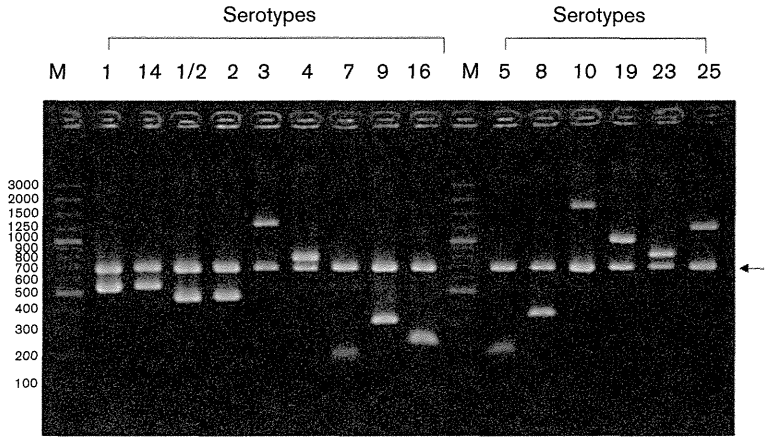

Fig. 1. PCR product bands produced by *S. suis* serotypes 1, 14, 1/2, 2, 3, 4, 7, 9 and 16 using the multiplex PCR primer set 1, and by *S. suis* serotypes 5, 8, 10, 19, 23 and 25 using primer set 2. The arrow indicates the species-specific *gdh* gene band (695 bp). M, Molecular marker (bp).

serotypes (serotypes 1/2, 1, 2, 3, 4, 7, 9, 14 and 16 in set 1 of the multiplex PCR, and serotypes 5, 8, 10, 19, 23 and 25 in set 2 of the multiplex PCR) were positive and they produced two bands, the species-specific band for the *S. suis* 695 bp *gdh* gene as expected, and the serotype-specific band for the *cps* gene. By contrast, the 18 other serotypes produced only the species-specific band (data not shown). As expected, this PCR could not differentiate serotypes 1 and 14, or serotypes 2 and 1/2, because the *cps* loci of these serotypes share high genetic similarity, although they are not identical (Kerdsin *et al.*, 2009; Smith *et al.*, 1999a; Wang *et al.*, 2011b).

Of 179 human isolates of *S. suis*, this assay confirmed 165 serotype 2 isolates as serotype 2 or 1/2, 12 serotype 14 isolates as serotype 1 or 14, and one serotype 5 isolate as serotype 5, but, as expected, it could not identify the single serotype 24 isolate (Table 2).

As shown in Table 3, the multiplex PCR assay also confirmed various serotypes among the 109 pig isolates. This assay could not identify the serotypes of 39 isolates, including serotypes 6 (n=4), 12 (n=1), 17 (n=1) and 24 (n=3), as well as serotypes untypable with antisera (n=30).

The specificity of the multiplex PCR for *S. suis* serotypes was tested using other *Streptococcus* species reference strains as described in Methods. No cross-reactivity was detected with any of these streptococcal species.

In this study, our multiplex PCR distinguished 15 serotypes of *S. suis* isolates from humans and pigs. This single assay correctly identified *S. suis* at the species level and differentiated its serotypes using the same system, so this multiplex PCR has advantages over previously reported PCR systems that detect only a limited range of serotypes (Kerdsin *et al.*, 2009; Marois *et al.*, 2004; Okwumabua *et al.*, 2003; Smith *et al.*, 1999a, b; Silva *et al.*, 2006; Wang *et al.*, 2011a; Wisselink *et al.*, 2002). Our multiplex PCR could not differentiate serotype 2 from serotype 1/2, or serotype 14 from serotype 1, so antisera are required to distinguish these serotypes. However, this multiplex PCR can reduce the cost and labour required to type the other 11 serotypes without using antisera. Our method could detect serotypes 2, 5 and

Table 2. Serotyping of 179 *S. suis* isolates from humans using multiplex PCR and antisera

