Flow Cytometric Analysis for Enterotoxin Exposed on Clostridium Perfringens Spores

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(Received 20 March 1998/Accepted 18 August 1998)

ABSTRACT. Flow cytometric method (FCM) with fluorescent-labeled anti-CPE antibody was applied to develop a rapid, specific, and convenient method to detect enterotoxin (CPE) exposed on the surface of spores of Clostridium perfringens. The results obtained indicate that FCM can specifically detect CPE exposed on C. perfringens spores for a short time. Thus, FCM is found to be a rapid, specific, and convenient assay method for detection of CPE exposed on C. perfringens spores, suggesting that it will be hopefully useful to diagnose food poisoning caused by C. perfringens. — KEY WORDS: Clostridium perfringens, enterotoxin, FCM.


Clostridium perfringens is a common food bacterium distributed widely in human foods, especially meat and poultry products [20]. Type A strains of C. perfringens produce an enterotoxin (CPE) which is the causative factor of human food poisoning [19]. CPE is synthesized during sporulation of C. perfringens vegetative cells and the lysis of sporulated cells liberates CPE into the intestinal tract [7]. Since CPE involves in food poisoning outbreaks caused by C. perfringens, several methods for detection of CPE have been developed over the past few decades [5, 12, 18, 21]. Although enzyme-linked immunosorbent assay (ELISA) is one of the common methods to detect CPE at the present time, it is not useful to analyze CPE exposed on the surface of spores of C. perfringens. On the other hand, flow cytometry method (FCM) is an established method for analysis of different types of cells from bacteria to mammalian cells in terms of accuracy, sensitivity, and rapidity. FCM has recently been reported to be a useful method to analyze bacteria in the environment [1, 11, 16] and food samples [4, 8, 13]. Thus, we attempted to confirm whether or not FCM is applicable to identify and/or analyze CPE exposed on the surface of spores of C. perfringens.

Vegetative cells of Staphylococcus aureus strain FRI-722 were prepared by incubating in thioglycollate medium (TGC medium, Nissui Seiyaku, Tokyo, Japan) at 37°C for 12 hr followed by culturing in brain-heart infusion broth (Difco Laboratories, Detroit, USA) containing 0.05% Na-thioglycollate at 37°C for 10 hr. Spore of C. perfringens strain 8239 (H-3) were prepared by the methods described previously [6]. Vegetative cells and spores were harvested by centrifugation at 10,000 × g for 15 min. The precipitate was suspended in 0.6% formalin at 30°C for 2 days. Then, the formalin-treated vegetative cells and spores were washed 3 times in 0.15 M phosphate buffered saline, pH 7.2 (PBS).

Purification of CPE produced by C. perfringens strain 8239 and preparation of rabbit anti-CPE serum were performed by the methods described previously [22, 23]. Rabbit antiserum was purified by gel filtration on Sephacryl S-300 (Pharmacia, Uppsal, Sweden). Purified antibody was labeled by the methods of Williams and Chase [24]. Thirty μg of fluorescein isothiocyanate, isomer 1 (FITC, Wako Pure Chemicals, Osaka, Japan) was added to 1 mg of antibody and incubated at 25°C for 1 hr. Then the mixture was applied to gel filtration on Sephadex G-25 (Pharmacia, Uppsal, Sweden). Further purification of FITC-labeled antibody was carried out by ion-exchange chromatography on DEAE-cellulose (DE-52, Whatman Paper Ltd, Maidstone, England).

Heat treatment of formalin-treated C. perfringens spores was carried out by incubating at 60°C for 30 min. The spores (10⁸) were also treated with 200 μl of rabbit anti-CPE serum by incubating at 37°C for 10 min. Binding of FITC-labeled anti-CPE antibody (40 μl) to either formalin-treated vegetative cells or spores (10⁹/ml) was done by incubating at 37°C for 10 min. After these treatment, the spores were washed in PBS by centrifugation at 10,000 × g for 10 min.

