

this work not to alter the case fatality rate, suggest that each year about 170 cases and 16 deaths from ulcer complications are attributable to its use in New South Wales. This estimate is lower than for non-steroidal anti-inflammatory drugs because aspirin is regularly used less often by subjects in this age group in New South Wales (D A Henry *et al*, 3rd international conference on pharmaco-epidemiology, Minneapolis, 1987).

The suggestion that corticosteroids increase the case fatality rate from complications of peptic ulcer is a data derived hypothesis and should be interpreted with caution. Furthermore, the estimate of the relative risk is approximate because of small numbers. Nevertheless, the possibility of a substantially increased case fatality rate for users of corticosteroids is supported by data from an extensive survey of gastrointestinal bleeding published by the American Society for Gastrointestinal Endoscopy.⁹ Interestingly, in our study the epidemiological data were backed by clinical evidence that the patients who died had suffered from adverse events which in some instances could be ascribed to the direct effect of corticosteroids, in particular overwhelming infections. Especially notable were two cases of staphylococcal septicaemia which were thought to be due to infection of intravenous cannula sites and thus by implication were preventable. On the basis of a meta-analysis of prospective randomised trials Messer *et al* concluded that corticosteroid treatment roughly doubled the incidence of haemorrhage from peptic ulcers.¹⁰ If our estimate of an increased fatality rate is correct then the risk of users of steroids developing and subsequently dying from ulcer complications might be about eight times higher than for non-users. Although this is a substantial increase for the individual, in population terms it is a small risk because of the low prevalence of use of steroids in the community.

Of the anti-inflammatory drugs, aspirin and non-steroidal anti-inflammatory drugs, particularly the latter, are the important causes of morbidity and mortality in the community. Unlike steroids their intrinsic toxicity is not high. Rather, difficulties arise because of

their extraordinarily wide use. Epidemiologically the relation is dominated by a high prevalence of the risk factor rather than a high relative risk of the disease. Thus the main thrust of attempts to reduce morbidity and mortality from non-steroidal anti-inflammatory drugs in the community should be to limit their use rather than to attempt to lower relative risk further by widespread coprescription of agents designed to "protect" the gastrointestinal mucosa, such as prostaglandins, sucralfate, or histamine H₂ antagonists. While these measures may prove to have a role in a limited number of individuals, the greatest need is for more information on the factors which control prescribing decisions so that we can identify opportunities for intervention.

This study was supported by a grant from the National Health and Medical Research Council of Australia. We thank Professor R Gibberd for his help and advice.

References

- 1 Langman MJS. Peptic ulcer complications and the use of non-aspirin non-steroidal anti-inflammatory drugs. *Adverse Drug Reaction Bulletin* 1986;120:448-51.
- 2 Somerville K, Faulkner G, Langman MJS. Non-steroidal anti-inflammatory drugs and bleeding peptic ulcer. *Lancet* 1986;i:462-4.
- 3 Blower AL, Armstrong CP. Ulcer perforation in the elderly and non-steroidal anti-inflammatory drugs. *Lancet* 1986;i:971.
- 4 Armstrong CP, Blower AL. Non-steroidal anti-inflammatory drugs and life threatening complications of peptic ulceration. *Gut* 1987;28:527-32.
- 5 Gilbert DA, Persing J, Silverstein FE, *et al*. National ASGE survey on upper gastrointestinal bleeding—Multivariate analysis of predictors of outcome. *Gastrointest Endosc* 1982;28:150-1.
- 6 Miettinen OS. Estimation of relative risk of individually matched series. *Biometrics* 1970;26:75-86.
- 7 Levy M. Aspirin use in patients with major upper gastrointestinal bleeding and peptic ulcer disease. *N Engl J Med* 1974;290:1158-62.
- 8 Coggon D, Langman MJS, Spiegelhalter D. Aspirin paracetamol and haematemesis and melaena. *Gut* 1982;23:340-4.
- 9 Silverstein FE, Gilbert DA, Tedesco FJ, *et al*. The National ASGE survey on upper gastrointestinal bleeding. II. Clinical prognostic factors. *Gastrointest Endosc* 1981;27:80-93.
- 10 Messer J, Reitman D, Sacks H, *et al*. Association of adrenocorticosteroid therapy and peptic ulcer disease. *N Engl J Med* 1983;309:21-4.

