Pathogenicity of *Propionibacterium acnes* Isolated from Kawasaki Disease Patients

—Cytopathogenic Protein (CPP) Isolated from *P. acnes* Culture Filtrates and Measurement of the Antibody against CPP—

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Summary: A culture filtrate of *Propionibacterium acnes* isolated from patients with Kawasaki disease was studied. After inoculation of the culture filtrate of these strains into human embryo liver cells, a cytopathogenic effect and vacuolation were recognized in the cells. From analysis of the culture filtrate, a specific cytopathogenic protein was found only in the culture filtrates from Kawasaki disease patients. This protein was approximately pI 7.0 and had a molecular weight of approximately 100,000 daltons. The amount of IgG antibody (from sera of Kawasaki disease patients) against this substance (CPP) was measured using the Enzyme-Linked Immunosorbent Assay (ELISA). There were 124 serum samples available from 95 patients with 2-8 days duration of illness, 100 samples from 67 patients with 14-33 days of illness, 61 samples from 42 patients with 11-22 months of illness and 148 samples of age-matched controls. There was a significant elevation in the ELISA values in the patients' groups, particularly in the acute or subacute stages of the illness, as compared with controls.

Key words: Kawasaki disease — propionibacterium *acnes* — CPP (cytopathogenic protein) — exotoxin — ELISA

Introduction

It has been twenty years since Kawasaki disease was first described (Kawasaki, 1967). The number of patients with Kawasaki disease is increasing, not only in Japan; but also in the US, Europe and Korea (Lee et al. 1980; Melish et al. 1982; MMWR, 1985; Takao, 1986). Epidemiological studies have suggested either an infectious etiology or certain immune responses to an infectious agent(s) (Yanagawa, 1979). However, the exact etiology of this disease has not been determined.

Since 1983, we have studied *Propionibacterium acnes* (*P. acnes*) isolated from

cervical lymph node biopsy specimens and blood samples of patients with Kawasaki disease (Kato et al. 1983; Kato et al. 1985). It has been suggested that *P. acnes* may be involved in the pathogenesis of this disease for the following reasons. 1) There has been a high incidence of *P. acnes* isolated from patients with Kawasaki disease, as compared with the control group. 2) Intraperitoneal inoculation of guinea pigs with this microorganism resulted in depilation, arteritis in the coronary arteries and focal myocarditis. 3) After inoculation of a culture filtrate of the microorganisms into human embryo liver cells, a cytopathogenic effect and vacuolation were

recognized in the cells. There was a higher incidence of these effects than with culture filtrates of the standard strain or other strains isolated from blood samples of patients with other illnesses. 4) When the culture filtrate was heated at 100°C for 30 minutes or treated with convalescent patients' serum, neither vacuolation nor cytopathogenic effects on fetal liver cells occurred. These results suggested that the strain isolated from the patients may produce a certain cytopathogenic substance. In this project, attempts were made to isolate this substance and measure the IgG antibody levels against it in the patients' sera.

Materials and Methods

Microorganisms

The method used for isolation and typing of *P. acnes* has been described previously by Kato et al. (1983). After initial isolation, the *P. acnes* strains were preserved in cooked meat broth at room temperature. The analyses of the culture filtrates were performed on microorganisms isolated from four patients with typical symptoms of Kawasaki disease, one patient with leukemia, one patient with congenital heart disease, house-dust mites collected from houses of Kawasaki disease patients and microorganisms in the standard strain (ATCC 11827). The same analysis was performed with the medium only, as a control.

Analysis of the culture filtrate and cytopathogenic substance

Microorganisms were grown in GAM broth (1.0 g/dl peptone, 0.3 g/dl soy-peptone, 1.0 g/dl protease peptone W, 1.35 g/dl peptide digest serum powder 'Nissui', 0.22 g/dl meat extract, 0.5 g/dl yeast extract, 0.12 g/dl liver extract, 0.3 g/dl glucose, 0.25 g/dl KH_2PO_4 , 0.3 g/dl sodium chloride, 0.5 g/dl soluble starch, 0.03 g/dl

L-cysteine-HCl, and 0.03 g/dl sodium thioglycollate in distilled water; pH 7.3 $\pm$ 0.1) for 4 to 10 days at 37°C.

The centrifuged supernatant was filtered through a 0.22 μm Millipore filter, and the filtrate was dialyzed after it was salted out using 80% ammonium sulfate. The concentrated culture filtrate was fractionated by gel filtration, and was further fractionated by Flat-Bed isoelectrofocusing (Flat-Bed IEF). The fractionate was inoculated into human embryo liver cells for observation of the cytopathogenic effect and vacuolation. The fractionate, induced vacuolations in the embryonic liver cells were analyzed by polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gel filtration was performed using an LKB Ultragel AcA44, and the filtrate was extracted with PBS, pH 7.2. An LKB Multiphor was used for Flat-Bed IEF. Fractionation was carried out using the Ultradex system and Ampholine, pH 3.5-10. LKB Multiphor and Ampholine PAG plate, pH 3.5-9.5, was used for PAGE, and the gel was stained with Coomassie Brilliant Blue after focusing. SDS-PAGE was performed by using microslab electrophoresis through a 12.5% acrylamide gel, and this gel was silver stained after Coomassie Brilliant Blue staining.

