Acta Med. Nagasaki 60: 13－19

Modest Expansion of Vß2$^+$CD4$^+$ T Cells and No Expansion of Vß7$^+$CD4$^+$ T Cells in a Subgroup of Kawasaki Disease Patients with Erythematic BCG Inoculation Site Lesions

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Background: The similarities between Kawasaki disease (KD) and superantigen (SA) diseases indicate that a microbial SA might cause KD. Viral diseases can trigger an endogenous SA.

Methods: We evaluated expression of Vß2 (responding to staphylococcal TSST-1) and Vß7 (responding to the endogenous SA induced by type-1 interferon or Epstein-Barr virus infection) on T cells from 70 KD patients along with the following control subjects: 18 non-vasculitic patients (NVs), 7 patients with anaphylactoid purpura (AP), and two with neonatal TSS-like exanthematous disease (NTED), a typical SA disease. We examined the correlation of clinical features of KD with Vß2$^+$ or Vß7$^+$CD4$^+$T cell populations.

Results: The Vß2$^+$CD4$^+$T cell rates were comparable between KD patients (9.9±2.9%) and NVs (9.0±1.8%), but were lower in AP patients (6.6±1.8%). However, the Vß2$^+$CD4$^+$T cell rate was significantly higher in KD patients with erythematic BCG inoculation site lesions (10.8±3.2%) than in those without (8.8±2.1%) and NVs (9.0±1.8%), but much lower than in NTED patients (25.2%, 16.9%). Multivariate linear regression analysis with elevation of Vß2 expression as a dependent variable revealed significant correlations with BCG. In contrast, Vß7$^+$CD4$^+$T cell rates were not significantly different between KD patients and other study subjects.

Conclusion: While we were unable to find evidence supporting the involvement of the endogenous SA in the pathogenesis of KD in this study, modest expansion of the Vß2$^+$CD4$^+$T cell population in a subgroup of KD with erythematic BCG inoculation site lesions implies the involvement of a microbial agent(s) different from TSST-1 as well as immunopathological heterogeneity of KD. (249 words)

ACTA MEDICA NAGASAKIENSIA 60: 13－19, 2015

Key words: Kawasaki disease, superantigen, Vß2, Vß7, toxic shock syndrome toxin-1, BCG

Introduction

Kawasaki disease (KD) is the most common acquired heart disease of childhood; however, its etiology remains unknown despite extensive investigation for decades.[1] KD shares many clinical features with superantigen (SA) diseases, such as toxic shock syndrome (TSS) and scarlet fever.[2] A number of bacterial pathogens, including, *Staphylococcus aureus,*[2,3] *Streptococcus pyogenes,*[3] and *Yersinia pseudotuberculosis*[4] cause KD-like illnesses and encode bacterial SAs (exotoxins).[5] In addition, *Epstein-Barr virus* (EBV) and interferon-alpha induce an endogenous SA en-

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Received October 1, 2014; Accepted February 9, 2015

coded by human endogenous retrovirus (HERV)-K18,[6] which stimulates Vβ7+ CD4+ T cells. EBV is another example known to cause KD-like illness,[7,8] and viral infections sometimes precede the onset of KD.[9] SAs stimulate large populations of T cells expressing particular variable T cell receptor β chain gene segments[10]. Interestingly, Abe et al. reported increased CD4+ T cells expressing particular T-cell receptors (TCR), Vβ2 (responding to TSST-1), and Vβ8 in KD.[11,12] Although their study indicated that KD is an SA disease caused by TSST-1 or related SAs,[13] no particular microbial exotoxin has been consistently associated with KD in subsequent studies. It is worthy of notice that 18 strains of gram-positive cocci found in the gut of KD patients have been shown to induce the expansion of Vβ2+ T cells *in vitro*.[14]

