Heat shock proteins and superantigenic properties of bacteria from the gastrointestinal tract of patients with Kawasaki disease

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Summary
We previously suggested that gut bacteria may be involved in the onset of Kawasaki disease (KD). In this study, we evaluated the production of heat shock proteins (hsps) and superantigens (sAgs) by microorganisms isolated from the jejunal mucosa of 19 children with KD in the acute phase and from 15 age-matched control children. We identified 13 strains of Gram-negative microbes from patients with KD; these microbes produced large amounts of hsp60 and induced pro-inflammatory cytokine production by peripheral blood mononuclear cells. The Gram-negative microbes also elicited endogenous hsp60 production, leading to the secretion of anti-inflammatory interleukin-10 (IL-10). We also identified 18 strains of Gram-positive cocci that had superantigenic properties and which induced the expansion of Vβ2 T cells in vitro. All bacteria identified in this study were antibiotic resistant. These data suggest that sAg and hsp60 produced by gut bacteria might be involved in KD.

Keywords: gastrointestinal tract; heat shock proteins; Kawasaki disease (mucocutaneous lymph node syndrome); superantigens

Introduction
Kawasaki disease (KD) is a multisystem vasculitis that primarily affects the coronary arteries of young children. A number of epidemiological and clinical observations suggest that KD is caused by an infectious agent, with suggestions ranging from Staphylococci, Streptococci, Mycoplasma or Chlamydia,1–4 to viruses such as adenovirus, parvovirus or Epstein–Barr virus.5–7 However, no single causative pathogen has been consistently demonstrated in the nasopharynx, oropharynx, skin, or faeces of patients with KD.9,10

Internal tissues of the airway or of the gastrointestinal (GI) tract may also be entry or colonization sites of the potential causative agents, but these have not been investigated in detail. Intense interest has recently centered on novel human coronavirus messenger RNA (mRNA) detected in the respiratory secretions of some children with KD;11 however, other investigators have not been able to confirm this finding.12,13

We have hypothesized that the mucosa of the upper GI tract could be involved in KD because of the role of the GI tract as an immunological organ constantly exposed to microorganisms and other agents. We have previously observed increased numbers of CD4+ T cells and human leukocyte antigen (HLA)-DR+ cells, and fewer CD8+ T cells, in the gut of patients with KD compared with controls.14 Consequently, we carried out a microbiologic investigation of the small intestine and showed that the range of bacterial species adhering to the lumen of the jejunum of patients with KD was quite different from that of controls.15 Notably, five strains of Streptococci and two strains of Staphylococci [both species are known to be common sources of superantigen (sAg)] were isolated only from KD patients. We have also investigated T-cell receptor (TCR) Vβ2 expression in the small intestinal mucosa of patients with KD, and found that Vβ2+ T cells were increased in the jejunal mucosa of KD patients compared with controls.16

Abbreviations: BCS, bacterial culture supernatants; ELISA, enzyme-linked immunosorbent assay; GI, gastrointestinal; hsp, heat shock protein; IFN, interferon; IL, interleukin; KD, Kawasaki disease; mAb, monoclonal antibody; mRNA, messenger RNA; PBMCs, peripheral blood mononuclear cells; PMF, peptide mass fingerprinting; SI, stimulation index; sAg, superantigen; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCR, T-cell receptor; TNF, tumour necrosis factor.
mucosa of KD patients and found that these cells were selectively increased in the mucosa of patients in the acute phase of KD compared with controls.

On the basis of these findings, we carried out microbiological and molecular biological studies focused on the biological activity of microorganisms detected on the alimentary tract surfaces of KD children in the acute phase of the disease. We focused, in particular, on heat shock protein (hsp) and superantigenic activity, in view of previous data indicating that they might have a role in KD.

### Materials and methods

#### Participants

The study received ethical approval from Juntendo University Hospital in Tokyo. All families of the patients and control subjects had given their consent to participate in the study.

Nineteen patients with KD (14 boys and five girls, 5 months to 8 years of age) were enrolled in our study between February 2004 and June 2006; their diagnoses were made in accordance with the clinical criteria for KD (Table 1). They had been hospitalized within 7 days of the onset of fever. All the patients except for one (patient no. 7) received intravenous γ-globulin at a dose of 2 g/kg. Coronary artery involvement was demonstrated in patients 1, 2 and 17. Giant aneurisms were found in patient 2, who died of myocardial infarction. The other patients had no evidence of persistent cardiac abnormal lesions, as assessed by echocardiography.

Jejunal swab specimens and peripheral blood samples were taken for analysis from all KD subjects and, as controls, from 15 patients with food-sensitive enteropathies in remission. We considered our control subjects to be a normal population with respect to the jejunal microflora for the reason stated in our previous studies.