Measurement of the IgG antibody against the cytopathogenic substance

IgG antibodies against the cytopathogenic substance in the sera of Kawasaki disease patients was measured by an indirect Enzyme-Linked Immunosorbent Assay (ELISA).

Serum specimens

1) IgG antibodies against the cytopathogenic substance in Kawasaki disease patients

There were 124 serum samples available,

from 95 patients ill for 2-8 days (mean 5.2 d.), 100 samples from 67 patients ill for 14-33 days (mean 24.3 d.), and 61 samples from 42 patients ill for 11-22 months (mean 17.8 m.). All samples were collected from patients with Kawasaki disease who were less than 4 years of age, and 148 samples of age-matched control sera were collected for the ELISA procedure.

2) Incidence of antibodies against the cytopathogenic substance in normal children and adults

The sera samples of the general population group were divided into four groups according to age, 0-4, 4-9, 9-14 years and adult, and the amounts of IgG antibodies against the cytopathogenic substance in the sera were measured by the ELISA procedure.

The procedure for ELISA

Polystyrene flat bottomed microtitra-

tion plates (Flow Laboratories, Inc.) were coated with 100 μ l of CPP (extracted from culture filtrates of three strains "A", "H", and "O") solution containing 50 μ g/ml of protein in a 0.05 M carbonate-bicarbonate buffer (pH 9.6). After an overnight (18 h) incubation at 4°C, the plates were washed four times with phosphate-buffered saline containing 0.05 % Tween 20 (PBS-T), and 100 μ l of test sera at optimal dilution (1:4) in PBS-T containing 2 % fetal bovine serum. The plates were incubated for 2 hours at 37°C, and washed four times with PBS-T. A 100 μ l sample of a peroxidase-conjugated rabbit antihuman immunoglobulin G (Miles Laboratories, Inc.) in a 1:2000 dilution in PBS-T was added. After incubation for 2 hours at 37°C, the plates were washed four times with PBS-T, and 100 μ l of 0.4 mg/ml o-phenylenediamine in citrate-phosphate buffer (pH 5.0) was added. The plates were kept in a dark chamber at room temperature for 30 minutes, and 50 μ l of 2.5 M H₂SO₄ was

Fig. 1. Gel-filtration analysis of culture filtrates. A small peak at 100,000 daltons molecular weight was recognized in the culture filtrates from patients with Kawasaki disease (MCLS).

Fig. 2. Preparative Flat-Bed electrofocusing of culture filtrates. Group (A) are controls; Lane 1, pure GAM broth. Lane 2, culture filtrate of *P. acnes* isolated from house-dust mite. Lane 3, culture filtrate of *P. acnes* isolated from patient with leukemia. Lane 4, culture filtrate of standard strain (ATCC 11827). Group (B) are patients; Lane 5-8, culture filtrates of *P. acnes* isolated from patients with Kawasaki disease.

Lane 5-8, the arrow indicates the specific band (CPP).

added to stop the reaction. The absorbance of these microtitration plates was measured at 486 nm by an ETY-96 ELISA ANALYZER (TOYO, Oriental Instrument LTD.)

Results

Analysis of the culture filtrate

The results of gel filtration at OD₂₈₀ nm are shown in Fig. 1. The peaks at approximately 200,000 and 20,000–30,000 daltons molecular weight were recognized in the culture filtrates of all strains and the control medium. There were no cytoplasmic vacuolations in embryo liver cells in these fractions. However, in the culture filtrates from the strains of Kawasaki disease patients, the small peak of approximately 100,000 daltons molecular weight was recognized, which caused the vacuolation in embryo liver cells. As is shown in Flat-Bed IEF (Fig. 2), a specific fractionation band in the range of approximately pI 7.0 was recognized in the culture filtrates from

all four of the Kawasaki disease patients, and these fractionations formed vacuolations in embryo liver cells (Fig. 3). However, there were no such bands in the fractions extracted from the four control strains (a patient with leukemia, a patient with congenital heart disease, house-dust mites in the homes of Kawasaki disease patients and the ATCC 11827 standard strain). These results are in accord with the results of the PAGE analysis. Furthermore, these fractionations were evaluated with SDS-PAGE, and consequently, the molecular weight was determined to be approximately 100,000 daltons. This protein was tentatively named the Cytopathogenic Protein (CPP).

Measurement of IgG antibodies against CPP

1) IgG antibodies against CPP in Kawasaki disease patients

The mean $\pm$ SD of the ELISA values are shown in Fig. 4. The serum samples used for the ELISA analysis were 63 samples

Fig. 3. Human embryonic liver cells inoculated with CPP. (A), the inoculated CPP was from a patient with Kawasaki disease. (B), the inoculated culture filtrate of *P. acnes* was isolated from blood of patient with leukemia. (C), there was no inoculation (control).