One of the most interesting clinical features of KD is Bacille Calmette-Guerin vaccine (BCG) inoculation site lesions.[15] BCG is included in the national immunization program in Japan and usually given to infants aged 3 to 6 months. Erythema and crust formation at the BCG inoculation site is not just a useful diagnostic tool for KD but also has implications for the pathogenesis of KD. Many researchers suspect a mycobacterial pathogen closely related to BCG as an etiological agent of KD.[16,17]

Other clinical features of interest include cervical lymphadenopathy and liver dysfunction.[18,19] The former is the least common among the major criteria of KD, but is often observed in EBV or other infections.[18] The latter is considered to be one of the factors predicting unresponsiveness to intravenous immunoglobulin (IVIG).[20] Although IVIG is effective against the development of coronary arterial lesions, it remains obscure how it works and is difficult to predict which patients may not respond to the therapy.

We hypothesized that KD is a syndrome caused by several different SAs, and that such etiological differences result in differences in clinical manifestations or responses to treatment. In this study we focused on two SAs: TSST-1, a representable microbial SA and an endogenous SA induced by type-1 interferon or EBV infection.

Materials and Methods

Patients

We conducted a prospective study between January 2007 and March 2009. KD was diagnosed according to a guideline by the Kawasaki Disease Study Group (Ministry of Health, Labour and Welfare).[21] Seventy KD patients (38 males, 32 females) were recruited at Nagasaki University Hospital and Nagasaki Harbor Medical Center City Hospital during the study period. Patients who had already received initial treatment at other hospitals were excluded. The patients' mean age was 22.9±15.8 months, ranging from 2 to 76 months. We showed KD patients' profile in Table 1.

Table 1. Kawasaki disease patients' profile

Gender (male : female)	38 : 32
Mean age (months)	22.9 ± 15.8
Age range	2 - 76
Treatment	
no IVIG (only aspirin)	11
scheduled IVIG only	46
additional IVIG	13
Cardiac complications：	
cardiac effusion	3
transient coronary dilatation	10
coronary aneurysm (regression)	1

The Harada scoring system was used to evaluate the risk of coronary artery aneurysms.[22] The standard therapy for KD patients consists of high-dose IVIG and oral administration of aspirin (30 mg/kg) during the febrile period. During 2007–2008, a daily infusion of 400 mg of IVIG per kg for five consecutive days was given. During 2008–2009, 2 g of IVIG per kg over 24 hours was given.[23] Patients with refractory KD required an additional dose of IVIG (1–2 g/kg), immunosuppressive drugs, or plasma exchange.[24] Only 11 patients were treated with aspirin, 46 received scheduled IVIG therapy, and 13 patients with refractory KD received additional IVIG therapy. Cardiac complications included three patients with pericardial effusion, 10 with transient coronary dilatation (coronary diameter >3 mm) [25] or a hyperechoic lesion of the vascular wall, and one with mild coronary aneurysm. Patients treated with aspirin only had no cardiac complications.

We recruited the following control subjects: 18 children (mean age 66.5±62.9 months, range 7 to 192 months) who were not suspected of any SA disease or vasculitic pathogenesis (non-vasculitic patients, NVs), 7 patients (mean age 73.4±32.5 months, range 20 to 114 months) with anaphylactoid purpura (AP), another systemic vasculitic syndrome of childhood, and two patients with neonatal TSS-like exanthematous disease (NTED), a typical SA disease[26] The majority of NVs were patients who underwent cardiac catheterization for congenital heart disease.

Definitions of clinical manifestations

Three selected clinical features, BCG inoculation site lesion, cervical lymphadenopathy, and liver dysfunction, were graded or defined. The BCG inoculation site lesion was defined as positive when erythema and crust formation was observed at the inoculation site.[15] Cervical lymphadenopathy was defined as the presence of lymph node(s) with a diameter of more than about 1.5 cm or easily palpable. Liver dysfunction was defined as serum alanine aminotransferase (ALT) over 100 IU/ml.[20]

Blood sample collection and flow cytometry

Peripheral blood was collected into a tube containing EDTA on 4–6 days from onset (pretreatment phase), 8–9 days from onset (post-treatment phase-1) and 10–14 days from onset (post-treatment phase-2).

