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学 位 論 文 名	Establishment of an immuno-polymerase chain reaction for the detection of tetanus toxin （破傷風毒素の免疫 PCR 法による検出）
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論 文 内 容 の 要 旨

〔 目　　的 〕

Tetanus is caused by the intoxication of a very potent neurotoxin, tetanus toxin. Therefore the detection of tetanus toxin in clinical specimens could lead the definitive diagnosis of the disease. However the diagnosis of tetanus relies on clinical manifestations because no laboratory means has been established for the detection of the toxin in the clinical specimen. To establish a very sensitive method for the detection of tetanus toxin, I have considered the use of immuno-polymerase chain reaction (IPCR) which combines the specificity of immunoassay and the sensitivity of PCR amplification.

〔 方法ならびに成績 〕

Monoclonal antibodies

Formalin treated tetanus toxin was used to immunize mice for producing monoclonal antibodies (mAbs). Splenocytes from the immunized mice were fused with 8Ag.653 mouse myeloma cells. Anti-tetanus-producing hybridomas were selected by ELISA and the positive hybridomas were cloned at least twice by limiting dilution. Six monoclonal anti-tetanus were selected on the basis of their strong reactivity to tetanus toxin. All the mAbs showed similar affinities (Kds) to tetanus toxin. ELISA using these individual mAb also showed similar minimum detectable concentration of the toxin as 10 ng/ml. However, the mixture of two mAbs increased the sensitivity of the ELISA. Among the combinations of the mAbs, the mixture of A9A6-G2D6 gave the lowest minimum detectable concentration of 1 ng/ml. Therefore, the mixture of A9A6-G2D6 was used in the following experiment of IPCR.

Detection of tetanus toxin by sandwich IPCR

The horse anti-tetanus was immobilized on micro-wells to capture the serially diluted tetanus toxin. Sequential incubations with the biotinylated monoclonal antibody, streptavidin, and the biotinylated reporter

DNA were performed. Then the wells were subjected to PCR amplification. The amplified PCR products were electrophoresed on a 1.5% agarose. The agarose gel was stained with ethidium bromide then scanned under Fluor Imager 595, and the intensity of the band was measured. Tetanus toxin at a concentration of 1 pg/ml produced an amplified reporter DNA band with the intensity significantly different to the intensity of PCR product without tetanus toxin ($P < 0.01$). This result shows that this IPCR is 1000 times more sensitive compared to the ELISA.

To clarify whether the IPCR system works on usual clinical specimens, such as serum and tissue extract, IPCR were also performed on tetanus toxin in mouse serum and muscle tissue extract. The intensity of the band produced by tetanus toxin at a concentration of 1 pg/ml was still significantly different to the band intensity of the PCR product without the toxin ($P < 0.05$). The results indicated that this IPCR worked on tetanus toxin in the serum and in the tissue extract with almost similar sensitivity to the toxin in the buffered saline.

〔 総　　括 〕

By using horse anti-tetanus serum as a first antibody and the mixture of two mAbs as a second antibody, the detection limit of this IPCR is 1.0 pg/ml (1,000 times more sensitive compared to the ELISA). This sandwich IPCR provides the most sensitive method, so far, to detect tetanus toxin. This IPCR also worked on tetanus toxin in the serum and in the muscle tissue extract without alteration of the detection limit, and to be a promising means for the detection of tetanus toxin in clinical specimens.

論文審査の結果の要旨

WHO の報告によれば 2005 年現在、破傷風による死亡者数は年間約 30 万人と報告されている。わが国においては DPT ワクチンの普及によって 1950 年以降著しく減少したものの、現在なお年間約 100 名の患者報告がある。破傷風は破傷風菌が産生する破傷風神経毒素による中毒症であり、体内に進入した毒素量によって重症度が決定される。しかし破傷風神経毒素はボツリヌス神経毒素と並んで最も致死性の高い毒素であることから、これまで破傷風患者から得られる臨床検体から毒素を検出できるほど高感度の毒素検出方法は無かった。本研究では、免疫 PCR 法を応用することによって、破傷風毒素を 1 pg/ml の濃度まで特異的に検出する方法を開発した。開発された免疫 PCR 法は定量性にも優れており、血清や組織抽出液中でも検出感度が低下することは無かった。また本検出方法に必要な機器および試薬は、単クローン抗体など特殊なものを除けば、広く普及しているものであることから、世界中とりわけ破傷風患者の多い発展途上国でも利用できると考えられる。本研究は臨床検体から破傷風毒素の検出することによって破傷風の重症度を早期に判定し、治療方法や予後の判定に役立たせる道を拓くものであり、学位の授与に値するものと認める。