Fig. 4. ELISA values for measurement of IgG antibodies against CPP (Cytopathogenic Protein). Significant elevations of ELISA values occurred in the patient with acute and subacute stages of Kawasaki disease.

from patients ill for 2-8 days (mean 5.2 days), 45 samples from patients ill for 14-33 days (mean 23.6 days), 42 samples from patients ill for 11-22 months (mean 19 months), and 102 samples of age-matched control sera used for measurement of IgG antibodies against CPP from the "A" strain of *P. acnes*. The ELISA values were elevated both in the group that was ill for 2-8 days and in the group that was ill for 14-33 days, and there were significant differences in these ELISA values and the values for the controls ($p<0.004$, $p<0.001$, as determined by the

Fig. 5. ELISA values for measurement of IgG antibodies against CPP in the general population. Under four years of age in normal children had few antibody titers. The antibody titers gradually increased with aging.

student's T test). The mean ELISA value for the 11-22 month group was similar to the control. The same results were obtained in a study with two other strains from Kawasaki disease patients ("H" strain and "O" strain).

2) Incidence of antibodies against CPP in normal children and adults

The ELISA values for normal children and adults were increased with age. This indicates that IgG antibodies against CPP were gaining with age (Fig. 5).

Discussion

Usually, *P. acnes* is not pathogenic in children. However, previous studies have suggested that *P. acnes* isolated from Kawasaki disease patients may have some pathogenicity (Kato et al. 1983; Kato et al. 1985). Inoculation of guinea pigs with *P. acnes* isolated from patients with Kawasaki disease resulted in depilation, hepatosplenomegaly, lymphadenopathy and coronary arteritis in the animals. And when human embryo liver cells were treated with culture filtrates of *P. acnes* strains from Kawasaki disease patients, vacuolation and cytopathogenic effects occurred in these embryonic liver cells. Next, attempts were made to isolate the cytopathogenic substance from culture filtrates, and specific fractionations were successfully extracted only from culture filtrates of *P. acnes* isolated from Kawasaki disease patients. This substance induced cytoplasmic vacuolation in human embryo liver cells. Our previous study demonstrated that when the culture filtrate was heated to 100°C for 30 minutes or treated with convalescent patients' serum, neither vacuolation nor cytopathogenic effects on the fetal liver cells occurred. These results have led to the speculation that this substance, designated Cytopathogenic Protein (CPP), may be a cytopathogenic or toxigenic protein, or an exotoxin. When serum levels of IgG antibodies against CPP were measured by ELISA, it was recognized that there were significant antibody elevations in the sera of Kawasaki disease patients. These results suggest that CPP may play some etiologic role in Kawasaki disease, and the data gathered in this study is in accord with previous studies that have discussed the agglutination test and cytoplasmic vacuolation in embryonic liver cells.

Epidemiological studies to date have reported several outbreaks in Japan (Yanagawa et al. 1979; Yanagawa, 1986). They have shown a regular interval of

three years between outbreaks. This suggests an infectious etiology. However, it is difficult to explain how Kawasaki disease can be epidemic if caused by *P. acnes* alone, because *P. acnes* is the principal indigenous bacterium of the human skin. Most of the infants and young children in the general population had no antibodies to the CPP, and the antibody titers gradually increased with aging (Fig. 5). This may explain why infants and younger children are suffered from Kawasaki disease. When infants and young children are affected by CPP, some of them who are susceptible to CPP may get Kawasaki disease. The three year interepidemic interval noted repeatedly in Japan or the United States indicates that after a large outbreak, this disease can not be propagated until the group of susceptible children is adequately increased through birth. The interepidemic interval of three years correlates exactly with the high risk age group.

When the serial antibody levels were also measured in patients with Kawasaki disease, two antibody titer patterns were noted. In one patient group, the high antibody titer was maintained for a long term, however in another patient group, the antibody titer declined during the early convalescent phase. The patients, who continue to have a high titer of antibodies to CPP, may have been repeatedly infected with this indigenous bacterium or CPP, and therefore were receiving numerous "booster" effects. It has been reported that 2 or 3% of the patients had recurrences (Yanagawa et al. 1979; Yanagawa, 1986). This could occur in the patient group in which the antibody titers declined during the convalescent stage.

Why and how *P. acnes* changes to produce an exotoxin like substance or gains its pathogenic effects, has not been clearly explained. Pathogenicity may be caused by a bacteriophage or plasmid, or other unknown factors. However, Yamamoto et

al. (1985) reported that no plasmid was present in the bacterial cells. Some other factor(s) may be involved in the etiology of Kawasaki disease in association with *P. acnes*. However, the fact that *P. acnes* isolated from the patients produces the CPP, and Kawasaki disease patients have significantly higher antibody titers against CPP suggests that *P. acnes* has some etiological role in Kawasaki disease. Further investigations on the etiology should be carried out.

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