#### Microbiologic study on the jejunal surfaces

Swab specimens of the jejunal surfaces were acquired from a piece of jejunal mucosa taken using a sterile paediatric Crosby-type capsule. Details of the microbiological examination are given elsewhere. None of the subjects or controls were treated with antibiotics before the jejunal biopsies were taken.

Bacteria were cultured in brain-heart infusion medium at 37°C for 24 hr. Bacterial culture supernatants (BCS) were collected by centrifugation and then filtered through a 0.22-μm pore membrane to remove the bacteria.

#### Peripheral blood samples

Blood samples were taken from all subjects when KD was at the acute phase (before drug treatment) and 12–16 days after the onset of fever (i.e. during the convalescent phase). Blood specimens from children with food-sensitive enteropathies in the convalescent phase were taken simultaneously with their biopsies.

### Peripheral blood mononuclear cell co-culture with BCS

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples using the standard procedure of density-gradient centrifugation, and 200 μl (containing 1 × 10⁶ PBMCs) was transferred, in triplicate, to each well of a round-bottomed 96-well plate (Becton Dickinson, Franklin Lakes, NJ) and then incubated, for 72 hr at 37°C in 5% carbon dioxide, in the presence or absence of BCS at a final concentration of 0.25%.

#### PBMC proliferation

For screening for the presence of hsps in BCS, BCS-induced proliferative activity of PBMCs, obtained from convalescent-phase KD patients, was compared with that of PBMC from controls because hsps could induce significant cell-proliferative activity; however, this cell-proliferative activity appeared to be weaker than that induced by sAg or by other potentially immunodominant proteins. Significant cell-proliferative activity of BCS, containing hsp, to PBMC was defined by a stimulatory index (SI) of more than 3.0.

#### Flow cytometric analysis

Superantigenic activity analysis in BCS has been described previously. We looked for expansion of Vβ2 and Vβ8 T cells because Vβ2 T-cell proliferation in KD patients has been reported previously, and Vβ8 T cells were known to be expanded by a clinical isolate of Streptococcus pyogenes toxin used as a positive control. Monoclonal antibodies (mAbs) were obtained from PharMingen (San Diego, CA). Data were analyzed using CELLQUEST® software (Becton Dickinson, San Jose, CA).

#### Western blot analysis

In order to detect bacterial hsps in BCS and human endogenous hsp molecules in the PBMC supernatant co-cultured with BCS, in comparison with controls, Western blot analysis was performed using plasma samples from the subjects as antibodies to those proteins. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in 10% gels (ATTO Corporation, Tokyo, Japan), as described previously.
Detection of bacterial hsp in plasma

Proteins in a BCS sample were separated by SDS–PAGE and electrotransferred to immobilon P, polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membrane was incubated at 37°C for 40 min in 100-fold dilutions of plasma samples obtained from patients in the acute and convalescent phases of KD, or from controls, as

Table 1. Summary of the subjects

| Patient no. | Age               | Gender | Days after | Coronary artery involvement | Significant strain detected (SI > 3.0) |
|-------------|-------------------|--------|------------|-----------------------------|-------------------------------------|
| 1           | 5 months          | M      | 5          | +                           | S. mitis (SI = 28.7)                |
| 2           | 6 months          | M      | 6          | +                           | S. mitis (SI = 12.6)                |
| 3           | 6 months          | M      | 6          | −                           | S. aureus (SI = 15.6)              |
| 4           | 6 months          | M      | 5          | −                           | S. mitis (SI = 25.4)                |
| 5           | 7 months          | M      | 5          | −                           | S. aureus (SI = 14.1)              |
| 6           | 9 months          | M      | 6          | −                           | S. oralis (SI = 12.8)              |
| 7           | 11 months         | M      | 5          | −                           | N. mucosa (SI = 4.9)               |
| 8           | 1 year 2 months   | M      | 6          | −                           | S. oralis (SI = 11.6)              |
| 9           | 1 year 10 months  | M      | 5          | −                           | S. aureus (SI = 14.3)              |
| 10          | 1 year 10 months  | M      | 6          | −                           | S. oralis (SI = 12.2)              |
| 11          | 1 year 11 months  | M      | 6          | −                           | S. sanguinis (SI = 12.0)           |
| 12          | 5 years 8 months  | M      | 6          | −                           | S. aureus (SI = 34.1)              |
| 13          | 6 years 9 months  | M      | 6          | −                           | S. sanguinis (SI = 11.9)           |
| 14          | 6 years 11 months | M      | 7          | −                           | S. mitis (SI = 53.3)               |
| 15          | 1 year 11 months  | F      | 6          | +                           | S. mitis (SI = 15.2)               |
| 16          | 2 years 11 months | F      | 6          | −                           | S. aureus (SI = 28.4)              |
| 17          | 4 years 11 months | F      | 5          | −                           | N. mucosa (SI = 9.4)               |
| 18          | 5 years 1 month   | F      | 7          | −                           | Veillonella spp. (SI = 11.4)        |
| 19          | 8 years 2 months  | F      | 7          | −                           | S. aureus (SI = 32.7)              |

1Aneurysm or dilatation of more than 4 mm in diameter of the coronary artery.

