Vasculitis induced by immunization with Bacillus Calmette-Guérin followed by atypical mycobacterium antigen: a new mouse model for Kawasaki disease

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Abstract
Kawasaki disease causes systemic vasculitis. The development of skin lesions at the vaccination site with Bacillus Calmette-Guérin (BCG) is an important diagnostic symptom. We hypothesized that infection with ubiquitous microorganisms immunogenically related to BCG might induce an immunopathologic reaction leading to the development of Kawasaki disease. Mice were first inoculated with BCG, and then secondarily inoculated 4 weeks later with crude extract from Mycobacterium intracellulare (cMI), an abundant atypical mycobacterium. Animals inoculated with BCG followed by cMI developed coronary arteritis with infiltration of inflammatory cells, whereas control animals inoculated with only cMI or BCG did not, suggesting that the immune response to the mycobacteria induced autoimmunity to the vascular wall. Intravenous injection with antibodies to peroxiredoxin II, a modulator of vascular remodeling and a suggested target for autoimmune vasculitis, also resulted in coronary arteritis, but only after prior inoculation with BCG. Tumor necrosis factor-α, MCP1 and interferon-γ production were significantly higher in the animals inoculated with BCG than in the control groups (P < 0.05). BCG immunization was required for the development of coronary arteritis, suggesting that these cytokines might play important roles. The results indicate that BCG induces primary autoimmunity and stimulates cytokine induction, and that atypical mycobacterial infection boosts the autoimmunity resulting in coronary arteritis.

Introduction
Kawasaki disease with subsequent coronary aneurism was first reported by the Japanese pediatrician Tomisaku Kawasaki in 1967 (Kawasaki, 1967, 1974). To date, many cases have been reported from over 60 countries (Burns & Glode, 2004). Several investigations have revealed that Kawasaki disease consists of systemic vasculitis with hypercytokinemia (Furukawa et al., 1988; Maury et al., 1988; Ueno et al., 1989; Matsubara et al., 1990; Eberhard et al., 1995; Asano & Ogawa, 2000). A number of reports indicate that the development of Kawasaki disease might be associated with bacterial or viral infections such as those caused by Staphylococcus aureus, hemolytic Streptococcus, Yersinia, Epstein–Barr virus, human herpesvirus-6, or human coronavirus (Kikuta et al., 1988; Akiyama & Yashiro, 1993; Leung et al., 1993; Abe et al., 1997; Ihira et al., 2002; Esper et al., 2005). However, the etiology of Kawasaki disease is still controversial.

Several investigations have demonstrated coronary arteritis in experimental animals, and in some of these, dilatation of the coronary artery developed (Murata, 1979; Lehman et al., 1983, 1988; Philip et al., 2004). However, these models were not directly applicable to the clinical phenomenon, as the methods of induction were not based on clinical observations.

Flare and crust formation at Bacillus Calmette-Guérin (BCG) inoculation sites is one of the typical symptoms of Kawasaki disease listed in the diagnostic criteria, and is probably an immunopathologic response (Sato et al., 1993; Kuniyuki & Asada, 1997; Ayusawa et al., 2005). Although not a principal symptom of Kawasaki disease, it allows for early diagnosis of most cases. This response indicates that the immune response to BCG antigens, which reside intradermally in vaccine recipients, might be boosted when the systemic vasculitis occurs.

In patients with Kawasaki disease, antibodies to the mycobacterial heat shock protein (HSP65) increase and
recognize the conserved peptide of the human homolog, suggesting that a cross-reactive immune response to BCG and human antigens might cause immunopathogenesis that leads to systemic vasculitis (Sireci et al., 2000; Yokota et al., 2006).

Autoantibodies, such as antineutrophil antibody (ANCA) and antiendothelial cell antibody (AECA), are known to be associated with autoimmune vasculitis (Beliza & Tervaert, 1997; Bosch et al., 2006). It has been reported elsewhere that AECA is found in patients with Kawasaki disease (Kane et al., 1994). Recently, Karasawa et al. have reported that autoantibodies to peroxiredoxin II are found predominantly in the sera of patients with autoimmune diseases who have systemic vasculitis (Karasawa et al., 2003; Ozaki 2004). A homolog of peroxiredoxin II is also conserved in many microorganisms, and this homolog is also expressed in BCG at a higher level than in Mycobacterium tuberculosis (Springer et al., 2001). Thus, cross-reactive immune responses, which might be triggered by a microorganism that possesses cross-reactive antigens, could be associated with the development of Kawasaki’s disease.

