Clostridium perfringens Enterotoxin is a Superantigen Reactive with Human T Cell Receptors Vβ6.9 and Vβ22

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Summary

Candidate superantigens were screened for their ability to induce lysis of human histocompatibility leukocyte antigen class II-positive targets by human CD8+ influenza-specific cytotoxic T cell (CTL) lines. Clostridium perfringens enterotoxin (CPET) induced major histocompatibility complex unrestricted killing by some but not all CTL lines. Using "anchored" polymerase chain reactions, CPET was shown to selectively stimulate peripheral blood lymphocytes bearing T cell receptor Vβ 6.9 and Vβ22 in five healthy donors. Vβ24, Vβ21, Vβ18, Vβ5, and Vβ6.1–5 appeared to be weakly stimulated. Antigen processing was not required for CPET to induce proliferation. Like the staphylococcal enterotoxins, CPET is a major cause of food poisoning. These data suggest that superantigenic and enterotoxigenic properties may be closely linked.

The term superantigen (SA) has been applied to a group of bacterial and retroviral proteins that stimulate large numbers of T lymphocytes in a manner distinct from that of classical antigens (1). SA bind to the TCR Vβ chain of both CD4⁻ and CD8⁺ T cells (2), are mitogenic at nM concentrations, and do not require processing (3). SA bind MHC class II molecules selectively and with high affinity (4, 5), although they may also bind other ligands (6). Staphylococcal toxic shock syndrome toxin 1 (TSST) is implicated in toxic shock (7), and the staphylococcal enterotoxins (SE) are potent emetics, although it is not clear if their emetic action is a direct result of their superantigenic properties (8).

In this report we show that the 34-kD enterotoxin produced by Clostridium perfringens (CPET) is a superantigen with a novel specificity for human TCR Vβ chains.

Materials and Methods

Purification of Enterotoxins. SEA and SEB were purified from culture supernatants by dye ligand affinity chromatography as described (9). TSST was a gift from Rossalyn Brehm (Public Health Laboratory Service, Wilts, UK). CPET was purified as described previously (10).

Cytotoxic T Cell Lines and Clones. Influenza A virus-specific CTL lines were generated as described previously (11). Influenza A matrix peptide 57–68 was used to generate HLA A2-restricted CTL from donor JM (HLA A2 B15,51 Dr11) (11), and nucleoprotein (NP) 380–391 to generate HLA B27-restricted CTL (12) from donors GR (HLA A3,31 B27 DR4) and SD (HLA A2,32 B27,49 DR4 DQ8). CTL clones were obtained by limiting dilution. TCR usage of JM, GR, and SD CTL and clones (Bowness, P., and A. J.

McMichael, manuscript in preparation) was studied using the PCR as described below. CTL lysis assays were performed as described previously (11).

Proliferation Assays. PBMC from donor NW (HLA A3,31 B8,27 DR3,14) were cultured with APC in 96-well plates at 10⁵ cells/well. As APC autologous PBL were fixed with 0.025% glutaraldehyde for 90 s before adding 0.2 M lysine, washing three times, and irradiating (5,000 rad). APC were incubated for 2 h with purified protein derivative 1:200 (PPD 100,000 U/ml; Evans Medical, Langhurst, UK), CPET, SEB, or PHA at 5 μg/ml before washing twice. After 5 d 1 μCi [³H]thymidine was added, cells were harvested after 6 h and counted by standard scintillation techniques.

Selection of Activated Lymphocytes. PBL, cultured for 72 h with CPET, TSST, or SEB at 100 ng/ml or PHA at 5 μg/ml, were washed with PBS and incubated on ice with the mAb 23A9.3 (a gift of C. Mawas, Institut National de la Recherche Médicale, Marseille, France), specific for the IL-2 receptor (IL-2R). Lymphocytes bearing the IL-2 receptor were selected with magnetic beads coated with goat anti–mouse Ig (Dynal, Wirral, UK). Fresh unselected PBMC or IL-2R-selected PHA-stimulated PBMC were used as controls.