Serotype using antiserum	*n*	Serotype using multiplex PCR										
		1	2	1/2	3	4	5	7	9	14	16	19
2	165		165	165								
5	1						1					
14	12	12								12		
24	1											

Table 3. Serotyping of 109 *S. suis* isolates from pig tonsils using multiplex PCR and antisera

Serotype using antiserum	*n*	Serotype using multiplex PCR										
		1	2	1/2	3	4	5	7	9	14	16	19
1	1	1							1			
2	9		9	9								
1/2	1		1	1								
3	10				10							
4	7					7						
5	2						2					
7	8							8				
9	8								8			
14	7	7								7		
16	15										15	
19	2											2
Other*	39											

*Serotypes 6 (n=4), 12 (n=1), 17 (n=1) and 24 (n=3), and untypable serotypes (n=30) determined with antisera were included.

14 in human isolates, and serotypes 3, 4, 5, 8, 9 and 16, which are frequently isolated from diseased pigs (Gottschalk *et al.*, 2010; Kerdsin *et al.*, 2011b; Nghia *et al.*, 2008; Schultsz *et al.*, 2012). The *cps* loci of the remaining 18 serotypes should be determined to facilitate the future development of PCR systems that can identify these serotypes.

ACKNOWLEDGEMENTS

This work was supported by research grants from the Department of Medical Sciences, Ministry of Public Health of Thailand, the Ministry of Health, Labour and Welfare of Japan, and The Japan Initiative for Global Research Network on Infectious Diseases launched by the Ministry of Education, Science, and Culture, Japan.

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Vaccine 30 (2012) 3304–3310

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

A possible relationship of natural killer T cells with humoral immune response to 23-valent pneumococcal polysaccharide vaccine in clinical settings

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ARTICLE INFO

Article history:
Received 30 July 2011
Received in revised form 28 February 2012
Accepted 4 March 2012
Available online 15 March 2012

Keywords:
Pneumococcal polysaccharide vaccine
Antibody production
NKT cells

ABSTRACT

Pneumococcal polysaccharide vaccine (PPV), a type-2 thymus-independent antigen, induces the activation of B cells by directly triggering their antigen receptors. Although this type of antigen generally does not undergo class switching from IgM to IgG, PPV has been known to induce IgG2 in vaccinated subjects, which suggests the possible involvement of certain innate immune lymphocytes supporting the activation of B cells and their class switching. In the present study, we addressed the possibility that natural killer (NK) T cells are involved in Ab production caused by PPV. We measured serum levels of IgG against pneumococcal capsular polysaccharides and the numbers of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ double negative (DN) invariant NKT (iNKT) cells and $CD3^+CD56^+$ NKT cells in the peripheral blood before and after PPV injection. IgG was increased after PPV injection, peaking at 4 weeks after injection in serotypes 6B, 19F and 23F and at 3 months in serotype 14. Low responders, whose serum concentrations of IgG peaked at less than double their original levels, constituted 16%, 13%, 13% and 16% of vaccinated subjects with regard to serotypes 6B, 14, 19F and 23F, respectively. A significant positive correlation was detected between an increase in DN iNKT cells and the elevation of anti-serotype 14 IgG; in serotype 19F, DN iNKT cells were more markedly increased in responders than in low responders. These results suggest that DN iNKT cells may be involved in IgG production caused by vaccination against pneumococcal capsular polysaccharides.

1. Introduction

Streptococcus pneumoniae is a major bacterial agent which causes community-acquired pneumonia as well as other invasive diseases, such as bacteremia and meningitis, which arise as complications of pneumonia in 15–30% of cases [1]. The incidence rate of pneumococcal bacteremia is 18 to 30 per 100,000 in the general population, but can be as high as 56 to 83 per 100,000, especially in people aged 65 years or over in the USA [2–5]. In Japan, pneumonia is the fourth leading cause of death, and *S. pneumoniae* is a leading causative agent of pneumonia, being detected in 23% of community-acquired pneumonia cases [6]. This bacterium is also frequently detected as an etiologic agent in secondary pneumonia arising as a complication of the flu [7–9]. Morens and co-workers have demonstrated that the majority of deaths in the 1918–1919 influenza pandemic resulted directly from secondary bacterial pneumonia caused by common upper respiratory-tract bacteria, among which *S. pneumoniae* was most frequently detected in autopsy lung samples [10].