As described above, many reports indicate that Kawasaki disease is associated with infection. Therefore, we hypothesized that infectious agents that have antigenic proteins homologous to those of BCG and the vascular wall might induce an immunopathologic reaction to both the lesion at the BCG vaccination site and the vascular wall.

In this study, we examined whether coronary arteritis develops from the combination of a primary immunization with BCG and a secondary immunization with a ubiquitous microorganism, Mycobacterium intracellulare (cMI), which should share cross-reactive antigens. In this model, we demonstrate coronary arteritis with cytokinemia, similar to that found in Kawasaki disease. Furthermore, we show that administration of an antibody to a component of the vascular endothelium, peroxiredoxin II, after BCG priming also induces coronary arteritis, indicating that an immunopathologic reaction causes the coronary arteritis and requires prior BCG immunization.

Materials and methods

Bacteria

The BCG (Tokyo strain) was purchased from Nihon BCG Inc. (Tokyo, Japan). Mycobacterium intracellulare (MI, JCM number 6384) was obtained from the Japanese Collection of Microorganisms (RIKEN BioResource Center, Saitama, Japan).

Antibody

We purchased antiperoxiredoxin II polyclonal antibody from Lab Frontier (Seoul, Korea.). This antibody was obtained from rabbits immunized with recombinant human peroxiredoxin II purified from Escherichia coli. The antibody also recognizes the mouse and rat homologs.

Preparation of crude M. intracellulare lysate

We followed the procedure of Lehman et al. to obtain crude lysate for injection (Lehman et al., 1983). MI was cultured in Middlebrook 7H9 broth (Difco, Detroit, MI) for 24 h at 37°C, and then harvested by centrifugation (1000 g, 10 min). After being washed three times with phosphate-buffered saline (PBS) (pH 7.4, 1000 g, 10 min), the bacteria were lysed by overnight incubation at room temperature with a 10-fold volume of 4% sodium dodecyl sulfate (SDS) (EM Science, Gibbstown, NJ), and then washed 10 times with PBS to remove the supernatant and SDS. The bacterial lysate was incubated sequentially with 250 μg mL⁻¹ RNAse, DNase I, and trypsin (Sigma, St Louis, MO) for 4 h. After centrifugation, the pellets were washed four times with PBS.

Crude lysate was then obtained by sonication of the pellet (5 g wet weight in 20 mL of PBS) on ice for 2 h (5-s pulse, then a 1-s pause, at a fixed frequency of 20 kHz). The sonicated preparation was then centrifuged for 1 h at 40 000 g, at 4°C, and the supernatant was harvested for injection. The wet volume of the ultracentrifugation pellet was then adjusted to a final concentration of 1 mg mL⁻¹ in PBS.

Animal experiments

Female C57BL/6J mice 3 weeks of age were purchased from the Sankyo Laboratory (Tokyo, Japan). BCG (0.4 mg in 0.05 mL of saline) or saline (as a control) was inoculated intradermally into the left flank. After observation of a nodular formation at the inoculation site, 0.1 mL of the purified protein derivative (PPD) was injected intradermally for the skin test to ascertain immunization. A cutaneous reaction was observed 48 h after the injection. Four weeks after the BCG immunization, the mice were intradermally injected with either 0.5 mg of crude MI lysates (cMI) in 0.5 mL of PBS or with PBS as a control, in either one or four daily dose(s). In other experimental groups, the mice were administered rabbit antiserum to mice peroxiredoxin II, or PBS as a control, through the tail vein 4 weeks after the BCG immunization. Ten days after cMI injection or the administration of peroxiredoxin II antibody, sera were collected from the jugular vein. Specimens for histologic analysis were prepared as described previously (Nakamura et al., 2000). Briefly, after the animals had been sacrificed under anesthesia, the heart was excised and embedded in OCT compound (Sakura Finetechinal Co., Ltd, Tokyo, Japan), and then stored at −30°C. Serial sections 7 μm in width were made using a cryostat.