2Significant strain is a strain in which culture supernatant could induce peripheral blood mononuclear cell (PBMC) proliferation equating to a stimulation index (SI) of more than 3.0.

F, female; M, male; A. iwoffii, Acinetobacter iwoffii; A. odontolyticus, Actinomyces odontolyticus; C. aquaticum, Corynebacterium aquaticum; E. cloacae, Enterobacter cloacae; L. cremoris, Lactococcus cremoris; N. flavescens, Neisseria flavescens; N. mucosa, Neisseria mucosa; S. acidominimus, Streptococcus acidominimus; S. adjacens, Streptococcus adjacens; S. aureus, Staphylococcus aureus; S. epidermidis, Staphylococcus epidermidis; S. mitis, Streptococcus mitis; S. oralis, Streptococcus oralis; S. sanguinis, Streptococcus sanguinis; X. maltophilia, Xanthomonas maltophilia.
the primary antibody. Membranes were incubated at 37\(^\circ\)C for 40 min with the secondary antibody (goat anti-human IgG–alkaline phosphatase conjugate, 1 : 1000 dilution; Sigma, St Louis, MO). Immune reactivity was visualized using Disodium 3-(4-methoxyspiro [1,2-dioxetane-3,2’-(5’-chloro) tricyclo [3.3.1.13,7]decan]-4-yl)phenyl phosphate (Roche, Basel, Switzerland).

Identification of hsp molecules

Proteins were visualized by staining with Coomassie\(^\text{R}\) Brilliant Blue. The target proteins were excised from the gels, trypsinized and analyzed using a matrix-assisted laser desorption time-of-flight mass spectrometer (Voyager DE Pro; Applied Biosystems, Tokyo, Japan). A list of the corrected mass peaks provided details for peptide mass fingerprinting (PMF). Proteins were identified in the National Center for Biotechnology Information (NCBI) database through PMF using the MS-FIT program in protein prospector (http://prospector.ucsf.edu).

Enzyme-linked immunosorbent assay

To measure the concentrations of cytokines and human endogenous hsp60 in PBMCs co-cultured with BCS, enzyme-linked immunosorbent assays (ELISAs) were performed using mAbs according to the manufacturer’s instructions. ELISA kits for interferon-\(\gamma\) (IFN-\(\gamma\)), tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interleukin-10 (IL-10) were purchased from Sanquin Research (Amsterdam, the Netherlands) and those for human endogenous hsp60 were obtained from Stressgen (Ann Arbor, MI). The detection limits were 0.5 IU/ml for IFN-\(\gamma\), 10 pg/ml for TNF-\(\alpha\), 4.0 pg/ml for IL-10 and 3 ng/ml for hsp60. Absorbance was read at 450 nm against air using a microplate reader (Bio-Rad, Hercules, CA).

Statistical analysis

The paired Student’s \(t\)-test was used to analyze statistical significance between subjects with KD and controls. Values of \(P < 0.05\) were considered significant.

Results

Screening of BCS for hsps

To detect hsp in BCS, BCS-induced proliferative activity on PBMCs was performed. All bacteria isolated from all KD patients gave culture supernatants that induced significant proliferative activity (SI > 3.0) in autologous PBMCs (Table 1). We performed antibiotic-sensitivity testing on the strains producing BCS that caused significant proliferation of PBMCs, considering the possibility of treatment of these bacteria if they partici-