(Accepted 16 September 1987)

Cytopathogenic protein in filtrates from cultures of *Propionibacterium acnes* isolated from patients with Kawasaki disease

SHOBUN TOMITA, HIROHISA KATO, TAMOTSU FUJIMOTO, OSAMU INOUE, YUKO KOGA, NORIKAZU KURIYA

Abstract

Propionibacterium acnes may have a role in Kawasaki disease. Filtrates from cultures of *P. acnes* isolated from cervical lymph node biopsy specimens and blood samples from patients with Kawasaki disease were studied and compared with samples from control subjects. After inoculation of human embryo liver cells with filtrates from the patients a cytopathogenic effect and vacuolation were seen. A specific cytopathogenic substance was found in only the filtrates of cultures from patients with Kawasaki disease; it was a protein of about isoelectric point 7.0 with a molecular weight of about 100 000 daltons. The amount of IgG antibody to this cytopathogenic protein was measured by enzyme linked immunosorbent assay (ELISA) in serum samples taken from 63 patients in the acute phase of Kawasaki disease (mean 5.2 (SD 1.1) days after onset of illness), 45 in the subacute phase (mean 23.6 (3.3) days), and 51 in the convalescent phase (mean 18.5 (4.1) months) and from 102 control subjects matched for age. Titres of IgG antibody were significantly raised in patients with

Kawasaki disease, particularly in the acute and subacute phases of the illness, compared with in the control subjects.

Titres of IgG antibodies to cytopathogenic protein were found to be low in normal children below the age of 4 years but they increased with age thereafter. This may explain why outbreaks of Kawasaki disease, which is most common in children aged under 4, occur every three years.

Introduction

Kawasaki disease was first described in 1967,¹ and the number of patients affected is increasing not only in Japan but also in the United States, Europe, and Korea.^{2,5} Epidemiological studies have suggested either an infectious aetiology or an immune response to an infectious agent(s),⁶ but the exact cause of the disease has not been determined.

In 1983 we isolated *Propionibacterium acnes* from a lymph node biopsy specimen from a 6 year old boy with typical symptoms of

Kawasaki disease on the 11th day of a second recurrence of the disease.^{7,8} *P. acnes* may be implicated in the pathogenesis of this disease for the following reasons: (1) the incidence of isolation of *P. acnes* from samples of cervical lymph nodes and blood from patients with Kawasaki disease compared with a control group has been high^{7,8}; (2) intraperitoneal inoculation of guinea pigs with this micro-organism resulted in depilation, lymphadenopathy, hepatosplenomegaly, and coronary arteritis^{7,8}; (3) guinea pigs sensitised to BCG had an increased footpad reaction to BCG after a subsequent intraperitoneal injection of *P. acnes*⁹; (4) filtrates of cultures of *P. acnes* induced a cytopathogenic effect and vacuolation when inoculated into human embryo liver cells and these effects were less commonly seen with filtrates of cultures of the standard strain or other strains isolated from blood samples of patients with other illnesses^{7,8}; and (5) when the filtrate from cultures of *P. acnes* was heated at 100°C for 30 minutes or treated with serum from convalescent patients neither vacuolation nor cytopathogenic effects occurred in fetal liver cells.^{7,8}

These results suggested that the strain isolated from patients with Kawasaki disease may produce a cytopathogenic substance. In this study we isolated this substance and measured the amount of IgG antibody against it in the serum of patients with Kawasaki disease and a group of control subjects.

Materials, subjects, and methods

MICRO-ORGANISMS

We used the method of Kato *et al* to isolate and type *P. acnes*.⁷ After initial isolation the strains of *P. acnes* were preserved in cooked meat broth at room temperature. We analysed filtrates from cultures of micro-organisms isolated from four patients with typical symptoms of Kawasaki disease, one patient with leukaemia, and one patient with congenital heart disease, as well as those from house dust mites collected from the houses of patients with Kawasaki disease and micro-organisms of the standard strain (ATCC 11827). Filtrates from the medium alone served as a control.