For flow cytometric assay, test samples were prepared as follows: 0.4 ml of formalin-treated vegetative cells or spores (10⁹/ml) was mixed with 50 μl of FITC-labeled purified rabbit anti-CPE antibody for 10 min at 37°C. FCM was performed in a FACStar™ (Becton Dickinson Immunocytometry Systems, Mountain View, California, U.S.A.) equipped with 5 W argon ion laser (Coherent Innova 90) tuned at 488 nm and 0.2 W. At least 10,000 cells were determined by a measurement of forward angle light scatter (FSC). Green fluorescence (from FITC, through a 530/30 nm filter, Becton Dickinson) from stained cells was detected in the 90° light slide scatter, which was recorded and displayed in a cyogram. Subcellular debris were excluded from the analysis by setting a suitable threshold on the basis of FCS. Data were processed with consort 30 Software in a Hewlett-Packard 900 series model 217 personal computer (Hewlett-Packard, Fort Collins, CO, U.S.A.). All other procedures were done according to the FACStar™ operation manual.

To study the specificity of FITC-labeled anti-CPE antibody, CPE exposed on C. perfringens spores was treated with unlabeled rabbit anti-CPE antibody prior to FITC-

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labeled anti-CPE antibody. After the treatment, the reactivity of the treated spores was analyzed by FCM. As shown in Fig. 1, the spores did not react with FITC-labeled anti-CPE antibody, indicating that CPE exposed on the surface of C. perfringens spores reacted with anti-CPE serum. To further study the specificity of FITC-labeled anti-CPE antibody, the reactivity of C. perfringens spores and S. aureus vegetative cells were analyzed by FCM. As shown in Fig. 2, the peak channel of CPE exposed on C. perfringens spores was found to be different from that of CPE-nonproducing S. aureus. These indicated that FITC-labeled anti-CPE antibody was a highly specific reagent to detect CPE exposed on the surface of C. perfringens spores.

Formalin-treated spores of C. perfringens were also analyzed by FCM after incubation with FITC-labeled and unlabeled rabbit anti-CPE antibodies. As shown in Fig. 3, CPE exposed on the surface of the spores appeared in a single peak. On the other hand, FITC-unlabeled spores appeared at the different position, showing that formalin treatment at 0.6% was found to be ineffective to the antigenicity of CPE exposed on C. perfringens spores.

To study the antigenicity of CPE exposed on the surface of C. perfringens spores after heat treatment at 60°C for 10 min, the reactivity of C. perfringens spores before and after the treatment were analyzed by FCM. As shown in Fig. 4, the peak channel of C. perfringens spores before the
treatment was found to be different from that after the
treatment, indicating that the heat treatment at 60°C for 30
min destroyed the antigenicity of CPE exposed on the
surface of C. perfringens spores. This is supported by the
previous report \cite{17} that CPE is a heat-labile protein.

The food industry needs an accurate, sensitive, and rapid
method to detect bacteria in food samples since the faster
the microbiological detection is carried out, the sooner
perishable foods can be released for sale. They also need
the method to reduce false negative results to permit the
release for sale of contaminated foods. To overcome such
problems, FCM is a useful method which will be automation
for in a factory environment. As described previously \cite{2, 3,
15}, the minimum concentration of bacteria detectable by
FCM has been reported to be $10^3$–$10^4$ bacterial cells/ml.

With two species of salmonellas, the minimum detectable
concentration by FCM has been to be $10^3$ml in pure cultures
\cite{9}, and $10^3$–$10^4$ml in milk and egg \cite{10, 14}. In all cases,
the total analysis time was about 30 min. Thus, FCM is
suggested to be more rapid method to detect and/or analyze
salmonellas than polymerase-chain reaction (PCR).

From the present findings, FCM is found to be a rapid,
specific, convenient method to detect and/or analyze CPE
on the surface of spores of C. perfringens. Thus, it will be
a useful method to detect food stuffs contaminated with C.
perfringens for prevention of food poisoning.

ACKNOWLEDGMENTS. This study was supported in part by Grant-in-Aid for Scientific Research (No. 09460146)
from the Ministry, Education, Science, Culture and Sports
of Japan and by The Japan Food Industry Center (JAFIC).