To prevent these pneumococcal diseases, 23-valent pneumococcal polysaccharide vaccine (PPV) is used for people aged 65 years or older and younger people with certain risk factors such as chronic cardiopulmonary diseases [11]. PPV is a type 2 thymus-independent (TI-2) antigen, which does not require helper T cells for the activation of B cells [12]. While thymus-dependent (TD) antigens activate B cells *via* engagement of CD40 by CD40L

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doi:10.1016/j.vaccine.2012.03.007

during cognate interaction with helper T cells, TI-2 antigens directly trigger surface immunoglobulin for the activation of B cells [13]. These different types of antigen produce distinct humoral immune responses: TD-antigens undergo class switching from IgM to IgG, which causes affinity maturation of Ab and induces memory B cell response; TI-2 antigens, on the other hand, do not [14]. Although PPV is a TI-2 antigen, Barrett and Ayoub [15] have found that it induces the restriction of production of IgG2 specific for pneumococcal polysaccharides. Snapper and co-workers [16] have reported that interferon (IFN)-γ contributes to Ab class switching to IgG3 in mice, which corresponds to IgG2 in humans, after PPV administration [15]. These findings suggest that a certain group of innate immune cells may be involved in the activation of B cells and Ab class switching caused by PPV.

Natural killer (NK) T cells, which express both αβ T cell antigen receptors and NK cell markers, have been identified as a novel lymphocyte population that acts in the innate stages of immune responses [17]. A major subset of NKT cells is the invariant NKT (iNKT) cells, which possess an extremely limited repertoire with antigen receptors consisting of Vα14-Jα18 in mice and Vα24-Jα18 in humans [18]. These cells recognize glycolipid antigens, such as α-galactosylceramide (α-GalCer), in the context of CD1d molecules on dendritic cells [19], which leads to the rapid production of IFN-γ and IL-4 [20,21]. iNKT cells are concentrated in the thymus, liver and bone marrow in mice [20,22] and occur at a rate of approximately 0.05% in human peripheral blood [23]. In our previous studies using a mouse model [24], iNKT cells were observed to play a critical role in neutrophilic inflammatory responses to and host defense against pneumococcal infection through production of IFN-γ. Interestingly, Kobrynski and co-workers have demonstrated that Ab production after PPV injection was completely abrogated in mice lacking iNKT cells [25]. These earlier observations raised the possibility that iNKT cells may contribute to Ab production and class switching caused by the administration of PPV.

In the present study, to address this possibility in a clinical setting, we analyzed the relationship between serum concentrations of Ab against pneumococcal capsular polysaccharides and the number of $CD4^+$, $CD8^+$ or $CD4^-CD8^-$ double negative (DN) iNKT cells in the peripheral blood of subjects who received PPV administration. We found that DN iNKT cell counts increased, and that this increase was positively correlated with the production of IgG against a certain serotype of *S. pneumoniae*.

2. Materials and methods

2.1. Subjects

Fifty-five outpatients with chronic respiratory diseases were vaccinated with 0.5 ml of PPV (Pneumovax®, Banyu Pharmaceutical Co., Tokyo, Japan), intramuscularly, at the Department of Respiratory Diseases, Katta General Hospital, Shiroishi-shi, Miyagi, Japan between July 2006 and August 2008 after giving informed consent. The PPV23 contained 25 μg each of 23 different types of pneumococcal polysaccharide antigen (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F). Serum samples were collected prior to vaccination and at 2 weeks, 4 weeks, 3 months, 6 months and 1 year after vaccination. The average age of these subjects was 74.4 years (range 61–88 years); 67.3% of subjects were male, 43.6% were smokers, and 10.9% were receiving glucocorticoid therapy. The most common chronic respiratory diseases among these patients were chronic obstructive pulmonary disease, bronchial asthma, bronchiectasis and old pulmonary tuberculosis (Table 1). This study was approved by the institutional ethics committees of Tohoku University, Sendai, Japan (#2005-233) and Katta General Hospital. We also paid the utmost attention to ensure that personal information was handled in compliance with our institutions' guidelines.