Anti-CD4 R-phycoerythrin (PE) (CALTAG ™ Laboratories, Buckingham, UK) and anti-Vβ2 or anti-Vβ7 fluorescein isothiocyanate (FITC) (IMMUNOTECH, Marseille, France) were added to each 100 μl of the fresh whole blood. After a 15-minute incubation on ice, lysing solution was added to lyse red blood cells. The remaining cells were washed with phosphate buffer saline (PBS) several times and resuspended in 1 ml of PBS. The FACScan™ system (Becton Dickinson, New Jersey, United States) was used to measure the proportion of Vβ2+ cells or Vβ7+ cells in CD4+ T cells.

Statistical Analysis

The statistical analysis was performed by *t*-test, paired *t*-test and multiple linear regression analysis with the stepwise method by using the JMP pro 10 software (SAS institute Inc., North Carolina, USA).

We analyzed differences in Vβ2+ or Vβ7+CD4+ T cells between KD patients and control subjects by *t*-tests, and time series in KD by paired *t*-tests. Next, we performed a univariate analysis to compare gender, BCG inoculation site lesion, cervical lymphadenopathy, liver dysfunction, therapy, cardiac complication, and Harada score. We analyzed these factors by a multiple linear regression analysis with the stepwise method. The data were expressed as means ± standard deviation.

Ethics

This study was approved by the Ethical committee of Nagasaki University School of Medicine.

Results

Comparison of Vβ2 and Vβ7 expression in CD4+ T cells between KD patients and control subjects

In the pretreatment phase, the rates of Vβ2+CD4+ T cells were not different between KD patients (9.9±2.9%)[27] and NVs (9.0±1.8%), slightly lower in AP patients (6.6±1.8%), and much higher in NTED patients (25.2, 16.9) (Table 2). The rates of Vβ7+CD4+ T cells were not significantly different between KD patients and control groups.

The rates of Vβ2+CD4+T cells in KD patients did not significantly change throughout the pretreatment (4-5 days after onset), post-treatment-1 (8–9 days after onset) and post-treatment-2 (10–14 days after onset) phases. On the other hand, the rates of Vβ7+CD4+ T cells in KD patients were slightly higher in the pretreatment phase (2.8±1.1) than in the post-treatment-2 phase (2.5±0.6) (p <0.05).

Comparison of Vβ2 and Vβ7 expression in CD4+ T cells between subgroups of KD patients

We subdivided KD patients according to selected clinical features or therapeutic responses, and reanalyzed Vβ2 or

Table 2. Vβ2 and Vβ7 expression levels in CD4+ T cells from children in various health conditions

	N	Vβ2 (%)		Vβ7 (%)
KD (pretreatment)	70	***9.9 ± 2.9***	a)	2.9 ± 1.3
NVs	18	***9.0 ± 1.8***	b) a)	3.0 ± 1.5
Allergic purpura	7	***6.6 ± 1.8***	b)	2.2 ± 0.6
NTED	2	25.2 / 16.9		3.6 / 1.8

NVs; Children who were not suspected of any SA disease or vasculitic pathogenesis Multiple comparison between the top three groups

In Vβ2, a) KD vs Anaphylactoid purpura, $p< 0.01$, b) NVs vs Anaphylactoid purpura, $p< 0.05$

Table 3. Vβ2 and Vβ7 expression levels in $CD4^+$ T cells from subpopulations of KD patients