REFERENCES

フローサイトメトリー法によるエンテロトキシン産生性ウエルシュ菌芽胞の解析（短報）——
楠 博文・胡 東・Piyankarage, Ramani H.・杉井俊二・植村 興(大阪府立大学農学部獸医学科)………………1357-1359

エンテロトキシン産生性ウエルシュ菌の同定・検出を簡便、特異的、迅速に行うため
に、フローサイトメトリー法で表面にエンテロトキシンをもつウエルシュ菌芽胞の解析
を試みた。その結果、抗エンテロトキシン抗体を用いたフローサイトメトリー法は特異
的で、エンテロトキシンを表面に持つウエルシュ菌芽胞を検出することができることが
判った。したがってフローサイトメトリー法を用いることにより、食中毒原因菌のエン
テロトキシン産生性のウエルシュ菌芽胞を短時間内に同定することが可能である。

外科学：
馬尾症候群の3症例に対するMRI検査の有効性（短報）——田賀淳夫1 - 2、田浦保雄1、
西本孝志1、滝口正文1・植口雅仁1(1)山口大学農学部附属家畜病院、2山口大学大
学院総合獣医学研究科、3滝口動物病院)………………1345-1348

馬尾症候群が疑われたイヌの3症例に対してMRI検査を実施し、馬尾およびその周
辺組織の検索を行った。その結果、MRIは造影剤を用いないとも馬尾の圧迫状態、硬膜
脂肪組織の消失、椎間板の変性といった馬尾症候群特有的病態変化を捉えることが
可能であった。それ故、MRI検査は馬尾症候群の診断に有効であることが示唆された。

ウイルス学：
移行抗体保有豚を用いた豚繁殖・呼吸症状候群(PRRS)ウイルスの実験感染——柴田
勲・森 正史・宇留野勝好(全農家畜衛生研究所)………………1285-1291

PRRSウイルス感染に及ぼす移行抗体の影響を調べるために、コンベンショナル豚と
SPF豚を用いて実験感染を実施した。10頭の移行抗体保有17日齢コンベンショナル豚
と6頭の抗体陰性SPF豚に107 TCID50のPRRSウイルスを鼻腔内接種した。2頭のコン
ベンショナル豚と4頭のSPF豚は非感染対照豚とした。感染後コンベンショナル豚では
発咳と発熱が認められ、平均増殖率は低下した。1頭は28日目に死亡し、肺から
Haemophilus parasuisが分離された。感染SPF豚でも発熱が認められたが、増殖率の減少は
観察されなかった。コンベンショナル豚の1頭を除いた全ての感染豚の血清からコン
ベンショナル豚で感染後1〜7週、SPF豚で1〜4週ウイルスが分離された。感染後の血清から
のウイルス分離開始時期がSPF豚で65日目で5頭および死亡した1頭のコンベンショナル豚からウイルス
が分離された。一方、非感染コンベンショナル豚と感染および非感染SPF豚の脾臓からウイルス
が分離されなかった。感染時、PRRSウイルスに対するコンベンショナル豚の間接蛍
光抗体価は1:20であった。感染後抗体価は徐々に低下し、感染3〜4週から上昇し9
週目では1:160から1:640であった。中和抗体価は感染時1:2から1:4で、感染後明らか
な抗体上昇は認められなかった。対照豚では何抗体とも徐々に低下した。1頭にSPF
豚では間接蛍光抗体価は感染後1〜2週で検出され、感染3〜5週で1:320から1:2,560を示し、
中和抗体価は感染後6〜8週で検出され、抗体価1:1から1:4で推移した。

インターフェロン-αの牛下痢・粘膜病ウイルスに対する抗ウイルス作用——泉対 博・高見
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山県立家畜保健衛生所、1農林水産省家畜衛生試験場北海道支所)………………1329-1333

ウイルス性持続感染症をサイトカインにより制御する可能性を検討するために、イン
ターフェロン(IFN)-αおよびγ、腫瘍破壊因子(TNF)-αおよびβの4種類のヒト自然型
サイトカインを使用し、牛下痢・粘膜病ウイルス(BVDV)に対する抗ウイルス作用を検
べた。様々な濃度のこれらのサイトカインで健康牛乳末相半球および牛胎児筋肉培養細