Table 1
Clinical characteristics of all subjects ($n = 55$).

	Number (%)
Males	37 (67.3)
Smoking	24 (43.6)
Alcohol abuse	1 (1.8)
Underlying diseases	
COPD	20 (36.4)
Bronchial asthma	13 (23.6)
Bronchiectasis	2 (3.6)
Old pulmonary tuberculosis	6 (10.9)
Chronic cardiovascular diseases	5 (9.1)
Immunosuppressive conditions	1 (1.8)
Chronic renal failure	1 (1.8)
Chronic liver diseases	1 (1.8)
Diabetes mellitus	8 (14.5)
Treatment with glucocorticoids	6 (10.9)
Home oxygen therapy	6 (10.9)

Mean age (yr) ± SD = 74.4 ± 6.6.

2.2. Measurement of anti-pneumococcal capsular polysaccharide Ab

Serotype-specific antibodies against 6B, 14, 19F and 23F (American Type Culture Collection, Manassas, VA, USA) were measured by means of a third-generation Enzyme-Linked Immunosorbent Assay (ELISA) as described previously [26] after absorption of non-specific antigens to cell wall polysaccharide (CWP: Statens Serum Institute, Copenhagen, Denmark) and serotype 22F (American Type Culture Collection). In brief, microtiter plates (MICROLON: Greiner Bio-One, Frickenhausen, Germany) were coated individually with 100 μl of a polysaccharide antigen: either 5 μg/ml of 6B, 2.5 μg/ml of 14, 5 μg/ml of 19F or 2.5 μg/ml of 23F, in PBS. After five hours of incubation at 37 °C, these plates were stored at 4 °C until use, which occurred within 6 months. Prior to testing, the sera from our patients and U.S. anti-pneumococcal reference serum [89-SF: kindly provided by Dr. Milan S. Blake (Food and Drug Administration, Silver Spring, MD, USA)] were also stored at −80 °C. Serum samples and 89-SF were diluted with an absorption buffer of 0.05% Tween-20 PBS to 1:50 and 1:100, respectively, and incubated at room temperature for 30 min. Next, serial two-fold dilution of these sera to 1:51200 were performed arbitrarily; the resulting solutions were added to the wells and incubated at 37 °C for 1 h. After the microtiter plates were washed, a detection antibody, consisting of AP-conjugated goat anti-human IgM or IgG (Southern Biotechnology Associates, Birmingham, AL, USA) diluted to 1:2000, was added to each well. *p*-nitro phenyl phosphate (Sigma–Aldrich, St. Louis, MO, USA) was dissolved with 1 mol/l of diethanolamin (Sigma–Aldrich) to a concentration of 1 mg/ml as a substrate solution. Then, after the plates were washed again, this substrate was added to the wells and incubated at room temperature. Sodium hydroxide was added at 3 M to stop the enzyme reaction, and the absorbance values were detected at 405 nm as well as at 600 nm for reference. The concentrations of IgM and IgG Abs were calculated on the basis of a reference standard based on the 89-SF absorbance and expressed as μg/ml.

2.3. Flow cytometric analysis of PBMCs

Peripheral blood mononuclear cells (PBMCs) were collected from patients before vaccination and at 2 weeks, 4 weeks, 3 months and 6 months after vaccination. After Fc receptors on the cell surface were blocked, PBMCs were stained with FITC-anti CD3 [Clone: UCHT1 (eBioscience, San Diego, CA, USA)] and PE-anti CD56 [Clone:

B159 (BD Biosciences, Franklin Lakes, NJ, USA)] mAbs and PE-α-galactosylceramide (α-GalCer)-conjugated CD1d tetramer. These cells were also stained with APC-anti-CD4 and -CD8 mAbs [Clones: RPA-T4 and RPA-T8 (eBioscience), respectively]. Isotype control IgG (eBioscience) for each Ab and PE-α-GalCer-unconjugated CD1d tetramer were used as references. Flow cytometric analysis was performed using a Cytomics FC500 cytometry system (Beckman Coulter, Fullerton, CA, USA). The number of NKT cells (/μl) was calculated as follows: white blood cell (WBC) counts (100/μl) × % of lymphocytes in WBC/100 × % of NKT cells in lymphocytes. The WBC counts and % of lymphocytes were measured in blood samples collected from the patients during routine examinations.

2.4. Statistical analysis

Ab concentrations in sera, fold increases after vaccination and number of NKT cells in peripheral blood are expressed as geometric means. The concentrations of serum Ab and degrees of change in NKT cell counts during the first 2 weeks after vaccination were compared between responders and low responders using the Mann–Whitney *U*-test. The concentrations of serum Ab between pre- and peak levels were compared using Wilcoxon *t*-test. The correlation between the degree of change from pre-vaccination to peak levels of anti-pneumococcal IgG and the degree of change in NKT cell counts during the first 2 weeks post-vaccination was tested using Spearman's correlation test. A p value less than 0.05 was considered significant.

3. Results

3.1. Serum levels of anti-pneumococcal Ab after vaccination

Initially, we measured the concentrations of IgM anti-pneumococcal Ab against serotypes 6B, 14, 19F and 23F in 15 subjects at various time intervals after pneumococcal vaccination. As shown in Fig. 1A, the pre-vaccination levels of IgM Ab were 0.91, 0.59, 1.04 and 0.26 μg/ml for serotypes 6B, 14, 19F and 23F, respectively, and these levels were not altered during the six months post-vaccination.

Next, we measured the concentrations of IgG anti-pneumococcal Ab against the same serotypes in 55 subjects. As shown in Fig. 1B, in contrast to IgM Ab, IgG Ab began to increase during the second week, reached its peak at the fourth week for serotypes 6B, 19F and 23F and at the third month for serotype 14, then decreased one year after vaccination. For all the serotypes, the peak values were significantly higher than the values measured before vaccination (1.60 *vs.* 4.53, 3.04 *vs.* 12.87, 2.98 *vs.* 7.73 and 1.69 *vs.* 6.32 μg/ml for serotypes 6B, 14, 19F and 23F, respectively). One year post-vaccination, IgG levels had decreased from the peak levels by 12.3%, 37.8%, 25.2% and 41.5% for serotypes 6B, 14, 19F and 23F, respectively.

3.2. Responders and low responders

The individuals who received PPV administration were divided into two groups based on their responsiveness, *i.e.* responders and low responders. Here, we defined responders as individuals whose peak IgG levels were more than twice their IgG levels before vaccination, and low responders as individuals whose serum IgG concentrations were less than 2 μg/ml before vaccination and whose peak IgG levels were less than twice their IgG levels before vaccination. By these definitions, 62%, 62%, 45%, and 65% of the 55 vaccinated individuals were responders, and 16%, 13%, 13%, and 16% were low responders, with regard to the serotypes 6B, 14, 19F and 23F, respectively. As shown in Table 2, for all serotypes, peak IgG levels were significantly higher than IgG levels measured before

Table 2
Serotype-specific antibody levels in responders and low responders.