During experiments, the body weight and rectal temperature of the mice were monitored at the time of BCG
inoculation and then every day upon and after the secondary immunization or antibody administration.

Histologic examination

The serial sections from base to apex of the heart were stained with hematoxylin and eosin to evaluate them for coronary arteritis. Eight sections from each animal were evaluated, and five eye-fields were observed for each section. The criteria for coronary arteritis were as follows: ‘mild’ was defined as infiltration of inflammatory cells surrounding the arterial walls; and ‘severe’ was defined as the infiltration of inflammatory cells within the layers of the arterial walls.

Cytokine analysis

The amounts of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-6, IL-10 and monocyte chemoattractant protein-1 (MCP-1) in animal sera were evaluated by the sandwich enzyme-linked immunosorbent assay (Mouse inflammation kit, BD Pharmingen, San Diego, CA).

Statistical analysis

To compare groups, the Wilcoxon test, χ² test and Fisher’s exact values were calculated using the JMP application (SAS Institute Inc., Cary, NC).

Results

Primary BCG immunization followed by secondary cMI immunization induced coronary arteritis

Three-week-old female mice were inoculated with BCG or saline. Four days after BCG immunization, nodular formations were observed in all the mice inoculated with BCG, but not in those inoculated with saline (Table 1). The PPD tests resulted in erythema formation with increased venular appearance in all the animals inoculated with BCG, but not in the saline-inoculated animals (Table 1). Four weeks after being immunized with BCG or saline, animals were intraperitoneally inoculated with cMI or PBS.

The histologic analysis was performed 4 weeks after the secondary inoculation. Mild coronary arteritis with mononuclear cell infiltration around the arterial wall was demonstrated in six of 120 sections (5%) from animals immunized with both BCG and cMI (B’T+I+, Fig. 1a, Table 2), but in only one of the 120 sections (0.8%) from animals immunized with BCG alone (B’T+, Table 2). Sections from animals immunized with cMI alone without BCG (B’T+) did not show coronary arteritis.

In another set of experimental groups (‘Four boosters’ in Tables), animals were inoculated four times daily with cMI or PBS following primary inoculation with BCG or saline.

Table 1. Nodal change and tuberculin reaction of injection site of mice (C57BL/6J) inoculated with BCG or saline

As a result, coronary arteritis, including one severe case (infiltration into the arterial wall), was demonstrated in significantly more heart sections (20/200, 10%) in animals that had both BCG and cMI immunization (B’T+I+) than in those in other groups (Fig. 1b; Table 2, P < 0.05 vs. 1% in B’T+, 0% in B’T+I, and 0% in B’T). When BCG or cMI immunizations were performed independently of each other, coronary arteritis was not observed or barely observed in mice treated with either BCG or cMI only. Thus, BCG immunization followed by multiple doses of cMI immunization significantly induced coronary arteritis. Body weight and body temperature were not affected by these immunizations (data not shown).

Development of coronary arteritis as a result of anti-peroxiredoxin II antibody administration requires BCG immunization

We examined whether the administration of antibodies to peroxiredoxin II induces coronary arteritis. Administration of rabbit antiserum against peroxiredoxin II alone resulted in coronary arteritis in only one of 200 sections examined (0.5%, Table 2). However, when mice were inoculated with BCG prior to antibody administration, coronary arteritis...
developed in 16 of 200 (8%) sections and at a significantly higher frequency than in the other control groups (P < 0.05, Fig. 1c, Table 2), similar to that observed with BCG and cMI immunization. Thus, antibody against peroxiredoxin II induced coronary arteritis, but only when administered after BCG immunization.

### Cytokine profiles in animals with vasculitis

Ten days after the cMI or PBS inoculation following BCG or saline inoculation, sera were obtained from the mice to determine cytokine levels. Levels of TNF-α, IFN-γ, and MCP-1, but not IL-10 or IL-6, were significantly higher in both the BCG-inoculated groups (B⁺I⁺ and B⁺I⁻) than in those mice treated with saline instead of BCG (Table 3, P < 0.05). None of these cytokines was elevated in animals inoculated with only cMI (without primary BCG inoculation; B⁻I⁻) compared with levels in control animals (Table 3, B⁻C⁻/C⁻).