ANALYSIS OF FILTRATES FROM CULTURES

Micro-organisms were grown for four to 10 days at 37°C in Gifu anaerobic medium broth (peptone 10 g/l, soy peptone 3 g/l, protease peptone W 10 g/l, peptide digest serum powder 13.5 g/l, meat extract 2.2 g/l, yeast extract 5 g/l, liver extract 1.2 g/l, glucose 3 g/l, potassium dihydrogen phosphate 2.5 g/l, sodium chloride 3 g/l, soluble starch 5 g/l, cysteine hydrochloride 0.3 g/l, and sodium thioglycollate 0.3 g/l in distilled water; pH 7.3 (SD 0.1)). The supernatant was centrifuged and filtered through a 0.22 µm Millipore filter; the filtrate was dialysed after being salted out with 80% ammonium sulphate. The concentrated filtrate was fractionated by gel filtration and further fractionated by flat bed isoelectrofocusing. The fractionate (containing 15 µg protein) was inoculated into human embryo liver cells to see the cytopathogenic effect and vacuolation. Vacuolations induced in these liver cells were analysed by polyacrylamide and sodium dodecylsulphate-polyacrylamide gel electrophoresis.

Gel filtration was performed using LKB Ultrogel AcA44, and the filtrate was extracted with phosphate buffered saline, pH 7.2. An LKB Multiphor was used for flat bed isoelectrofocusing. Fractionation was carried out with the Ultradex system and Ampholine, pH 3.5-10. LKB Multiphor and Ampholine plates, pH 3.5-9.5, were used for polyacrylamide gel electrophoresis, and the gel was stained with Coomassie brilliant blue after focusing. Sodium dodecylsulphate-polyacrylamide gel electrophoresis was performed using microslab electrophoresis through a 12.5% acrylamide gel, which was stained with silver after staining with Coomassie brilliant blue.

MEASUREMENT OF THE IgG ANTIBODIES TO CYTOPATHOGENIC SUBSTANCE

IgG antibodies against the cytopathogenic substance extracted from filtrates of cultures of *P. acnes* isolated from the patients with Kawasaki disease were measured in serum samples by an indirect enzyme linked immunosorbent assay (ELISA). Polystyrene flat bottomed microtitre plates (Flow Laboratories Inc) were coated with 100 µl of the cytopathogenic substance and a solution containing protein 50 mg/l in a 0.05M

carbonate-bicarbonate buffer (pH 9.6). After incubation at 4°C overnight the plates were washed four times with 0.05% Tween 20 in phosphate buffered saline; 100 µl of test serum diluted 1 in 4 with 0.05% Tween 20 and 2% fetal calf serum in phosphate buffered saline was added and the plates incubated for two hours at 37°C. The washing procedure and incubation at 37°C were repeated. After a further four washes 100 µl of a rabbit antihuman IgG conjugated with peroxidase (Miles Laboratories Inc) was added at a dilution of 1 in 2000 with 0.05% Tween 20 in phosphate buffered saline. After two hours' incubation at 37°C the plates were again washed four times.