Serotype	Time point	Geometric mean concentrations (μg/ml) (95% CI)			Geometric mean increase from pre-vaccination to peak concentration (*n*-fold) (range)		
		All subjects (n = 55)	Responders[a]	Low responders[b]	All subjects (n = 55)	Responders[a]	Low responders[b]
6B	Pre	1.60 (1.20–2.14)	1.08 (0.76–1.52)	1.33 (1.10–1.60)			
	Peak	4.53 (3.44–5.95)**	4.56 (3.04–6.84)**	2.02 (1.57–2.59)*#	2.83 (0.89–78.89)	4.24 (2.00–78.89)	1.52 (0.11–1.92)
14	Pre	3.04 (1.96–4.70)	2.20 (1.38–3.50)	0.74 (0.35–1.60)			
	Peak	12.87 (8.46–19.59)**	21.14 (13.88–32.19)**	1.11 (0.52–2.37)$	4.24 (0.68–120.18)	9.61 (2.11–120.18)	1.49 (0.86–1.93)
19F	Pre	2.98 (2.20–4.03)	2.04 (1.41–2.95)	1.06 (0.86–1.30)			
	Peak	7.73 (5.70–10.49)**	11.23 (7.55–16.72)**	1.51 (1.18–1.93)$	2.60 (0.81–49.48)	5.51 (2.03–49.48)	1.43 (1.09–1.90)
23F	Pre	1.69 (1.18–2.43)	1.43 (0.95–2.15)	0.61 (0.35–1.06)			
	Peak	6.32 (4.18–9.55)**	8.96 (5.44–14.76)**	0.83 (0.49–1.40)$	3.73 (0.97–60.62)	6.25 (2.03–60.62)	1.36 (0.97–1.67)

*p < 0.05, **p < 0.01, compared with pre-vaccination level; #p < 0.05, $$p$ < 0.01, compared with peak level in responders.
[a] Responders are 34, 33, 26 and 36 subjects for serotypes 6B, 14, 19F and 23F, respectively.
[b] Low responders are 9, 8, 10 and 9 for serotypes 6B, 14, 19F and 23F, respectively.