| Case | Significant strain | ABPC | CFDN | MINO | EM | ST | FOM |
|------|-------------------|------|------|------|----|----|-----|
| 1    | Neisseria mucosa  | I    | I    | I    | R  | S  | I   |
| 2    | Acinetobacter iwoffii | I    | I    | S    | I  | S  | I   |
| 3    | Enterobacter cloaceae | R    | S    | R    | S  | I  |    |
| 4    | Neisseria mucosa  | I    | I    | I    | R  | S  | I   |
| 5    | Staphylococcus aureus | R    | R    | R    | I  | S  |    |
| 6    | Enterobacter cloaceae | R    | R    | S    | R  | S  | I   |
| 7    | Neisseria mucosa  | I    | I    | I    | R  | S  | I   |
| 8    | Streptococcus oralis | R    | R    | S    | S  | I  |    |
| 9    | S. aureus        | R    | R    | R    | S  | I  |    |
| 10   | Neisseria mucosa  | I    | I    | I    | R  | S  | I   |
| 11   | Streptococcus sanguinis | R    | R    | R    | S  | I  |    |
| 12   | S. aureus        | R    | S    | S    | R  | S  | S   |
| 13   | Neisseria mucosa  | I    | I    | I    | R  | S  | I   |
| 14   | Streptococcus mitis | R    | R    | R    | S  | I  |    |
| 15   | Neisseria mucosa  | I    | I    | I    | R  | S  | I   |
| 16   | S. aureus        | R    | S    | S    | R  | S  | S   |
| 17   | Neisseria mucosa  | I    | I    | I    | R  | S  | I   |
| 18   | Veillonella      | R    | R    | S    | S  | I  |    |
| 19   | S. aureus        | R    | R    | R    | S  | I  |    |

A significant strain was defined as a strain of which the culture supernatant could induce cell proliferative activity of peripheral blood mononuclear cell (PBMC) of more than 3.0 stimulation index (SI). ABPC, ampicillin; CFDN, cefdinir; EM, erythromycin; FOM, fosfomycin; I, intermediate; MDSTO, minocycline; R, resistant; S, sensitive; ST, sulfamethoxazole/trimethoprim.

To determine superantigenic activity in PBMCs stimulated with BCS, the expression of TCRs V\(\beta\)2 and V\(\beta\)8 was determined on cultured cells. Streptococcus sanguinis of KD patients 11 and 13, Streptococcus oralis of patients 6, 8 and 10, and Streptococcus mitis of patients 1, 2, 4, 14, 15, 17 and 18, and all staphylococcal strains of patients 3, 5, 9, 12, 16 and 19 produced an expansion of T-cell populations expressing V\(\beta\)2 (Fig. 1). None of the other strains, including Neisseria mucosa, induced selective V\(\beta\) expansion in T-cell populations from children with KD or from controls.

Demonstration of antibodies to bacterial hsps in plasma

The antibody responses to bacterial products that induced significant proliferative activity, as shown in Table 1, in
host plasma, were analyzed by Western blotting using plasma obtained from 19 patients during acute and convalescent stages of KD. Some plasma samples obtained from patients during the convalescent stage of KD, after treatment with immunoglobulin, showed vigorous antibody reactivity against 60 000–70 000 molecular weight (MW) proteins that were not seen in acute plasma; however, interestingly, this phenomenon was not observed in

**Figure 1.** The expression of T-cell receptors (TCRs) Vβ2 and Vβ8 on harvested cells, determined using three-colour flow cytometry. The percentage of Vβ2+ T cells in peripheral blood mononuclear cells (PBMCs) isolated from convalescent-phase patients with Kawasaki disease (KD) was significantly increased when co-cultured with Gram-positive cocci isolated from all the KD patients, except for patient no. 7, in comparison with the positive control (Streptococcus pyogenes), which was in contrast to the results with Gram-negative microbes from patients 1, 2, 3, 4, 6, 7, 10, 12, 13, 14, 15, 17 and 18. No statistical difference was observed among those groups regarding expression of the Vβ8 repertoire, except for the positive control.

**Figure 2.** Antibodies to bacterial heat shock proteins (hsp) are present in plasma. The plasma obtained during the convalescent phase of Kawasaki disease (KD), from patients 1, 2, 3, 4, 6, 7, 10, 12, 13, 14, 15, 17 and 18, showed vigorous antibody reactivity against 60 000–70 000 molecular weight (MW) proteins after immunoglobulin treatment; this antibody reactivity was absent in plasma obtained during the acute phase of KD. (a) (‘A’, acute plasma; ‘C’, convalescent plasma). For superantigenic strains such as Staphylococcus aureus, obtained from patients 5 and 16 with KD, and Streptococcus oralis, Streptococcus sanguinis and Streptococcus mitis from patients 8, 11 and 14 with KD, respectively, a small quantity of 60 000–70 000 MW proteins had already been noted in plasma obtained from the acute phase, or none was seen in both of the acute convalescent phase plasma (b). The reactivity shown in Fig. 2a was not detected in the control plasma samples to any bacterial products originated from their own or KD jejunal flora. Representative reactivity in several samples (control patient no. 1, KD patient nos 4 and 14) is demonstrated (c).
the BCS originating from superantigenic strains, in which a small quantity of 60 000–70 000 MW proteins was seen in plasma from patients in the acute phase of KD (Fig. 2a,b). Furthermore, in control plasma samples this reactivity was not seen to any bacterial products originating from the jejunal mucosa of control or KD patients. Because of a lack of blood samples it was impossible to examine all the combinations and therefore representative reactivity patterns of several samples are demonstrated in Fig. 2c.

