Mitogenic Activity of Clostridium perfringens Enterotoxin in Human Peripheral Lymphocytes

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ABSTRACT. Clostridium perfringens enterotoxin (CPE) was found to possess interferon (IFN)-producing and mitogenic activities to human peripheral blood mononuclear cells. Both activities were demonstrated only in the T lymphocyte-rich fraction from healthy volunteers. The IFN produced appeared to be γ-type since the activity of the IFN was neutralized by antiserum against human IFN-γ. With formalin-treated CPE, the IFN-producing and mitogenic activities were weakly found. Similar findings were also obtained in the mouse lethality and cytotoxicity to Vero (African green monkey) cells, suggesting that the biological activities of the CPE molecule may be existing on the similar (or the same) sites. From these findings, human peripheral T cells may be one of useful reagents to study the mode of action of CPE since CPE was found to be a T cell mitogen which is supposed to be a superantigen. — KEY WORDS: Clostridium perfringens, enterotoxin, interferon, mitogen.

Clostridium perfringens enterotoxin (CPE) is associated with outbreaks of food poisoning. The enterotoxin acts on the intestinal tract and causes diarrhea. The disturbances in fluid transport have been demonstrated in experimental animals administered with CPE. The in vitro investigations using certain cell lines such as Vero (African green monkey) and HeLa cells have also been carried out to elucidate the mechanism of action of CPE [5, 8–10]. Although bacterial exotoxins show their own specific toxicities, some of bacterial exotoxins such as staphylococcal enterotoxin type A (SEA) and streptolysin O have been shown to possess mitogenic and interferon (IFN)-γ-producing activities to cultured mononuclear cells from human peripheral blood [2]. Thus, it is very helpful to study immunological response of lymphocytes induced by these bacterial exotoxins to analyze their mode of action. However, few reports on immune response of peripheral blood mononuclear cells (PBMC) induced by CPE are demonstrated although CPE has recently been reported selectively stimulate human T cells bearing receptors with Vβ6.9 and Vβ22 [1], suggesting that CPE is a T cell mitogen as found for the cases with other bacterial exotoxins and different antigens. To compare the mode of action of CPE with other bacterial exotoxins, therefore, we studied the IFN-γ-inducing activity as well as mitogenic activity of CPE in cultured PBMC.

MATERIALS AND METHODS

Mitogens and enterotoxins: Concanavalin A (Con A), phytohemagglutinin (PHA) and pokeweed mitogen (PWM) were purchased from Pharmacia Fine Chemicals Inc., Piscaraway, NY., Difco Laboratories, Detroit, MI and from Grand Island Biological Co., Grand Island, NY, respectively. We purified CPE from sporulating cultures of C. perfringens type A strain NCTC 8239 by the method of Sakaguchi et al. [12], and SEA from cultures of Staphylococcus aureus strain FRI-722 by the methods of Shinagawa et al. [13], respectively.

Formalin-treatment of CPE: CPE was dialyzed at 30°C against 0.1 M phosphate buffer, pH 7.0, containing 0.4% formalin and then excess formalin was excluded by dialysis against 0.1 M phosphate buffer, pH 7.0 for 24 hr at 4°C.

Assay methods for biological activities and antigenicity of untreated and formalin-treated CPE: Lethal activities of untreated and formalin-treated CPE were determined by intravenous injections into mice (DDY) weighing 15–20 g as described elsewhere [12]. Cytotoxic activities of untreated and formalin-treated CPE were estimated by percent of rounding of Vero cells by the method of McDonel and McClane [11]. The potency of antigenicity of formalin-treated CPE was titrated by the reversed passive hemagglutination (RPHA) test [18], and compared with that of untreated CPE on the basis of protein content.

