR V gene expression, a stimulation index was calculated as the percentage of T cells expressing an individual V region post-stimulation, divided by the percentage of T cells expressing the same V region prestimulation.

Statistical analysis

Statistically significant differences between different groups of patients were determined by the Mann–Whitney *U*-non-parametric test (two-tailed test). A level of significant difference was accepted at $P < 0{\cdot}05$.

RESULTS

Decreased cytotoxic activity of $V\beta8^+$ T cells in CD

Freshly isolated PBMC from patients with CD or controls did not have detectable cytotoxic activity under the conditions used in the anti-CD3-triggered cytotoxicity assay against P815 (data not shown) [21]. Differences with previous reports may reflect different target cells used or different culture conditions [8]. Therefore, the cytotoxic potential of peripheral blood T cell subsets was measured after *in vitro* stimulation with Con A and IL-2 for 5–7 days. This stimulation resulted in significant anti-CD3-mediated killing of the P815 target cells. Cytotoxicity in the absence of antibody or in the presence of an irrelevant antibody such as anti-CD8 was <10%, indicating that lectin- or lymphokine-activated killers contributed little to the observed killing. The anti-CD3-mediated cytotoxic activity in Crohn's patients and controls ($61{\cdot}0 \pm 5{\cdot}1$% and $59{\cdot}5 \pm 6{\cdot}6$% killing ± s.e.m., respectively) was not statistically different (see Fig. 1).

The cytotoxic activity of peripheral blood T cell subsets

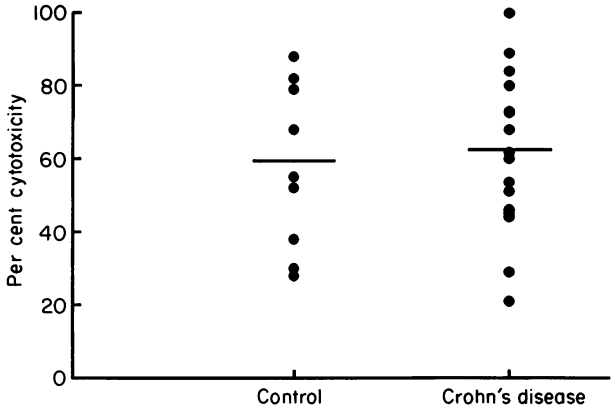

Fig. 1. Anti-CD3-triggered cytoxicity in Crohn's disease peripheral blood is similar to that in normal controls. Peripheral blood mononuclear cells (PBMC) from Crohn's disease ($n = 11$) and control patients ($n = 17$) were cultured for 5–7 days with concanavalin A (Con A) and IL-2 and tested for anti-CD3-triggered cytotoxicity against the P815 cell line, as described in Patients and Methods ($P = 0{\cdot}4$).

expressing TCR Vβ8, Vβ5a, Vα2a, Vβ6a or Vβ12a was measured individually using MoAbs specific to each TCR V region gene product after culture for 5–7 days with Con A and IL-2, as described above. The cytotoxicity of each T cell subset in patients with CD was compared with that in control individuals. The results indicate that PBMC from CD and control patients had similar redirected cytotoxicity with anti-TCR Vβ12a, Vβ5.a, Vα2a (see Fig. 2), or Vβ6a (data not shown). On the other hand, the cytotoxicity observed with anti-Vβ8 in PBMC from CD patients was significantly decreased compared with controls (Fig. 2). This was not related to the isotype of the anti-Vβ8 MoAb, since anti-Vβ12 was also IgG2b and did not show any difference in CD patients.