Identification of bacterial hsp60 using plasma from KD patients in the convalescent stage

The 60 000–70 000 MW target proteins were identified in the NCBI database through PMF using the MS-Fit program. All were identified as hsp60 originated from each organism with high sequence coverage of more than 30% (Table 3). Interestingly, those hsp60 molecules were derived from only Gram-negative strains of bacteria in KD patients 1, 2, 3, 4, 6, 7, 10, 12, 13, 14, 15, 17 and 18, whereas some Gram-positive cocci yielded no hsps or the others had already secreted them before the onset of KD. The most frequently detected protein was hsp60, hitherto reported to be derived from *Neisseria* spp. in convalescent plasma from 8 of 19 (42%) patients with KD.

| Patient no. | Bacterial hsp 60 |
|-------------|-----------------|
| 1           | Heat shock protein 60 (*Neisseria* spp.) |
| 2           | Heat shock protein 60 (*Acinetobacter* spp. ADP1) |
| 3           | Heat shock protein 60 (*Enterobacter* doacae) |
| 4           | Heat shock protein 60 (*Neisseria* spp.) |
| 5           | ND |
| 6           | Heat shock protein 60 (*Enterobacter* doacae) |
| 7           | Heat shock protein 60 (*Neisseria* spp.) |
| 8           | ND |
| 9           | ND |
| 10          | Heat shock protein 60 (*Neisseria* spp.) |
| 11          | ND |
| 12          | ND |
| 13          | Heat shock protein 60 (*Neisseria* spp.) |
| 14          | ND |
| 15          | Heat shock protein 60 (*Neisseria* spp.) |
| 16          | ND |
| 17          | Heat shock protein 60 (*Neisseria* spp.) |
| 18          | Heat shock protein 60 (*Veillonella*) |
| 19          | ND |

ND, not detected.

Human endogenous hsp 60 secreted by peripheral blood mononuclear cells co-cultured with BCS

It has been previously suggested that the increased expression of endogenous hsp may contribute to the abnormally activated immune system of KD patients via molecular mimicry between bacterial hsps and human hsps.17–19 To demonstrate the production of endogenous hsp by PBMCs reacting to each strain of bacteria, the presence of human hsp60 in PBMC culture supernatants, co-cultured with BCS which induced significant proliferative activity, was investigated by ELISA. The production of endogenous hsp60 was clearly evident in the supernatants originating from Gram-negative microbes compared with those from Gram-positive microbes and controls (Fig. 3).

Demonstration of amino acid sequence homology between bacterial hsps and human hsps

We analyzed homology between hsp60 derived from bacteria such as *Neisseria*, *Acinetobacter* and human endogenous hsp60 according to the NCBI database, CLUSTALW W Multiple Sequence Alignments and the manufacturer’s reference. Homology between the 66–86 amino acid sequence of bacterial hsp60, originating from both *N. mucosa* and *Acinetobacter iwoffsii*, and the 90–110 amino acid sequence of human hsp60 (50%), presumably recognized by effector T cells in the disease,19 was found, as shown in Table 4, indicating cross-reactivity between...
those two molecules related to a possible pathogenic role of hsp-specific T cells in KD. Likewise, high homology was observed between the 222–230 amino acid sequence of bacterial hsp60 and the 246–254 amino acid sequence of human hsp60 (67%), which are demonstrated to have binding specificities to HLA-DR4. 24 Similarly, the 259–267 amino acid sequence of bacterial hsp60 and the 283–291 amino acid sequence of human hsp60 showed homology (56%), and are known to show high binding affinity to HLA-DR8.

### Cytokine production in the PBMC culture supernatant cultured with BCS

The cytokine response of PBMCs to bacterial products showed that pro-inflammatory cytokines IFN-γ and TNF-α were elicited in patients with KD, but not in the controls (Fig. 4). As a recent report proposed that increased levels of IL-10 induced by self-hsp60 molecules could lead to anti-inflammatory process in children with juvenile idiopathic arthritis, we also investigated the concentration of IL-10 in supernatants. 25 Interestingly, microbial products, except for superantigen (sAg), elicited a significantly higher IL-10 response in PBMCs from patients with Kawasaki disease, unlike those from the controls (P < 0·01).