PBMC cultures and mitogenic activity: Venous blood was drawn from healthy volunteers aged between 21 and 40 years. PBMC were isolated by layering whole blood onto Ficoll-Hyphaque (Pharmacia) followed by centrifugation for 30 min at 400 xg. The PBMC-rich fraction was obtained with Pasteur pipette and washed three times with PBS. Finally, the PBMC were suspended in RPMI 1640 (M.A. Bioproducts, Walkersville, MD) supplemented with 0.004% gentamycin (Shering Co., Kenilworth, NY), 2 mM glutamine and 10% fetal calf serum (M. A. Biproducts) to give a final concentration of 1 × 106 cells/ml. One hundred and eighty µl of the cell suspension was added to each well of flat-bottomed microtiter plate (Falcon, Cockeysville, MD), and incubated for 3 or 6 days at 37°C in an atmosphere of 5%
CO₂ in air. Five cultures received 20 µl of each one of test mitogen at an appropriate time of the incubation. After incubation, two of them were stored at -70°C and used for IFN assay. The rest three cultures received 0.5 µCi of [³H]-thymidine (specific activity 5.0 Ci/mmol, Radiochemical Centre Ltd., Amersham, Buckinghamshire, England) at 20 hr before the end of the incubation, and continued to incubate. After incubation, cells were harvested with a multiple sample harvester (Skatronas, Lierbyen, Norway) and submitted for liquid scintillation counting. The T cell-rich fraction from PBMC was obtained by the rosette forming with sheep red blood cells [17].

**IFN assay:** The IFN assay for the culture supernatants of human PBMC was carried out referring to the quantitative prevention of vascular stomatitis virus (VSV) infection to the culture of human embryonal fibroblast (HEL) grown in the culture medium of one volume of Eagle’s minimum essential medium (MEM, Flow Laboratories, Mclean, VA) mixed with the same volume of 199 (Microbiological Associates, Walkersville, MD) supplemented with fetal calf serum at 10%. The culture filtrate separated from the culture of human PBMC was made two-fold dilution with the culture medium containing reduced fetal calf serum to 2% in the wells of the microplates in which HEL was growing. The microplates were reset in an incubator with an atmosphere of 5% CO₂ in air, kept for one day at 37°C, and finally each well was received VSV of TCID₅₀ (50% Tissue Culture Infecting Dose). The results were read after two days of incubation for the prevention of the viral cytopathogenic effect. The potency of IFN was determined using purified standard fibroblast IFN (IFN-β) (Toray Industries, Inc., Kamakura), and expressed as an international unit.

**Characterization of IFN:** Rabbit antisera against human IFN (IFN-α and IFN-γ) were provided from NIH and Institute for Biochemical Research, Hayashibara Co., Inc. (Okayama, Japan), respectively. Fifty µl of the culture supernatants containing approximately 50 units of IFN was added to equal volume of the antiserum having potency to neutralize 100 units of IFN-α or IFN-γ. The mixture was incubated for 1 hr at 37°C and submitted for IFN assay.

**Protein estimation:** Protein contents were determined by the method of Lowry et al. with bovine serum albumin as a standard [7].

**RESULTS**

**Effect of CPE on [³H]-thymidine uptake and IFN production in PBMC:** [³H]-thymidine uptake of PBMC grown in the presence of CPE and other mitogens at different incubation periods is shown in Fig. 1. The maximum in the [³H]-thymidine uptake was observed on the 5th day with CPE, whereas on the third day with other mitogens. After 4-days of incubation with CPE, PBMC produced 160 to 320 units of IFN (Table 1). The IFN-inducing activity of CPE was similar to those of PWM and Con A, but slightly lower than that of SEA. Within 4-days of incubation, IFN production reached a plateau.

With different amounts of CPE, we examined [³H]-thymidine uptake and IFN production of peripheral lymphocytes. As shown in Fig. 2, dose response curves for both activities were plotted; maximum responses were observed with CPE in between 0.1 to 1.0 µg/ml, whereas decreased responses were obtained with CPE at more than 5 µg/ml.

**Responses of PBMC subpopulations to CPE:** To determine which type of human PBMC was responsible for the mitogenic activity induced by CPE, T cell-rich population was separated by rosette forming methods and tested for [³H]-thymidine uptake and IFN production. Both

![Fig. 1. Time course of [³H]-thymidine uptake of human PBMC induced by various mitogens. PBMC were cultured as described in MATERIALS AND METHODS. [³H]-thymidine was added to the PBMC cultures at 20 hr before harvesting. Human PBMC were cultured in the absence of mitogens; □□□□, or in the presence of the following mitogens, PHA (30 µg/ml); □□□□, Con A (5 µg/ml); □□□□, SEA (0.01 µg/ml); □□□□, and CPE (1.0 µg/ml); □□□□.