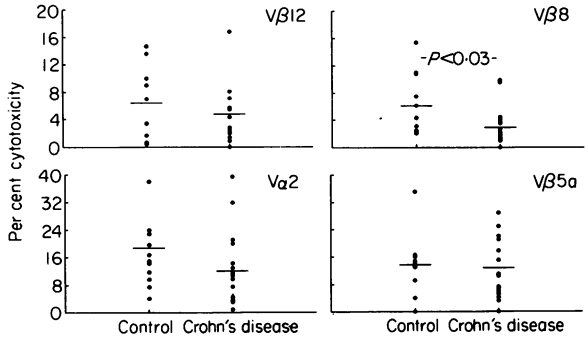

Fig. 2. Anti-T cell receptor (TCR) Vβ8 antibody-triggered cytotoxicity is selectively decreased in Crohn's disease. Peripheral blood mononuclear cells (PBMC) from Crohn's disease ($n = 11$) and control patients ($n = 17$) were cultured for 5–7 days with concanavalin A (Con A) and IL-2 and tested for anti-TCR-triggered cytotoxicity using MoAbs specific for the TCR V region gene product indicated. P815 killing was measured as described in Patients and Methods. The *P* values of the difference between the cytotoxicity generated with anti-Vβ12, Vβ8, Vα2, and Vβ5 were 0·096, 0·029, 0·126, 0·301, respectively.

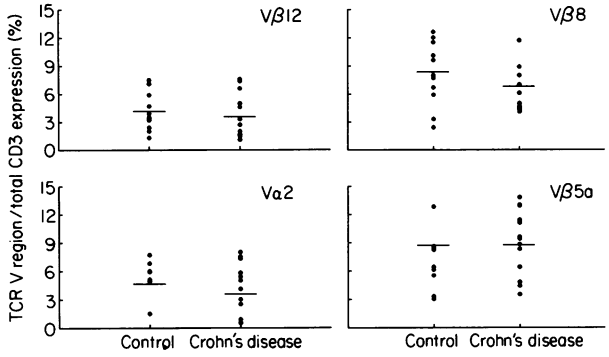

Fig. 3. Expression of T cell receptor (TCR) V gene products on total $CD3^+$ T cells after culture is not altered in Crohn's disease. Peripheral blood mononuclear cells (PBMC) cultured with concanavalin A (Con A) and IL-2 for 5–7 days were stained with FITC-conjugated anti-TCR V region-specific MoAbs and PE-conjugated anti-CD3. Two-colour flow cytometry analysis was performed to determine the distribution of TCR V gene expression as a per cent of total CD3-expressing cells.

Vβ8 TCR expression in cultured peripheral blood T cells is not altered in CD patients

In order to determine if the changes in the cytolytic activity detected in the TCR Vβ8 T cell subset, reflected changes in the proportion of T cells expressing this Vβ gene product after culturing with Con A, we analysed the distribution of TCR expression on total $CD3^+$ and $CD8^+$ T cells by two-colour flow cytometry. The percent expression of each of the TCR V region gene products on $CD3^+$ (Fig. 3) or on $CD8^+$ T cells (Fig. 4) in PBMC from Crohn's patients, after a 5-day culture, was similar to that in controls. In particular, the expression in Crohn's patients and controls of the Vβ8 TCR on total $CD3^+$ ($7{\cdot}1 \pm 1{\cdot}1\%$ *versus* $8{\cdot}4 \pm 0{\cdot}9\%$, respectively) and on $CD8^+$ T cells ($4{\cdot}3 \pm 1{\cdot}4\%$ *versus* $5{\cdot}9 \pm 1{\cdot}8\%$, respectively), was not statistically different.

Soluble bacterial superantigens stimulate cytotoxic activity in peripheral blood T cells

A possible explanation for the selective decrease in Vβ8 T cell cytotoxicity and the previously described changes in the

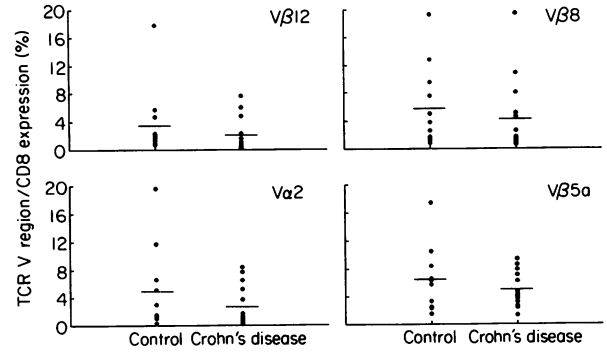

Fig. 4. Expression of T cell receptor (TCR) V gene products on total $CD8^+$ T cells after culture is not altered in Crohn's disease. Peripheral blood mononuclear cells (PBMC) cultured with concanavalin A (Con A) and IL-2 for 5–7 days were stained with FITC-conjugated anti-TCR V region-specific MoAbs and PE-conjugated anti-CD8. Two-colour flow cytometry analysis was performed to determine the distribution of TCR V gene expression as a per cent of total CD8-expressing cells.