### Discussion

It has been postulated that the primary cause of KD is an infectious agent that elicits cytokine secretion; these cytokines target vascular endothelial cells, producing cell-surface neoantigens, and antibodies produced against these antigens may then target the vascular endothelium, resulting in a cascade of events leading to vascular damage. Most of the searches for an aetiologic agent in KD have been carried out for a single infectious agent. However, a more plausible underlying principle is that there is cooperation between different mechanisms of different causative bacteria, and a final common pathway of immune activation is responsible for the clinical disorder and complications. 8,26 Recently, the issue has been raised as to whether the provoking antigen in KD might be a conven-

### Table 4. Peptide sequences of a representative bacterial heat shock protein 60 (hsp60) epitope and a corresponding human self-hsp60 epitope, as recognized by T cells and binding to major histocompatibility complex (MHC) class II molecules

| Origin of the hsp60 peptide | Amino acid sequence recognized by T cells | Map position |
|-----------------------------|------------------------------------------|--------------|
| Neisseria mucosa hsp60      | FENMGAGQMVKETNDVAG                     | 66–86        |
| Human self-hsp 60           | YKN IGAKLQDVANNTNEEAG                   | 90–110       |
| A. iwoffii hsp60            | FENMGAGQVLKETNDVAG                     | 66–86        |
| Human self-hsp 60           | YKN IGAKLQDVANNTNEEAG                   | 90–110       |
| Amino acid sequence hinging to HLA-DR4 |                           |              |
| Neisseria mucosa hsp60      | LFDKKISNI                              | 222–230      |
| Human self-hsp 60           | LSEKKISSI                              | 246–254      |
| A. iwoffii hsp60            | LEVKKISNI                              | 222–230      |
| Human self-hsp 60           | LSEKKISSI                              | 246–254      |
| Amino acid sequence hinging to HLA-DR8 |                           |              |
| Neisseria mucosa hsp60      | LATLVVNNI                              | 259–267      |
| Human self-hsp 60           | LSTLVLNRL                              | 283–291      |
| A. iwoffii hsp60            | LATLVVNNI                              | 259–267      |
| Human self-hsp 60           | LSTLVLNRL                              | 283–291      |

The sequences of bacterial hsp60 derived from Neisseria mucosa and Acinetobacter iwoffii peptides 66–86, 222–230 and 259–267, respectively, and those of the corresponding human hsp60 90–110, 246–254 and 283–291 peptides are shown. Identical residues are underlined.

Figure 4. Cytokine production [determined using enzyme-linked immunosorbent assays (ELISAs)] of peripheral blood mononuclear cells (PBMCs), co-cultured with bacterial culture supernatants (BCS). Interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) production was induced in a BCS obtained from a significant strain, but not in the control supernatants (P < 0·01 versus controls; those P values were omitted in the figure for a tidy view). Microbial products except for superantigen (sAg) elicited a significantly higher interleukin-10 (IL-10) response in PBMCs from patients with Kawasaki disease, unlike those from the controls (P < 0·01).
tional antigen or a sAg. Although the debate continues regarding the mechanism of initial immune activation, the most likely scenario is that sAg and conventional peptide antigens work together to direct a persistent immune response leading to coronary artery damage. A unifying model proposed that a microbe with superantigenic activity initiates massive activation of the developing immune system. A subpopulation of sAg-responsive T cells is rescued from apoptosis as a result of interaction with costimulatory signals between antigen-presenting cells and a conventional peptide antigen. This peptide antigen may be derived from itself or an infectious mimic of itself. The immune response is perpetuated locally where the self-antigen is found on the coronary vessels.

In terms of the current study we have provided data consistent with two of these hypotheses, namely that bacteria unique to the upper bowel of patients with KD have sAg activity and that supernatants of these bacteria contain hsp60, which induce T-cell division and production of pro-inflammatory cytokines. One may be concerned with the description of *S. oralis*, *S. sanguinis* and *S. mitis* as superantigenic strains because they are generally not known as superantigenic strains. Very few reports on the sAg activity of *S. mitis* have been published, and sAg activity for *S. oralis* and *S. sanguinis* has not yet been confirmed, except for some interesting findings suggesting the possibilities of toxic shock-like syndrome caused by viridans streptococci to which those strains belong and sAg-related toxigenic conversion between streptococci species. However, these findings did not directly support the production of sAg by those strains. Further investigations in this respect are obviously required. Autologous PBMCs may be problematic because T cells bearing a certain TCR would be stimulated in an oligoclonal manner and would eventually become anergic. However, we demonstrated that PBMCs obtained from patients during the convalescent phase of KD had the capacity to respond to sAg, probably because some T-cell subsets bearing Tβ2 were not anergized or the T cells had never been exposed to sAg in these young children. The reason why the IFN-γ and TNF-α production induced by BCS containing sAg was not markedly higher than that induced by BCS without sAg may be explained likewise in that a handful of T cells which respond to sAg in the convalescent sample could produce moderately high amounts of those cytokines.