Table 1. Interferon production of human PBMC grown in the presence of CPE or other mitogens

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>Concentration (µg/ml)</th>
<th>IFN production (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>CPE</td>
<td>1</td>
<td>160–320</td>
</tr>
<tr>
<td>PHA</td>
<td>30</td>
<td>160–320</td>
</tr>
<tr>
<td>Con A</td>
<td>4</td>
<td>80–160</td>
</tr>
<tr>
<td>PWM</td>
<td>5</td>
<td>160–320</td>
</tr>
<tr>
<td>SEA</td>
<td>0.1</td>
<td>640–1280</td>
</tr>
</tbody>
</table>

PBMC were incubated for 4 days in the presence of CPE or other mitogens at indicated concentrations; culture supernatants were assayed for IFN production. PHA: phytohemagglutinin; Con A: concanavalin A; PWM: porkweed mitogen; SEA: staphylococcal enterotoxin A.
activities of CPE were observed to be distributed mostly to T cell-rich fraction as found for the case with other T cell mitogens such as PHA and SEA (Table 2).

**Table 2.** $^3$H-thymidine uptake and interferon production of human PBMC subpopulations grown in the presence of CPE and other mitogens

<table>
<thead>
<tr>
<th>PBMC subpopulation</th>
<th>Mitogen</th>
<th>$^3$H-thymidine uptake (1×10^4 cpm)</th>
<th>IFN production (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell rich fraction</td>
<td>PHA</td>
<td>12.8</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>SEA</td>
<td>12.6</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>CPE</td>
<td>7.5</td>
<td>240</td>
</tr>
<tr>
<td>Non T cell fraction</td>
<td>PHA</td>
<td>1.8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>SEA</td>
<td>1.5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>CPE</td>
<td>7.5</td>
<td>10</td>
</tr>
</tbody>
</table>

Culture conditions used were the same as those in the legend of Table 1.

**Table 3.** Characteristics of IFN produced in PBMC by different inducers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IFN induced by CPE (units)</th>
<th>IFN induced by SEA (units)</th>
<th>IFN-α (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Antibody to IFN-α</td>
<td>80</td>
<td>80</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Antibody to IFN-γ</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>80</td>
</tr>
</tbody>
</table>

IFN-α*: human leucocyte IFN-α.

**DISCUSSION**

CPE acts on the cytoplasmic membrane of animal cells leading to histological damage, metabolic disturbances, inhibition of macromolecular synthesis, and leakage of intracellular substances [3, 8, 9, 14]. However, there are few reports on cellular response of human and animal
lymphocytes against CPE. Recently CPE has selectively stimulated human peripheral lymphocytes bearing T cell receptor Vβ6.9 and Vβ22 [1], indicating that CPE acts as a T cell mitogen and/or a bacterial superantigen. The present study confirmed [3H]-thymidine uptake and IFN-γ production of human peripheral T lymphocytes with CPE. These two activities of human PBMC were observed in culture media incubated with CPE at concentrations of between 0.1 µg/ml and several µg/ml. With CPE at a concentration of 5 µg/ml or more, on the other hand, both [3H]-thymidine uptake and IFN-γ production of human PBMC were reduced, suggesting that CPE at more than 5 µg/ml may show its cytotoxicity to the PBMC.

Although staphylococcal enterotoxins as bacterial superantigens have shown their mitogenic activities to human peripheral lymphocytes [19], different findings have been obtained. SEA is a strong inducer of IFN-γ [6], whereas staphylococcal enterotoxins type B (SEB) and C (SEC) were weaker inducers of IFN-γ (Nagata et al., unpublished data). Furthermore, SEB toxoid has lost the emetic activity to monkey, but retained its mitogenic activity to mouse lymphocytes [15]. Trypsinization of SEC gave two peptide subunits; one was involved in the mitogenic activity, whereas the other one was associated with the emetic activity [16]. These findings were different from those with CPE obtained in the present studies since treatment of CPE with formalin reduced its all biological activities including both cytotoxicity, and mitogenic and IFN-γ-producing activities. In the previous studies [4], trypsinization has induced enhancement in the biological activity of CPE although such enzyme treatment has cleaved a small piece of peptide fragment. Although both CPE and staphylococcal enterotoxins show enterotoxigenicity and bacterial superantigenicity with novel T cell receptor (TCR) Vβ specificities but no structural similarity, their TCR Vβ specificities have been shown to be different [1].

From the present findings, CPE was found to be a T cell mitogen and also a inducer of IFN-γ. Either human or animal peripheral T lymphocytes and/or both will be very useful tools to elucidate the mode of action of CPE.

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REFERENCES