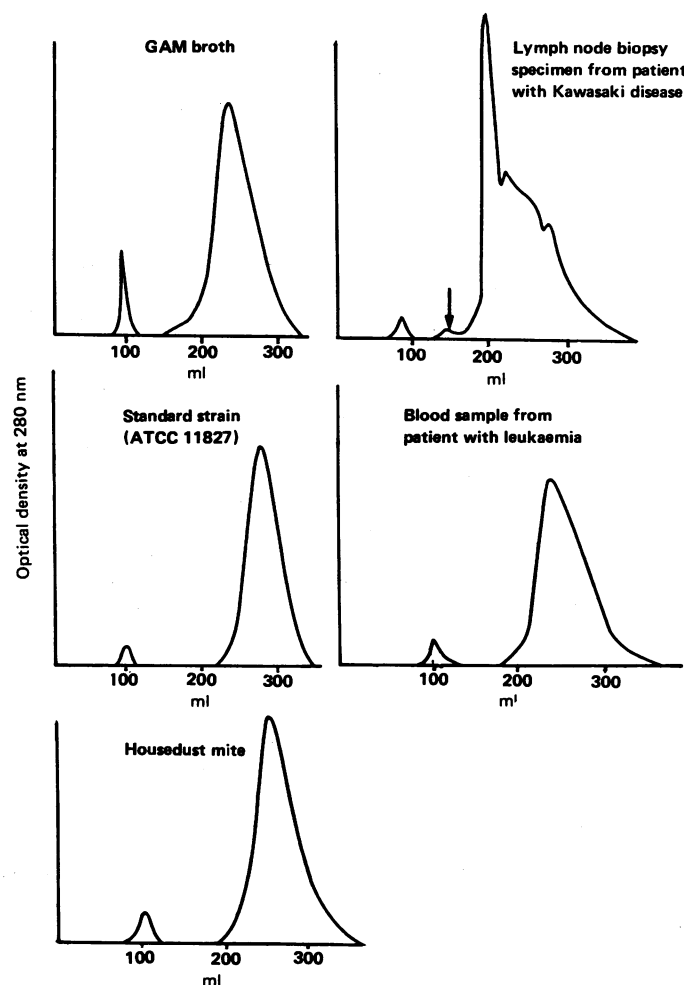


FIG 1—Results of gel filtration analysis of filtrates from cultures of *P. acnes*. Column was Ultrogel AcA44; bed dimensions 2.5×58 cm; eluent phosphate buffered saline, pH 7.2; sample volume 4 ml; and flow rate 30 ml/min. The small peak (arrow) at 100 000 daltons molecular weight was seen only in filtrates of cultures from patients with Kawasaki disease.

Then 100 µl of 0.04% *o*-phenylenediamine in citrate-phosphate buffer (pH 5.0) was added, and the plates were incubated in the dark at room temperature for 30 minutes. The reaction was stopped by adding 50 µl 2.5M sulphuric acid, and the absorption of the plates was measured at 486 nm by an ETY-96 ELISA Analyzer (TOYO, Oriental Instruments Ltd).

Serum samples were collected from 63 patients two to eight days (mean 5.2 (SD 1.1) days) after the onset of Kawasaki disease (acute phase), from 45 patients 14-33 days (23.6 (3.3) days) after the onset of disease (subacute phase), and from 51 patients 11-22 months (18.5 (4.1) months) after the onset of disease (convalescent phase). All patients were less than 4 years old (mean 2.0 (1.6) years). Samples were also taken from 102 subjects (mean age 1.8 (1.2) years), who served as controls for the ELISA procedure. There was no difference in age between the patients and the controls ($p > 0.5$). The prevalence of antibodies to the cytopathogenic substance in normal children and adults was determined in serum samples from 168 subjects from the general population aged 0-35 years (mean 6.0 (7.6) years). Finally, serum samples were collected from three patients with recurrent Kawasaki disease during the first eight days of the initial attack and the recurrence to measure serial antibody titres.

Results

ANALYSIS OF FILTRATES FROM CULTURES

Figure 1 shows the results of gel filtration at an optical density of 280 nm. Peaks around 200 000 and 20 000-30 000 daltons molecular weight were seen in the filtrates from cultures of all strains and the control medium, which did not induce cytoplasmic vacuolations in embryo liver cells. In the filtrates from cultures of strains from patients with Kawasaki disease, however, a small peak of about 100 000 daltons molecular weight was seen, which caused vacuolation in embryo liver cells.

Flat bed isoelectrofocusing (fig 2) showed a specific fractionation band with an isoelectric point about 7.0 in filtrates of cultures from all four of the patients with Kawasaki disease; this induced vacuolation in embryo liver cells (fig 3). There were no such bands in the fractions extracted from the four control strains (fig 2).

These results agreed with those obtained with analysis of polyacrylamide gel electrofocusing. Furthermore, these fractionations were evaluated with sodium dodecylsulphate-polyacrylamide gel electrofocusing, which showed the molecular weight to be about 100 000 daltons. We tentatively named it cytopathogenic protein.

IgG ANTIBODIES TO CYTOPATHOGENIC PROTEIN

The table shows the mean titres of IgG antibodies to cytopathogenic protein measured by ELISA in the 159 patients in the acute, subacute, or convalescent phase of Kawasaki disease and the control group of 102 patients. When the values in the four groups were compared by analysis of

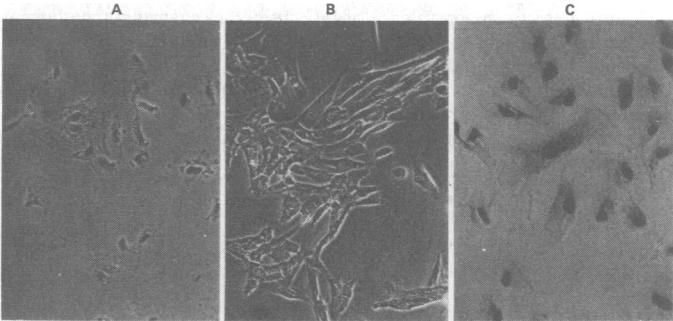


FIG 3—Effect of inoculating human embryonic liver cells with cytopathogenic protein. In A cells were inoculated with cytopathogenic protein from a patient with Kawasaki disease and showed a strong cytopathogenic effect and vacuolation. In B cells were inoculated with filtrates from cultures of *P. acnes* isolated from blood of a patient with leukaemia. In C cells were not inoculated.