PCR Analysis of TCR Vβ Gene Usage. Total cellular RNA was extracted from CTL, unstimulated PBL, or activated lymphocytes. cDNA was synthesized using an oligo dT primer and anchored PCR (AnPCR) was performed using primers and conditions described previously (13). Amplification products were digested with BglII and NotI and cloned in a modified M13mp18 vector. Clones were either sequenced using T7 DNA polymerase or blotted onto nitrocellulose and hybridized with radiolabeled oligonucleotide probes for Cβ (5′-CGACTTCGGGTGGGAACAC); Vβ6.9 (5′-TGGAGAAAGATCCCTTAG); Vβ22 (5′-AGTGAAATTTGAT-CCATCAG); and Vβ 24 (5′-AAAGCAGAAAGAAGTGTT).
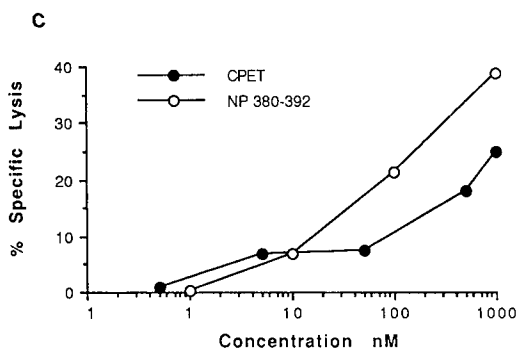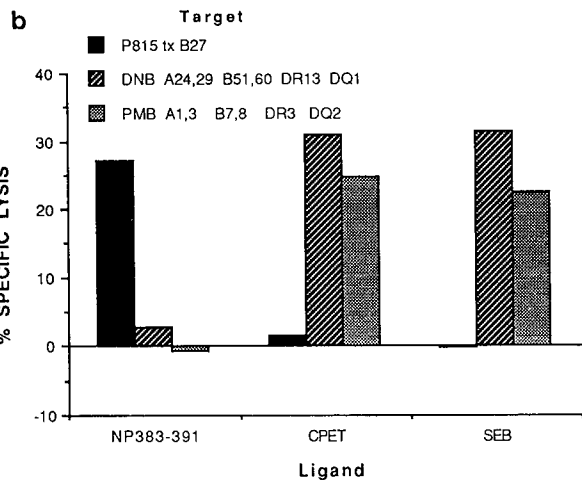
Figure 2. Proliferation of NW PBL after 5-d culture with autologous irradiated APC, with or without prior glutaraldehyde fixation, incubated with PPD, CPET, SEB, or PHA (5 μg/ml). Mean and SD of quadruplicate [³H]thymidine uptake estimations are shown.