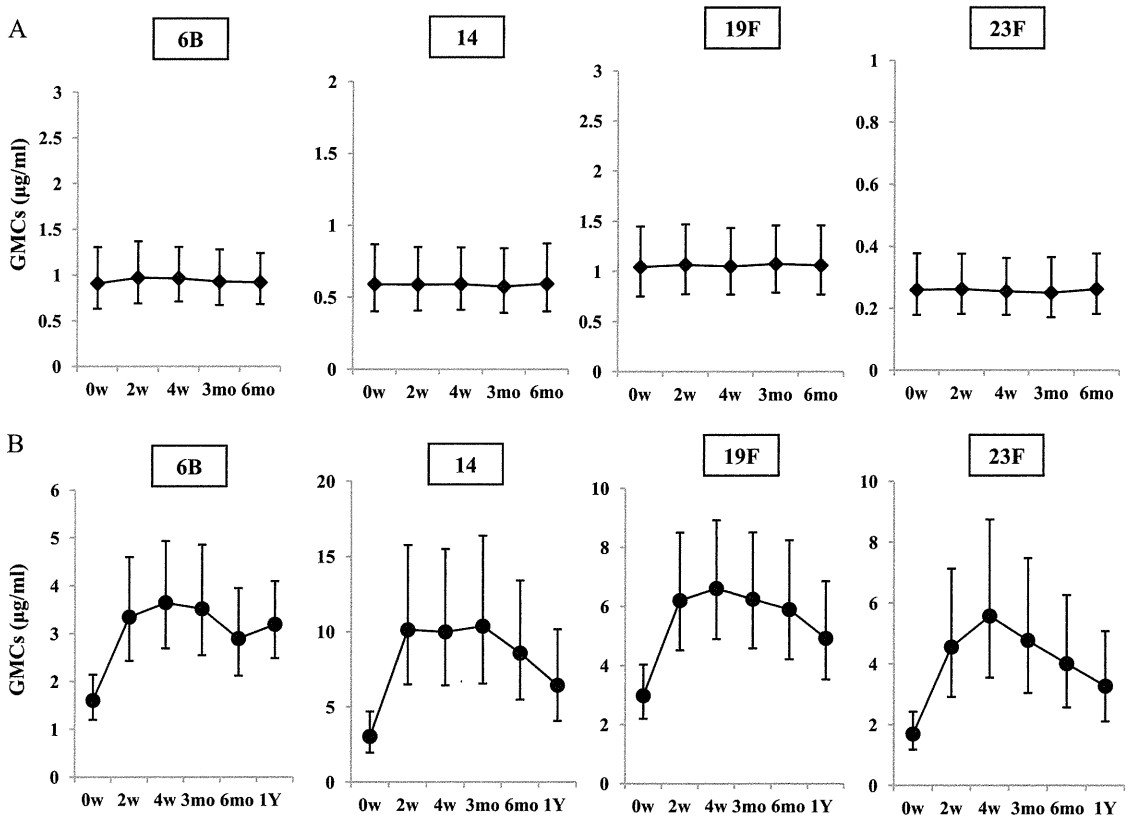

Fig. 1. Serum levels of anti-pneumococcal polysaccharide Abs after PPV injection. Concentrations of IgM (A: $n=15$) and IgG (B: $n=55$) Abs against each serotype of pneumococcal capsular polysaccharide in sera were measured at indicated time points after PPV administration. Data are shown as the geometric mean concentrations and 95% confidence intervals. GMCs, geometric mean concentrations; 0w, pre-vaccination; 2w, 2 weeks; 4w, 4 weeks; 3 mo, 3 months; 6 mo, 6 months; 1y, 1 year post-vaccination.

vaccination in the responder group, whereas no such significant increase in IgG concentration was observed in the low responder group, except for serotype 6B [pre-vaccination: 1.33 (95% CI was within 1.10–1.60) *vs.* peak: 2.02 (95% CI was within 1.57–2.59) ($n=9$, $p<0.05$)].

3.3. Alteration in the number of NKT cells in the peripheral blood after pneumococcal vaccination

We analyzed the number of NKT cells in the peripheral blood before vaccination and 2 weeks, 4 weeks, 3 months and 6 months after vaccination in 24 individuals, in whom the surface antigens on lymphocytes could be tested. NKT cells were identified as the lymphocytes positively stained with α-GalCer-CD1d tetramer or expressing both CD3 and CD56, and α-GalCer-CD1d tetramer$^+$ lymphocytes were further divided into $CD4^+CD8^-$ ($CD4^+$ iNKT), $CD4^-CD8^+$ ($CD8^+$ iNKT) and $CD4^-CD8^-$ (double negative: DN iNKT) subsets. As shown in Fig. 2, iNKT cell subsets did not show significant elevation in their cell count at any time point after vaccination, although increased iNKT cell counts were observed during the first two weeks in 11 or 12 individuals (data not shown).

3.4. NKT cell counts and serum levels of anti-pneumococcal Ab

In order to address the possible role of NKT cells in the humoral response to the pneumococcal vaccine, we analyzed the relationship between the degree of change in NKT cell counts during the first 2 weeks post-vaccination and the degree of change in serum anti-pneumococcal IgG levels from pre-vaccination to their peak. As shown in Fig. 3, a significant positive correlation was detected between increases in DN iNKT cells and increases in anti-serotype 14 IgG, and there were tendencies toward positive correlations between changes in $CD8^+$ iNKT and DN iNKT cell counts and increases in anti-serotype 19F IgG levels ($p=0.069$ and 0.067, respectively), and between changes in DN iNKT cell counts and increases in anti-serotype 6B and 23F IgG levels ($p=0.062$ and 0.082, respectively). By contrast, $CD4^+$ iNKT, $CD8^+$ iNKT and $CD3^+CD56^+$ cells showed neither a positive nor a negative correlation with changes in the serum levels of anti-pneumococcal IgG in all of the serotypes except for 19F in $CD8^+$ iNKT and $CD3^+CD56^+$ cells.

Finally, we compared changes in DN iNKT cell counts between responders and low responders, because these cells showed a tendency toward a positive correlation with Ab responses to PPV. As shown in Fig. 4, in serotype 19F, the increase in DN iNKT cells was significantly more marked in responders than in low responders. This tendency was also observed in serotypes 6B, 14 and 23F, although it was not statistically significant.