proportion of Vβ8 T cells in freshly isolated PBL and mesenteric lymph nodes from patients with CD, is that these patients have been exposed to a 'Vβ8 selective' superantigen *in vivo*. Therefore, we examined the effect of soluble bacterial superantigens on the cytotoxic activity of the Vβ8 T cell subset in CD. PBMC were stimulated with 1 μg/ml of SEB or SEE for 3 days, and the cytotoxicity generated in these cultures was compared with that generated after Con A stimulation. Stimulation of PBL with SEB or SEE alone generated strong levels of anti-CD3-triggered cytotoxicity (data not shown). Under each of these conditions, the cytotoxicity measured in the absence of antibody or in the presence of an irrelevant antibody remained less than 12%, with no statistical difference between CD and control patients, again suggesting that stimulation of natural killer (NK) or LAK activity was minimal.

The effect of stimulation with SEE or SEB on the cytotoxic activity of TCR Vβ8- or Vβ12-expressing subsets was then examined in CD patients. In both Crohn's patients and controls, stimulation with SEE and SEB led to the expansion of T cells expressing Vβ8 and Vβ12, respectively (see Fig. 5). In addition, SEE caused some expansion of Vβ12 cells in both patient groups. The effect of soluble superantigen on the cytotoxic activity of T cells from Crohn's patients was very different. In keeping with the effect on T cell subset expansion, SEB stimulation of PBMC led to a selective increase in the cytotoxic activity of Vβ12 T cells in both patient groups (Fig. 6). On the other hand, SEE stimulation led to an increase in cytotoxic activity of Vβ8 T cells in control patients, but not in patients with CD (Fig. 6). This was in spite of a measured expansion of Vβ8 T cells seen in Crohn's PBMC cultured with SEE (Fig. 5). SEE stimulation also increased the cytotoxic activity of Vβ12 T cells in both Crohn's patients and controls (Fig. 6).

DISCUSSION

This study examined for changes in the cytotoxic activity of T cells bearing different TCR V gene products using a MoAb-redirected cytotoxicity assay. Redirected cytotoxicity reflects the cytotoxic potential of a given T cell population realized by the fact that the MoAb serves to bridge the effector cell with the target cell, cross-linking the TCR and inducing activation of the T cell [7,21]. Under these conditions, the cytotoxic ability measured is restricted to those T cells expressing the TCR recognized by the MoAb. The data show that the cytotoxic activity of the Vβ8 T cell subset was significantly reduced in patients with CD. This was not due to disease or