Subpopulation	Number	Age (months)	Vβ2 (%)	Vβ7 (%)
Gender				
Male	38	21.8 ± 16.7	10.0 ± 3.0	2.6 ± 0.8
Female	32	24.3 ± 15.1	9.8 ± 2.8	3.1 ± 1.4
BCG inoculation site [1]				
present	39	***17.2 ± 10.2***	***10.8 ± 3.2***	2.7 ± 0.9
absent	29	***32.0 ± 17.7***	***8.8 ± 2.1***	3.0 ± 1.3
Cervical lymphadenopathy[2]				
present	12	***36.4 ± 15.8***	9.5 ± 3.4	3.0 ± 1.3
absent	58	***20.1 ± 14.4***	10.0 ± 2.8	2.7 ± 1.1
Liver dysfunction				
present	21	29.2 ± 16.8	9.6 ± 2.6	3.1 ± 1.6
absent	49	20.2 ± 14.7	10.1 ± 3.0	2.7 ± 0.9
Therapy				
only ASA	11	34.2 ± 21.1	9.9 ± 2.6	3.2 ± 2.0
ASA + IVIG	46	19.8 ± 12.7	9.9 ± 3.2	2.6 ± 0.7
repeated IVIG	13	24.5 ± 17.4	10.0 ± 2.2	3.2 ± 1.2
Cardiac complication				
present	14	20.9 ± 15.4	10.5 ± 3.4	2.8 ± 1.2
absent	56	23.4 ± 16.0	9.8 ± 2.8	2.8 ± 1.1
Harada Score				
Positive (≥ 4)	38	20.5 ± 14.4	9.7 ± 3.2	3.0 ± 1.3
Negative (≤ 3)	32	25.8 ± 17.1	10.2 ± 2.5	2.6 ± 0.9

[1] Two patients who had not been immunized with BCG were excluded. Vβ2 with BCG inoculation site present vs absent, $p < 0.01$. Age with BCG inoculation site present vs absent, $p < 0.001$

[2] Age with cervical lymphadenopathy present vs absent, $p < 0.001$

Table 4. Estimated parameters and multiple linear regression analysis contributing Vβ2 expression in $CD4^+$ T cells from KD patients

Parameters	Regression Coefficient	Standard Error	p value
BCG lesion (positive vs negative)	1.0	0.34	< 0.01

Vβ7 expression levels in $CD4^+$ T cells (Table 3). Among selected clinical features, only the presence of BCG inoculation site lesion was associated with increased Vβ2 expression in the pretreatment phase. When 39 KD patients with and 29 without BCG inoculation site lesions were compared, the rates of $V\beta2^+CD4^+$ T cells in the pretreatment phase were significantly higher in the former (10.8±3.2%) than in the latter (8.8±2.1%). ($p < 0.01$) And the rates of $V\beta2^+CD4^+$ T cells in KD patients with BCG inoculation site lesion (10.8±3.2) were also higher than in NVs (9.0±1.8%). ($p < 0.05$) KD patients with BCG inoculation site lesions (17.2±10.2 months) were younger than those without them

(32.0±17.7 months), and those with cervical lymphadenopathy (36.4±15.8 months) were older than those without it (20.1±14.4 months).

Next, we examined expression levels of Vβ2 and Vβ7 in association with responsiveness to treatment or cardiac complications (Table 3). There was no significant difference in their expression levels.

Stepwise multivariate linear regression analysis with elevation of Vβ2 expression as a dependent variable revealed significant correlations with positive or negative BCG inoculation site lesion (Table 4). There was no difference in Vβ7 expression in CD4+ T cells between the two subgroups. On the other hand, neither cervical lymphadenopathy nor liver dysfunction was associated with increases in Vβ2 or Vβ7 expression levels.

Disscussion

Many investigators have advocated KD as a syndrome that involves various etiologies and genetic factors.[28,29,30] Natividad et al. have recently hypothesized involvement of both superantigen and genetic factors in KD.[31] The hypothesis that KD is an SA disease has attracted many researchers, and a number of studies report expansion of CD4+ T cells expressing a particular T-cell receptor(s), implying activation by a SA(s). In particular, expansion of Vβ2+ cells by stimulation with TSST-1 has been shown by several studies, and is strengthened by the following results: (1) isolation of TSST-1 secreting *S*. aureus from the throat, rectum, axillar, and groin of KD patients,[2] and (2) apparent protection of young infants from KD by maternally transferred neutralizing antibody against TSST-1.[32]

Our data showed expansion of Vβ2+CD4+ T cell population at pretreatment in KD patients with erythematous BCG inoculation site lesion, but not those with cervical lymphadenopathy or liver dysfunction. This is the first report to investigate the association of the T cell repertoires with the respective clinical characteristics of KD.