Similarly, the administration of antibodies to peroxiredoxin II without prior BCG inoculation (B⁻P⁺) also did not result in elevated cytokine levels (Table 3).

The IFN-γ level was significantly higher in animals given antibody to peroxiredoxin II following BCG immunization than it was in animals immunized with BCG alone (Table 3, P < 0.05).

The average values of TNF-α, IFN-γ and MCP1 were higher in the B⁺I⁺ and B⁺P⁺ groups than in the B⁺I⁻ and B⁺P⁻ groups, although these differences were not statistically significant.

### Discussion

As flare and crust formation is observed in patients with Kawasaki disease, the immune response to BCG might be associated with the development of systemic vasculitis, a hallmark of this disease. As described above, autoantibodies
to peroxiredoxin II and the human homolog of mycobacterial HSP65 are found in patients with Kawasaki disease (Sireci et al., 2000; Karasawa et al., 2003; Yokota et al., 2006). Many reports also indicate that Kawasaki disease is associated with infection. This suggests that an immunopathologic reaction triggered by unknown microbial agents might be associated with the development of Kawasaki disease.

We hypothesized that a ubiquitous microorganism that shares a cross-reactive antigen with BCG might induce an immunopathologic reaction to both BCG and vascular antigens. Any microorganism could be a candidate, as long as it shares an antigenically related component with BCG and the endothelium. Under this assumption, we did not inoculate animals with BCG twice. Instead, we used an extract from an atypical mycobacterium as a booster immunogen to demonstrate this phenomenon experimentally. This hypothesis was also supported by the experiment involving the use of antibody to one of the endothelial components.

In this model, four booster doses resulted in an effect superior to that of one booster. As nonreplicating immunogens tend to be rapidly degraded by phagocytes in the absence of an adjuvant (Harlow & Lane, 1988), multiple immunizations might be required for more apparent booster immunity.

TNF-α, IFN-γ, and MCP-1, cytokines that are elevated in Kawasaki disease (Furukawa et al., 1988; Maury et al., 1988; Matsubara et al., 1990; Wong et al., 1997), were induced after BCG immunization. When the mice were inoculated with atypical mycobacterial antigen alone, coronary arteritis did not develop. This indicated that BCG immunization may induce cytokine production and provide the cytokine levels necessary for the development of coronary arteritis, and this idea was also supported by the experiment involving the use of antibody to one of the endothelial components.

Administration of antibody to peroxiredoxin II alone barely induced coronary arteritis. When animals were first primed with BCG, the administration of the antibody resulted in significant coronary arteritis, similar to that observed after multiple cMI inoculations following BCG immunization.

Seventy per cent of Kawasaki disease is observed in patients who are less than 3 years of age and presents shortly after BCG immunization. Several reports indicate that BCG may reside in the host for a few years after inoculation (Gasiorek-Chrzan, 2001). This clinical observation might support the importance of BCG as the first immunogen and as the immune modulator that provides the circumstances for the development of Kawasaki disease.