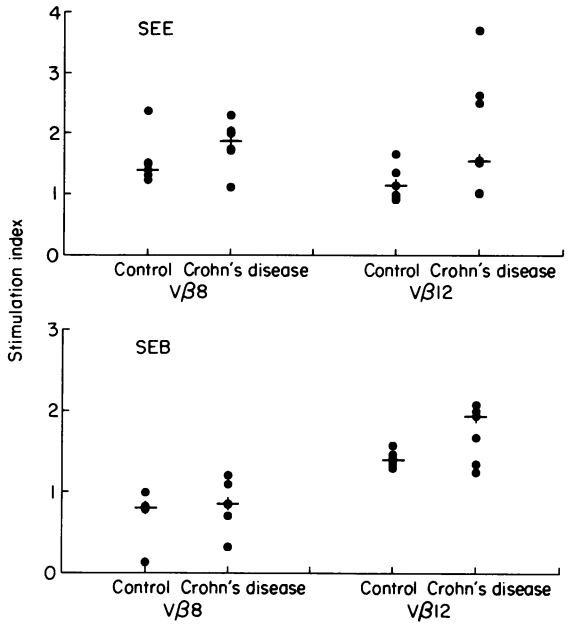

Fig. 5. Staphylococcal enterotoxins E (SEE) and B (SEB) induce expansion of Vβ8 and Vβ12 T cell subsets in peripheral blood mononuclear cells (PBMC), respectively, in both Crohn's and control patients. SEE also induced Vβ12 T cell expansion in both groups. PBMC cultured in the presence of concanavalin A (Con A), SEE or SEB as described in Patients and Methods, were stained for the expression of Vβ8 or Vβ12 T cell receptor (TCR). The stimulation index was calculated by dividing the mean expression of the given TCR after culture with the enterotoxin SEE or SEB by the expression seen after culture with Con A alone. Individual experiments are represented on the scatter plot, and the median for each group is represented by the hatch mark.

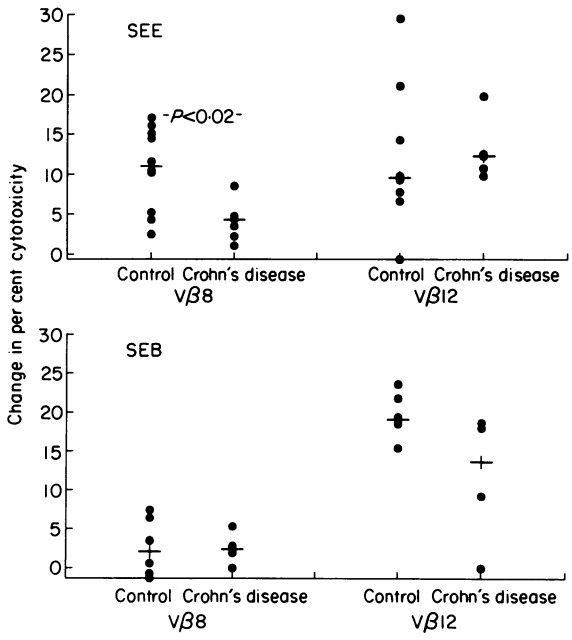

Fig. 6. Staphylococcal enterotoxin E failed to increase cytotoxicity of Vβ8 T cells in Crohn's disease patients. Peripheral blood mononuclear cells (PBMC) cultured in the presence of concanavalin A (Con A), staphylococcal enterotoxins E (SEE) or B (SEB), as described above, were tested for redirected cytotoxicity using anti-Vβ8 or anti-Vβ12 T cell receptor (TCR). The change in cytotoxicity for each patient is depicted as a dot on the scatter plot, and the median for each group is represented by the hatch mark. The SEE-stimulated cytotoxicity in Vβ8 T cells from patients with Crohn's disease was statistically less than that determined for controls ($P < 0{\cdot}02$).

treatment-induced down-regulation of total cytotoxic activity as measured with anti-CD3; or to a decrease in the proportion of total $CD3^+$ or $CD8^+$ T cells expressing Vβ8. It is unlikely that this was due to the influence of the individual's MHC class II haplotypes on TCR repertoire [22], since the bias for the usage of Vβ8 region gene product in CD was not linked to MHC genes alone [20]. The fact that among the T cell subsets tested with the seven MoAbs only $V\beta8^+$ T cells exhibited a difference in cytotoxic function, indicates that there was selectivity based on the Vβ expression, and this would suggest a superantigen effect.