Previous theories have proposed that hsp60 signals mediate T-cell activation through Toll-like receptor 4 on antigen-presenting cells, including GI dendritic cells, although there are also reports to the contrary. Heat shock protein 60 molecules may also function as conventional peptide antigens. It is an attractive concept that this cross-reactivity between specific epitopes of bacterial and human hsp60 could play a role in vascular damage characteristic of KD, as humoral and cellular reactivity against hsp65/63 has been described in more common vascular diseases such as atherosclerosis, which may share a common pathogenic mechanism involving the immune response against hsp65/63; however, the etiologic agents that produce bacterial hsp, leading to the onset of KD, are unknown.

To identify the possible aetiologic pathogen(s) and to elucidate the pathogenic mechanisms, we focused on microbiota that can produce hsp60 and are present on the GI tract surfaces of children with acute KD on the basis of the findings obtained from our previous studies. The agents demonstrated here are rich in variety, consistent with our hypothesis that many different agents may be related to the onset of KD. Based on our findings in this study, the most likely candidates for bacteria associated with the pathogenesis might be Gram-negative microbes, such as *N. mucosa*, in cooperation with plural Gram-positive cocci having superantigenic activity. Furthermore, bacterial hsp60–70 seemed to possess a certain homology, together with self-hsp. Recent studies suggest that an interaction between bacterial and self-hsp antibody responses to non-self epitopes might be promoted by the concomitant generation of anti-inflammatory cytokines, such as IL-10, induced by self-epitopes.

Accumulating evidence from epidemiological or immunopathological studies has suggested that hsp60 autoantibodies can cross-react with bacterial and self-hsp and may induce cytotoxic damage of stressed endothelial cells, resulting in coronary atherosclerosis. These theories seem applicable to the pathogenesis of coronary lesions of KD, in view of the high expression of hsp60 in endothelial cells, together with the serological detection of bacterial and self-hsp in KD patients, demonstrated by some investigators. Vascular surface-expressed hsp60, transferred from the cytoplasm or mitochondria following induction by bacterial hsp60 stimulation, can be recognized by circulating anti-hsp autoantibodies or cytotoxic T lymphocytes targeting autoantigens.

The incidence rate of coronary lesions in patients with KD may depend upon how strongly causative agents can induce the initial immune activation which elicits autoreactive T cells and, importantly, how much self-hsp molecules they can evoke from the cytoplasm or mitochondria to the vascular surface. Interestingly Gram-negative microbes appeared to trigger more self-hsp than Gram-positive cocci. Furthermore, Gram-negative microbes, such as *N. mucosa*, which co-exist with Gram-positive cocci, have been isolated in KD patients with vascular involvements.

The amino acid sequences of *Neisseria* hsp60 (221–231 and 255–269), which were found in this study, had high homology (60%, 50%, respectively) with self-hsp60 (246–256 and 280–294), which are core epitopes of a protein with a high capability of binding human class II
molecules, respectively. These sequences were demonstrated to have high affinity with HLA-DRB1*0802 and *0401, respectively, by DR-peptide binding assays. Interestingly, these DR alleles are frequently found specifically in the Japanese population (24-5%, 40-4%, respectively). This may account for the high incidence of KD among Japanese people. Other explanations suggest Toll-like receptor 4 or hsp polymorphism, both of which can affect the production control of self-hsp as an autoantigen.

We included children with food-sensitive enteropathies as controls; however, it is not entirely clear whether they are appropriate controls. Such types of studies may be inevitably under limitations because it is nearly impossible to conduct them in age-matched healthy controls, for ethical reasons.

Immunoglobulin is helpful in the acute phase of the illness, but there is a failure rate of approximately 10% and an adverse effect rate close to 30%. Additionally immunoglobulin is expensive, at approximately $100 per gram. Antibiotic treatments targeting the causative bacteria may yield a great deal of reliability and economical benefits for the treatment for KD if the more detailed pathogenic mechanisms of the disease can be defined by further studies.

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Disclosures

The authors have no conflict of interest.