Mean titres of IgG antibodies to cytopathogenic protein by ELISA at optical density of 486 nm in patients with Kawasaki disease and in controls. Values are means (SD)

Phase of Kawasaki disease			
Acute (n=63)	Subacute (n=45)	Convalescent (n=51)	Controls (n=102)
0.93 (0.21)	1.00 (0.22)	0.85 (0.23)	0.82 (0.26)

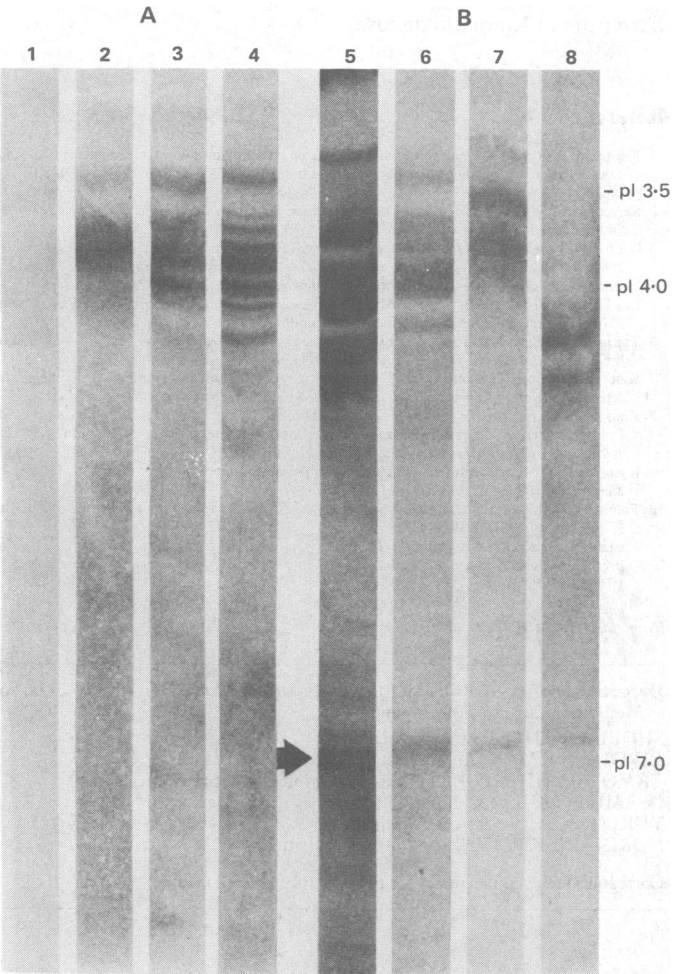


FIG 2—Preparative flat bed isoelectrofocusing of filtrates from cultures of *P. acnes*. Group A are controls: 1 is pure Gifu anaerobic medium (GAM) broth, 2 is filtrate from culture of *P. acnes* isolated from house dust mite, 3 is filtrate from culture of *P. acnes* isolated from a patient with leukaemia, and 4 is filtrate from culture of standard strain (ATCC 11827). Group B (5-8) are filtrates from cultures of *P. acnes* isolated from patients with Kawasaki disease; arrow indicates the specific band for cytopathogenic protein. pI=Isoelectric point.