With the availability of technology that allows for the assessment of changes in TCR variable gene usage [11,12,14], the question of whether changes in T cell repertoire and defects in the mechanisms of tolerance induction can explain the role of T cells in autoimmunity has been addressed. The ability of superantigens to influence T cell development through the induction of clonal deletion and anergy not only provides a model to study repertoire selection in these diseases, but also suggests a means by which an organism can influence the immune system [23]. CD is an inflammatory disease where the initiation of disease may be far removed in time from the chronic phase in which most patients are diagnosed and followed. The notion that an antigenic stimulus or a superantigen may have triggered the onset of the disease leaves open the possibility that the remnant of such an initiating event may be found in changes in the expression of the TCR repertoire. In addition, it is possible that changes resulting from superantigen exposure may be reflected in changes in the function of T cell subsets with or without changes in TCR V gene expression. This is highlighted by studies in mice that show that the earliest response to a superantigen is that of T cell proliferation and expansion of a restricted T cell subset based on the TCR V gene product. This is quickly followed by anergy and loss of function in this subset, which can persist for a long time [24,25], and subsequent elimination of the subset via apoptosis [18,26,27]. Similar temporal changes have been described in patients with acute Kawasaki's disease, where the initial stages of the disease can be easily diagnosed, and are characterized by a short lived expansion of Vβ2 and Vβ8 TCR [28], followed by a deletion of this T cell subset. Therefore, our initial hypothesis was that measuring alterations in the proportion of T cells expressing different TCR V gene products may fail to detect significant changes relevant to the pathogenesis of CD. Further insight into the possibility of a superantigen involvement in CD could be gained through examining changes in the function of the different T cell subsets.

The possibility that the alteration in function of Vβ8 T cells reflects exposure to a superantigen was further explored by examining the *in vitro* effect of SEB and SEE on the cytotoxic activity of T cell subsets. The data show that SEB increased the cytotoxicity of Vβ12-expressing T cells selectively in both CD patients and controls, while SEE increased the cytotoxicity of Vβ12 and Vβ8 PBL T cells, but only in the control patients. In CD, SEE failed to stimulate the cytotoxic function of Vβ8 T cells, in spite of the fact that SEE expanded this T cell subset. Staphylococcal enterotoxins have been shown to affect human T cell function [15,29] and modulate the responsiveness T cell clones to antigen without altering their proliferative capacity to IL-2 [30]. On the other hand, studies that have examined the effect on cytotoxic activity have shown that the induction of anergy, as reflected by the lack of proliferation, can occur without interfering in the cytotoxic function of T cell lines [31,32]. This suggests that TCR activation involved in these effector functions, proliferation and cytotoxicity, can be altered independently of one another. The finding that SEE was able to induce expansion of the Vβ8 T cell subset suggests that TCR-mediated signalling was not completely abolished. Nonetheless, it is tempting to speculate that the lack of a response by Vβ8 T cells to bacterial superantigens reflects an altered functional state that could be the result of a previous encounter with a superantigen selective for Vβ8 T cells.

In the animal model of experimental allergic encephalomyelitis, the biased expression of T cells bearing specific TCR V gene products is compartmentalized and is seen only at the initial stages of the disease [33]. This may related to locally available antigen-presenting cells. In the intestine, epithelial cells have been shown to present superantigens to peripheral and lamina propria T cells [34], although the degree to which this leads to an expansion of different T cell subsets based on restricted Vβ TCR expression was not shown. In addition there may be a heterogeneity in T cells reactive to the superantigen [35]. Therefore, it remains possible that superantigen exposure in the intestine may result in a different TCR repertoire of cytotoxic mucosal T cells, although a recent study suggests that in both normal and CD lamina propria T cells express a similiar TCR repertoire [36].

The mechanism by which alterations in cytotoxic function of $V\beta8^+$ T cells might contribute to the pathogenesis or clinical manifestations of CD is unclear. The overlap in cytotoxic activity of Vβ8 T cells between Crohn's patients and controls may represent differences in time from exposure to superantigen stimuli. Nontheless, we conclude that these findings indicate a previous exposure to a superantigen that may or may not be of bacterial origin, but that influences Vβ8 T cell function selectively. The possibility remains that these changes in Vβ8 function are a result of the chronic intestinal inflammation with breakdown of the intestinal barrier to bacterial products. Alternatively, T cells involved in autoimmune disease have been shown to be resistant to tolerance induction [37]. It is therefore possible that the Vβ8 T cells with altered cytotoxic function represent a clone of T cells that are involved in the autoimmune response, and have escaped tolerance induction, leaving only the functional changes as a marker of this abrogated attempt at tolerance [37].

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