ence of superantigens at nanomolar concentrations (2, 14, and P. Bowness, unpublished observations), regardless of their antigenic specificity. Influenza A-specific CD8⁺ oligoclonal CTL lines bearing different Vβ elements were used to screen potential superantigens. Such an experiment is shown in Fig 1 a. CPET induced killing of autologous targets in the absence of virus antigen by GR CTL (Vβ24, 21, 7, 6, and 5), but not with JM CTL (Vβ17). This suggested that CPET was a superantigen with specificity for the TCR Vβ expressed by GR CTL, but not JM CTL. Further experiments demonstrated that CPET-induced CTL killing is not MHC restricted. SD CTL (from which clones bearing Vβ6, 7, and 21 were derived) killed only HLA B27 positive targets in the presence of cognate peptide but killed HLA-mismatched allogeneic B cell lines (but not P815 cells lacking MHC class II) in the
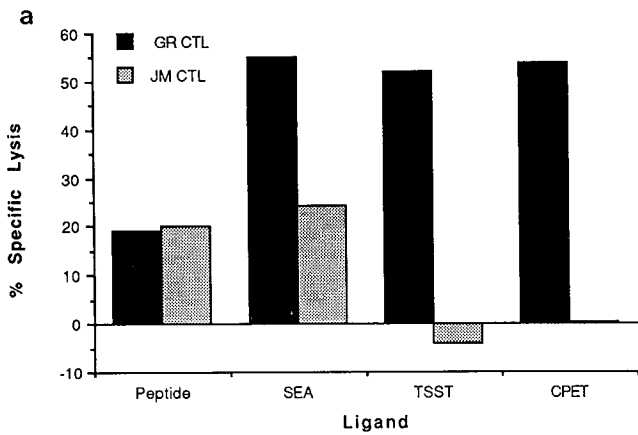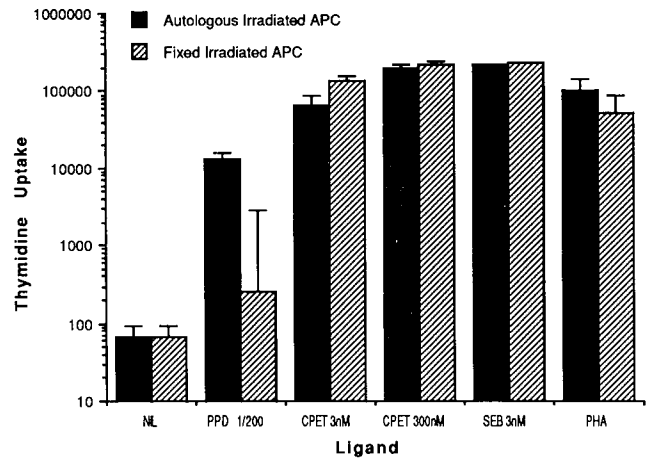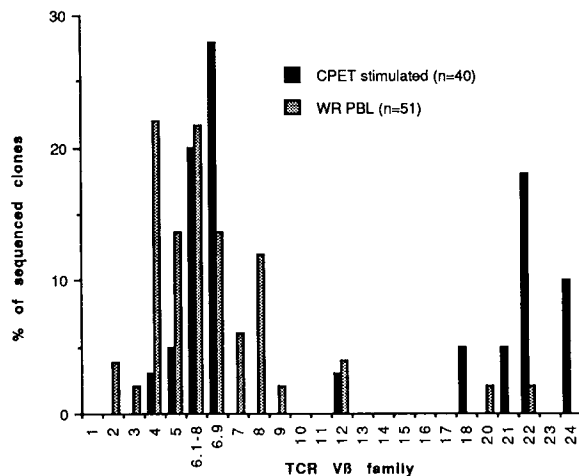
Figure 1. (a) Lysis of autologous target cells by JM and GR CTL in the presence of cognate peptide or bacterial toxins. Peptides (influenza matrix 57-68 for JM, nucleoprotein 380-391 for GR) were at 1 μM. SEA, TSST, and CPET were at 25 nM. The E/T ratio was 2:1 for JM CTL, and 5:1 for GR CTL. (b) Lysis by SD CTL (HLA 2,3 B27,49 DR4 DQ8) of P815 cells transfected with HLA-B27 or of HLA mismatched target cells in the presence of influenza nucleoprotein peptide, CPET or SEB at 1 μM. The E/T ratio was 1.5:1. (c) Lysis of autologous targets by GR CTL in the presence of influenza nucleoprotein 380-391 or CPET in the concentrations shown. The E/T ratio was 2:1.

Figure 3. TCR Vβ usage of CPET-stimulated lymphocytes and PBL from WR. Numbers and percentages of sequenced clones of AnPCR products are shown. The Vβ6 family is divided into Vβ6.1-8 and Vβ6.9. TCR Vβ usage of PBL from donor GR adapted from (21) with permission, and Rosenberg, W. R., unpublished data.

Results and Discussion

CPET Facilitates Cytolysis by Some but Not All CTL. It has been demonstrated previously that CD8⁺ CTL with suitable TCR Vβ kill MHC class II-positive cells in the pres-

Table 1. *TCR Vβ Usage of Lymphocytes Activated by PHA or Superantigens from Five Unrelated Donors*

	Cβ	Vβ6.9		Vβ22		Vβ24	
	No.	No.	Percent	No.	Percent	No.	Percent
Donor 1							
CPET	141	34‖	24	20§	14	9*	6
PHA	146	8	6	5	3	5	4
Donor 2							
CPET	80	23‖	30	14§	18	5‡	6
PHA	325	26	8	18	6	5	2
Donor 3							
CPET	53	5‡	9	6‡	11	ND	
PHA	102	2	2	1	1		
Donor 4							
CPET	201	24§	12	17§	9	ND	
PHA	137	3	2	1	1		
Donor 5							
CPET	244	17‖	7	14‖	6	ND	
SEB	366	3*	1	8‡	2.2		
TSST	509	2*	0.4	0*	0		
PHA	435	4	1	1	0.2		