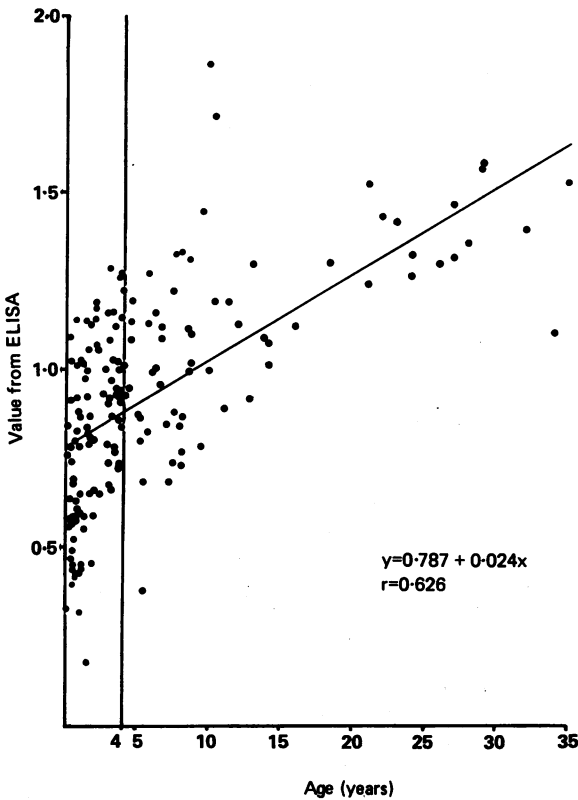


FIG 4—Values from ELISA measuring IgG antibodies to cytopathogenic protein in optical density of 486 nm in general population. (Kawasaki disease is most common in children aged under 4 (indicated by vertical line).)

variance the differences were significant ($F_{(3,257)}=7.7169, p<0.001$). Thus we made multiple comparisons of the values in these groups individually by Student's *t* test. The values were raised both in the group that had been ill for two to eight days and in the group that had been ill for 14-33 days and were significantly different from those in the controls ($p<0.005$ and $p<0.0001$ respectively). The mean value in the patients in the convalescent phase was similar to that in the controls.

Figure 4 shows the prevalence of IgG antibodies against cytopathogenic protein in the general population. The values for children younger than 4 years old were not raised, but they increased with age thereafter.

Figure 5 shows that in all three patients with recurrent Kawasaki disease studied the antibody titres were raised in the acute and subacute phases but fell in the convalescent phase of the first attack. In two patients, however, the titres rose again when the illness recurred, to values higher than those during the first attack.

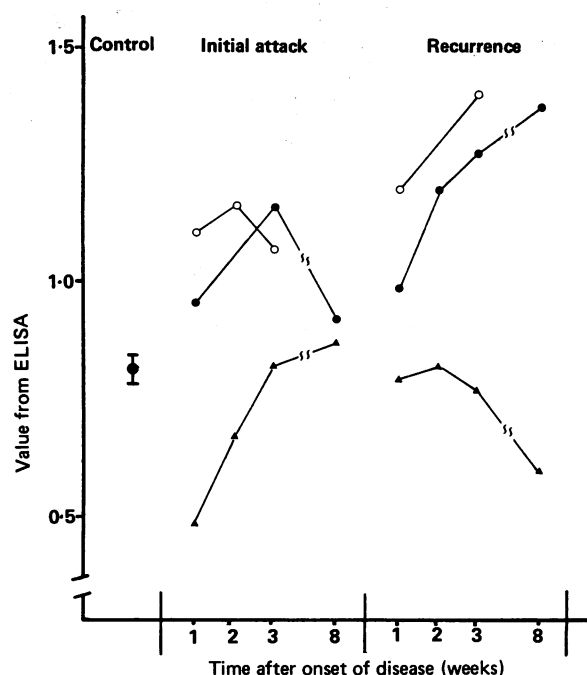


FIG 5—Values from ELISA measuring IgG antibodies to cytopathogenic protein at optical density of 486 nm in three patients with recurrent Kawasaki disease. Value for control is mean (SE).

Discussion

P. acnes is not usually pathogenic in children, but previous studies have suggested that when isolated from patients with Kawasaki disease it may have some pathogenicity.^{7,9} Experiments with *P. acnes* isolated from patients with the disease suggested that it was producing a cytopathogenic substance. Our results suggest that this substance, which we called cytopathogenic protein, may be a cytopathogenic or toxigenic protein, or an exotoxin. Serum titres of IgG antibodies to cytopathogenic protein measured by ELISA were significantly raised in the serum samples from patients with Kawasaki disease, which suggests that cytopathogenic protein may have some aetiological role in the disease. This agrees with previous studies assessing the agglutination test and cytoplasmic vacuolation in embryonic liver cells.^{7,9}

Epidemiological studies have reported several outbreaks of Kawasaki disease in Japan, which have occurred at regular intervals of three years, which suggests an infectious cause. It is, however, difficult to explain how Kawasaki disease can be epidemic if caused by *P. acnes* alone because *P. acnes* is the principal indigenous bacterium of human skin. We found that most infants and young children in the general population had no antibodies to the cytopathogenic protein and that the antibody titres gradually

increased with age. This may explain why it is mainly infants and younger children who suffer from Kawasaki disease. The three year interval between epidemics noted repeatedly in Japan and the United States indicates that after a large outbreak the disease cannot recur until the susceptible group of children has increased adequately. It correlates exactly with the age group at greatest risk.