It is not clear why expansion of the Vβ2+CD4+ T cell population was modest as compared to NTED, a typical SA disease[26] and why positive BCG inoculation site lesion was associated with expansion of the Vβ2+CD4+ T cell population. It is apparent that not only TSST-1 producing *Staphylococci* but also other microorganisms are capable of inducing Vβ2+CD4+ T cell population. Yamashiro and colleagues have recently identified Vβ2+CD4+T cells infiltrating in the jejunal mucosa and superantigenic exotoxins produced by bacteria colonizing the small intestinal mucosa of KD patients.[14,33] There must be several unknown microorganisms producing an agent(s) inducing Vβ2+CD4+T cells, although much less efficiently than TSST-1. Positive BCG inoculation site lesion has been associated with human heat shock protein (HSP) 63.[16] It has also been reported that T cells obtained from KD patients recognized an epitope from HSP65 and cross-reacted with the corresponding peptide sequence of human HSP63.[34] Since HSP65 was reported to be a marker of coronary heart disease,[35,36] it may also involve cardiac complications of KD. We hypothesize that a subgroup of KD is involved in a microbial agent(s) that express an SA to stimulate Vβ2+CD4+T cells and have a homologue of HSP63.

Although we hypothesize that expansion of Vβ2+CD4+T cell population is associated with acute-phase immunopathogenesis of this specific subgroup of KD, the rates of the Vβ2+CD4+T cells did not significantly change throughout the clinical course in this study. It is possible that observation period (up to 10-14 days from the onset) in this study was shorter than in other reports[11, 12] and might not be long enough to confirm settling down of these cell population.

In contrast, KD patients with severe clinical features, cardiac complications or high Harada scores did not exhibit expansion of the Vβ2+CD4+T cell population (Table 3). Thus, expansion of the Vβ2+CD4+T cell population is characteristic of only a subgroup of KD with positive BCG site lesion and not of all KD patients, suggesting that KD is immunopathologically heterogeneous and that risk factors for development of KD and those for its progression to more severe forms with cardiac complications may be different.

Expansion of the Vβ7+CD4+T cell population was not observed in KD patients. These cells population may be induced by EBV or interferon-alpha through expression of endogenous SA, and their relation to KD were unknown. Interestingly, a previous study showed expansion of Vβ7+CD8+T cells, but not of Vβ2+CD4+ T cells in KD patients[37]. Further studies may be needed to clarify the involvement of endogenous SA or its inducers such as EBV in the immunopathogenesis of KD.

Study limitation

The standard IVIG therapy for KD was changed in 2008; previously, 400 mg of IVIG per kg was given for 5 consecutive days, but a single IVIG infusion of 2 g per kg was given thereafter. Since the single IVIG infusion therapy was more effective to decrease cardiac complications, incidence of cardiac complications decreased during the latter half of the study period.

Conclusion

Our data could not find any evidence supporting the involvement of endogenous SA in the pathogenesis of KD. Modest expansion of the Vβ2+CD4+T cell population in a subpopulation of KD implies involvement of a microbial agent(s) different from TSST-1. Such an agent(s) appears to have less potent superantigenic activity and may behave like human HSP63. Apparent immunopathological heterogeneity observed among KD patients has implications for a better understanding of this clinically important disease. The pathological conditions of anaphylactoid purpura appear to be different form KD.

Acknowledgements

We thanked the pediatric stuff of Nagasaki Harbor Medical Center City Hospital for recruit patients, Dr. Norio Tominaga for his valuable comments and Ms. Taeko Akase for her technical assistance.

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