References

1 Matsubara K, Fukaya T, Miwa K et al. Development of serum IgM antibodies against superantigens of Staphylococcus aureus and Strepococcus pyogenes in Kawasaki disease. Clin Exp Immunol 2006; 143:427–34.
2 Barton M, Melbourne R, Morais P, Christie C. Kawasaki syndrome associated with group A streptococcal and Epstein–Barr virus co-infections. Ann Trop Paediatr 2002; 22:257–60.
3 Wang JN, Wang SM, Liu CC, Wu JM. Mycoplasma pneumoniae infection associated with Kawasaki disease. Acta Paediatr 2001; 90:594–5.
22 Pietra BA, Inocencio JD, Giannini EH, Hirsch R. TCR Vβ family repertoire and T-cell activation markers in Kawasaki disease. J Immunol 1994; 153:1881–8.
23 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680–5.
24 Southwood S, Sidney J, Kondo A et al. Several common HLA-DR types share largely overlapping peptide binding repertoires. J Immunol 1998; 160:3363–73.
25 Kamphuis S, Kuis W, Jager W et al. Tolerogenic immune response to novel T-cell epitopes from heat-shock protein 60 in juvenile idiopathic arthritis. Lancet 2005; 365:50–6.
26 Yeung RSM. Pathogenesis and treatment of Kawasaki disease. Curr Opin Rheumatol 2005; 17:617–23.
27 Rowley AM. The etiology of Kawasaki disease: superantigen or conventional antigen? Pediatr Infect Dis J 1999; 19:69–70.
28 Choi IH, Chwae YJ, Shim WS, Kim DS, Kwon DH, Kim SJ. Clonal expansion of CD8+ T cells in Kawasaki disease. J Immunol 1997; 159:481–6.
29 Rowley AM, Eckerley CA, Jack HM, Shulman ST, Baker SC. IgA plasma cells in vascular tissue of patients with Kawasaki syndrome. J Immunol 1997; 159:3946–5.
30 Leung DY, Gately M, Taumble A, Ferguson-Darndle B, Schievert PM, Picken LJ. Bacterial superantigens induce T-cell expression of the skin selective homing receptor, the cutaneous lymphocyte associated antigen via stimulation of interleukin 12 production. J Exp Med 1995; 181:747–75.
31 Matsushita K, Uchiyama T, Igarashi H, Ohkuni H, Nagaoka S, Kotani S, Takada H. Possible pathogenic effect of Streptococcus mitis superantigen on oral epithelial cells. Adv Exp Med Biol 1997; 418:683–8.
32 Lu HZ, Weng XH, Zhu B, Li H, Yin YK, Zhang YX, Haas DW, Tang YW. Major outbreak of toxic shock-like syndrome caused by Streptococcus mitis. J Clin Microbiol 2003; 41:3051–5.
33 Soto A, Evans TJ, Cohen J. Proinflammatory cytokine production by human peripheral blood mononuclear cells stimulated with cell-free supernatants of Viridans streptococci. Cytokine 1996; 8:300–4.
34 Vojtek I, Pirzada ZA, Henriques-Normark B, Mastny M, Janapatla RP, Charpentier E. Lysogenic transfer of group A Streptococcus superantigen gene among Streptococci. J Infect Dis 2008; 197:225–34.
35 Pockley AC. Heat shock proteins as regulators of immune response. Lancet 2003; 362:469–76.
36 Osterloh A, Veit A, Gessner A, Fleischer B, Breloer M. Hsp60-mediated T cell stimulation is independent of TLR4 and IL-12. Int Immunol 2008; 20:433–43.
37 Wick G, Perschinka H, Xu Q. Autoimmunity and atherosclerosis. Am Heart J 1999; 138:5444–9.
38 Xu Q, Willeit J, Marosi M et al. Association of serum antibodies to heat-shock protein 65 with carotid atherosclerosis. Lancet 1993; 341:255–9.
39 Zhu J, Quyyumi AA, Rott D, Csako G, Wu H, Halcox J, Epstein SE. Antibodies to human heat-shock protein 60 are associated with the immune component of atherogenesis. Circulation 2001; 103:1071–5.
40 Mayr M, Metzler B, Kiechl S, Willeit J, Schett G, Xu Q, Wick G. Endothelial cytotoxicity mediated by serum antibodies to heat shock proteins of Escherichia coli and Chlamydia pneumoniae: immune reactions to heat shock proteins as a possible link between infection and atherosclerosis. Circulation 1999; 99:1560–6.
41 Schett G, Xu Q, Amberger A, Van Der Zee R, Recheis H, Willeit J, Wick G. Autoantibodies against heat shock protein 60 mediate endothelial cytotoxicity. J Clin Invest 1995; 96:2569–77.
42 Mandal K, Afzal AR, Brecker SJ, Ploniecki J, Xu Q, Jahangiri M. Association of serum soluble heat shock protein 60 with toll-like receptor 4 polymorphism and severity of coronary artery disease. Heart 2006; 92:683–5.
43 Temple SEL, Cheong KY, Ardlie KG, Sayer D, Waterer GW. The septic shock associated HSPA1B1267 polymorphism influences production of HSPA1A and HSPB1. Intensive Care Med 2004; 30:1761–7.
44 Kumarapeli ARK, Wang X. Genetic modification of the heart: chaperones and the cytoskeleton. J Mol Cell Cardiol 2004; 37:1097–109.
45 Klassen P, Rowe PC, Gafiñ A. Economic evaluation of intravenous immune globulin therapy for Kawasaki disease. J Pediatr 1993; 122:532–42.