When serial antibody titres were measured in patients with Kawasaki disease two patterns were noted: in one group of patients a high antibody titre was maintained for a long time, but in another the titre fell during the early convalescent phase.¹⁰ The patients with maintained high titres of antibodies to cytopathogenic protein may have been repeatedly infected with this indigenous bacterium or releasing this protein and therefore receiving numerous "booster" effects. Epidemiological studies showed that 2% or 3% of the patients had recurrences of Kawasaki disease,^{2,6} which could occur in the group of patients in whom the antibody titres fell during the convalescent phase. We measured serial antibody titres in three patients from the time of illness to its recurrence. For two of them the titres fell in the convalescent phase after the first illness and increased again when the illness recurred, which agrees with our hypothesis.

Why and how *P. acnes* changes to produce a substance similar to an exotoxin or develops its pathogenic effects is not known. Pathogenicity may be caused by a bacteriophage or plasmid or other unknown factors, although Yamamoto *et al* reported that no plasmid was present in the bacterial cells.¹¹ The isolation of the strain of *P. acnes* producing the cytopathogenic protein in patients with Kawasaki disease and their significantly higher antibody titres against this protein, however, suggest a causal role for this bacterium in Kawasaki disease.

References

- 1 Kawasaki T. Acute febrile mucocutaneous syndrome with lymphoid involvement with specific desquamation of the fingers and toes in children. *Journal of Allergy* 1967;16:178-222. (In Japanese.)
- 2 Shigematsu I, Yanagawa H, Kawasaki T. *Kawasaki disease—epidemiological data book*. Tokyo: Soft Science Publications, 1986.
- 3 Centres for Disease Control. Multiple outbreaks of Kawasaki syndrome—United States. *MMWR* 1985;34:33-5.
- 4 Melish ME, Hicks RV, Reddy V. Kawasaki syndrome: an update. *Hosp Pract* 1982;17:99-106.
- 5 Lee DB, Cho SH, Lee KS, Lee BC. An epidemiologic and clinical study of Kawasaki's disease in Korea. *Journal of the Japanese Paediatric Society* 1980;84:949-50.
- 6 Yanagawa H, Shigematsu I, Kusakawa S, Kawasaki T. Epidemiology of Kawasaki disease in Japan. *Acta Paediatrica Japonica* 1979;21:1-10.
- 7 Kato H, Fujimoto T, Inoue O, *et al*. Variant strain of *Propionibacterium acnes*: a clue to the aetiology of Kawasaki disease. *Lancet* 1983;ii:1383-8.
- 8 Kato H, Fujimoto T, Koga Y, Inoue O, Tomita S. Pathogenicity of variant strain of *Propionibacterium acnes* in Kawasaki disease: a possible role of toxin production. *Japanese Journal of Paediatric Medicine* 1985;17:705-13. (In Japanese.)
- 9 Inoue O. *Propionibacterium acnes*: a study as a possible etiological agent of Kawasaki disease. *Kurume Med J* 1984;47:155-69. (In Japanese.)
- 10 Tomita S. Pathogenicity of *Propionibacterium acnes* isolated from Kawasaki disease patients. *Kurume Med J* 1986;33:173-80.
- 11 Yamamoto S, Kondo M, Urakawa T, Shingu M. A toxin released from variant strains of *Propionibacterium acnes* isolated from patients with Kawasaki disease. *Kurume Med J* 1985;32:29-36.

(Accepted 3 September 1987)

Department of Paediatrics and Child Health, Kurume University School of Medicine, Kurume 830, Japan

SHOBUN TOMITA, MD, research fellow
HIROHISA KATO, MD, professor of paediatrics
TAMOTSU FUJIMOTO, MD, assistant professor of paediatrics
OSAMU INOUE, MD, clinical fellow
YUKO KOGA, PHD, research assistant
NORIKAZU KURIYA, PHD, medical statistician

Correspondence and requests for reprints to: Professor Kato.