**Table 3. Serum levels of inflammatory cytokines**

|                | n | TNF-α       | IFN-γ       | MCP-1       | IL-10     | IL-6     |
|----------------|---|-------------|-------------|-------------|-----------|----------|
| One booster    |   |             |             |             |           |          |
| B⁺I⁺          | 3 | 15.9±3.8    | 4.9±0.8     | 31.5±2.4    | 2.8±4.8   | 2.9±1.9  |
| B⁺I⁻          | 3 | 14.7±2.8    | 4.2±5.1     | 43.3±9.3    | 0.0±0.0   | 2.3±0.4  |
| B⁻I⁺          | 3 | 9.8±1.4     | 3.1±3.0     | 29.1±4.4    | 0.0±0.0   | 1.8±1.7  |
| Four boosters  |   |             |             |             |           |          |
| B⁺I⁺          | 5 | 27.4±22.4   | 10.7±7.6    | 61.3±27.7   | 2.7±6.0   | 6.6±2.9  |
| B⁺I⁻          | 5 | 17.3±3.0    | 8.4±3.9     | 51.0±9.1    | 1.3±1.8   | 6.8±1.8  |
| B⁻I⁺          | 5 | 11.4±4.9    | 2.7±1.1     | 42.4±4.6    | 2.2±3.0   | 6.2±2.9  |
| B⁻I⁻          | 5 | 8.1±1.3     | 0.7±0.9     | 38.1±5.8    | 2.7±4.0   | 5.8±1.4  |
| Anti-PrxII     |   |             |             |             |           |          |
| B⁺P⁺          | 5 | 25.5±13.9   | 9.5±2.2     | 83.0±9.8    | 0.0±0.0   | 5.7±1.2  |
| B⁺P⁻          | 5 | 18.2±3.7    | 5.9±1.7     | 67.8±11.0   | 0.9±2.0   | 5.5±1.2  |
| B⁻P⁺          | 5 | 9.1±2.2     | 1.0±1.1     | 48.1±9.9    | 0.4±0.8   | 4.8±0.9  |
| B⁻P⁻          | 5 | 7.3±0.6     | 1.3±0.8     | 38.1±5.7    | 0.0±0.0   | 3.9±1.4  |

Data are expressed as mean±SD (pg mL⁻¹).

One booster: an experimental primary inoculation followed by one cMI inoculation or its substitute.

Four boosters: an experimental primary inoculation followed by four cMI inoculations or their substitutes.

Anti-PrxII: an experimental primary inoculation followed by administration of antiperoxiredoxin II antibody or its substitute.

B⁺, BCG administered; B⁻, saline administered as a substitute for BCG; I⁺, cMI administered; I⁻, saline administered as a substitute for cMI; P⁺, antiperoxiredoxin II antibody administered; P⁻, saline administered as a substitute for antiperoxiredoxin II antibody.

*Significant difference from control (B⁺P⁺).

Significant difference from control and B⁻I⁻ group.
although, except for the IFN-γ level in the B⁺P⁺ group compared with the B⁺P⁻ groups, these differences were not statistically significant. Immunopathologic inflammation may further enhance cytokine production.

Peroxiredoxin II has antioxidant activity is distributed in many types of cell, including those of the endothelium, and is located in both the cytoplasm and the cell membrane (Rhee et al., 2001; Lee et al., 2003; Choi et al., 2005). Peroxiredoxin II may be a regulator of platelet-derived growth factor and remodeling (Choi et al., 2005). In patients with autoimmune diseases who have systemic vasculitis, antibodies to peroxiredoxin II have been demonstrated (Karasawa et al., 2003). Thus, peroxiredoxin II might be an important target of systemic vasculitis. This molecule is conserved in mammals and microorganisms, including mycobacteria (Hofmann et al., 2002). Antibodies that are cross-reactive to this molecule after a microbial infection might induce destruction of the endothelium and also inhibit its function during the healing process.

Another report indicated that antibodies against HSP may cause cross-linking of HSP molecules, resulting in the subsequent production of IL-8 and TNF-α (Yokota et al., 2006). Thus, autoantibodies against molecules that might hamper endothelial repair and stimulate the cytokine network might be associated with the development of Kawasaki disease. As these molecules (peroxiredoxin II and HSP) are conserved between many microorganisms and humans, microorganisms that possess these homologs might also induce similar immunopathogenic reactions.

In our study, none of the animals developed coronary aneurysm. Blood pressure in mice might be too low to allow development of the aneurysm. Flare and crust formation was not observed at the site of BCG inoculation after cMI immunization. As several studies have indicated that Kawasaki disease might be associated with genetic background (Takeuchi et al., 1997; Quasney et al., 2001; Nishimura et al., 2003; Ouchi et al., 2003; Fukazawa et al., 2004; Kariyazono et al., 2004), we may need to examine the host’s genetic background associated with Kawasaki disease in order to produce stronger reactions. This might also explain why none of the animals was febrile. These approaches, investigations into infectious agents/immunogens and host factors, might be critical for clarifying the etiology of Kawasaki disease in detail.

In conclusion, BCG inoculation followed by booster immunizations with an atypical mycobacterium antigen resulted in coronary arteritis in mice. This model may lead to a better understanding of Kawasaki disease.

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