Represents TCR frequencies after culture of PBL for 72 h with CPET, PHA, SEB, or TSST; assigned by oligonucleotide probing of M13-cloned AnPCR products. The donor HLA types were: donor 1: A11,33 B35,65 DR1,4; donor 2: A3,31 B27 DR4; donor 3: A1,3 B7,8 DR3; donor 4: A2,29 B44 DR7; and donor 5: A1,2 B7,8.
* $p > 0.05$ compared with PHA.
‡ $p < 0.05$ (Fisher's exact probability).
§ $p < 0.005$.
‖ $p < 0.0005$ (χ^2 with Yates' correction).

presence of CPET (Fig. 1 *b*). In contrast, two NP-specific HLA B27 restricted clones bearing Vβ7.1 (derived from GR and SD CTL) were CPET unresponsive (data not shown). CPET induced killing at nanomolar concentrations (Fig. 1 *a*) that was dose dependent (Fig. 1 *c*), and caused modulation of CD3 from the CTL cell surface (data not shown).

Glutaraldehyde-fixed Cells Can Present CPET. PBMC proliferated in a dose-dependent manner when incubated with CPET at nanomolar concentrations (data not shown). Thymidine uptake was consistently 10-fold higher than with PPD or tetanus toxoid, peaked 2 d earlier (data not shown), and was not inhibited by glutaraldehyde fixation of APCs (Fig. 2), indicating that antigen processing is not required.

CPET Selectively Stimulates TCR Vβ6.9 and Vβ22. The Vβ distribution of CPET-stimulated lymphocytes from donor WR, estimated by sequencing 40 M13-cloned AnPCR products of lymphocyte cDNA, was markedly different from that of unstimulated PBL (Fig. 3). 19 of 40 sequences were of the Vβ6 family, and of these, 61% were Vβ6.9 (14) (compared with only 39% of PBL Vβ6 (W. Rosenberg, unpublished data). Four rare Vβ families: Vβ18, 21, 22, and 24 (15), also appeared to be selectively stimulated by CPET. Of these, the most prominent was Vβ22 (15), which made up 18% of sequences from CPET stimulated cells, as compared with < 2% of stimulated PBL. The stimulation of Vβ6 and Vβ21 was consistent with the cytolysis by GR and SD CTL (Fig. 1, *a* and *b*).

To confirm these findings using larger numbers, PBL from WR and four other unrelated donors were stimulated with CPET or PHA (and in one case also SEB and TSST), and after AnPCR recombinant M13 plaques were screened with oligonucleotide probes specific for the three TCR Vβ that appeared most strongly stimulated: Vβ6.9, Vβ22, and Vβ24. The results are shown in Table 1. Vβ6.9 and Vβ22 were significantly stimulated by CPET in all five individuals tested ($p < 0.05$ as compared with PHA). Vβ24 numbers were significantly increased after CPET stimulation in one of two donors tested. In the one individual tested, TSST stimulated neither Vβ6.9 nor Vβ22, but SEB was found to stimulate Vβ22 to a low but significant extent.

AnPCR has not previously been used to study the TCR repertoire of SA-stimulated PBL. Unlike the "V region family specific" PCR techniques used previously (16), AnPCR gives quantitative information about all Vβ families and subfamilies. Hence, CPET is the only SA for which a complete profile of Vβ stimulation has been obtained. These findings also support the proposition that one SA may have different affinities for different Vβ elements (17).

It is possible that the novel SA characterized here is not CPET itself, but a contaminant of the preparations used. However, such contamination (which is not necessarily avoided by using recombinant preparations) is unlikely here because CPET was active at nanomolar concentrations and gave only a single visible band after SDS gel electrophoresis under nondenaturing conditions (10).

C. perfringens is the second or third most common cause of food poisoning in the USA (18), and the characteristic clinical features of abdominal pain and diarrhea are thought to be caused by CPET. CPET has also been implicated in cases of antibiotic-induced diarrhea, infantile diarrhea, and sudden infant death syndrome (reviewed in reference 19), as well as in the pathogenesis of rheumatoid arthritis (20). This study shows that CPET is a superantigen with novel TCR Vβ specificity. It is intriguing that although it has no obvious structural similarity with the staphylococcal enterotoxins, CPET shares both superantigenic and enterotoxigenic functions.

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