4. Discussion

In the present study, serum levels of anti-pneumococcal IgG increased after pneumococcal vaccination, peaking in the fourth week for serotypes 6B, 19F and 23F and in the third month for serotype 14; in 45–65% of vaccinated subjects, these levels increased more than two-fold. There were also low responders, however, producing smaller quantities of anti-pneumococcal Ab; these constituted 16%, 13%, 13% and 16% of our 55 subjects for serotypes 6B, 14, 19F and 23F, respectively. Of the low responders, 15 showed a low response to one of the four serotypes examined, nine showed a low response to two serotypes, and one showed a low response to three serotypes, indicating that 45% of our 55 subjects were low responders for at least one serotype. Although there is no standardized definite on of a low responder, our results appear

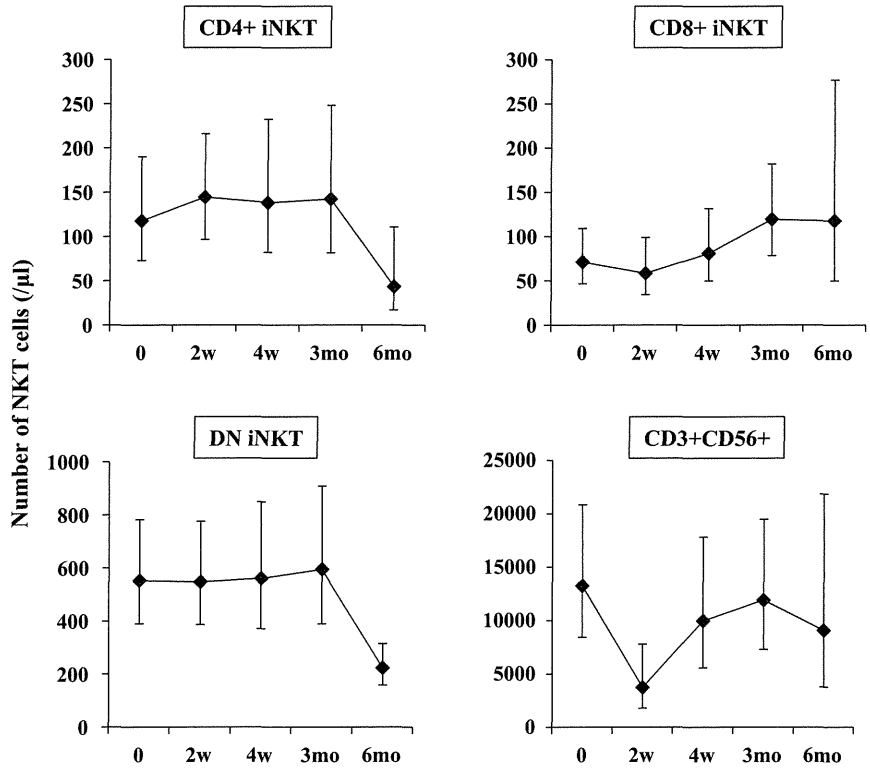

Fig. 2. NKT cells in the peripheral blood after PPV injection. Number of NKT cells in the peripheral blood was examined before PPV administration and 2 weeks, 4 weeks, 3 months and 6 months after PPV administration in 24 individuals. NKT cells were identified as the lymphocytes positively stained with α-GalCer-CD1d tetramer or expressing both CD3 and CD56, and α-GalCer-CD1d tetramer$^+$ lymphocytes were further divided into $CD4^+CD8^-$ ($CD4^+$ iNKT), $CD4^-CD8^+$ ($CD8^+$ iNKT) and $CD4^-CD8^-$ (double negative: DN iNKT) subsets. Data are shown as the geometric means and 95% confidence intervals in each NKT cell subset.

to be in accordance with those of previous investigations, which indicate that 16–31% of vaccinated subjects are low responders, whose anti-pneumococcal Ab levels increase less than two-fold for two among four to seven analyzed serotypes [27–29].

Previous studies have shown NKT cells to be involved in immune responses to TI-2 antigens, as a possible source of the secondary stimulatory signal for B cell activation [25] as well as in protection against pneumococcal infection [24]. These earlier observations suggest that NKT cells may play a certain role in the clinical effects of anti-pneumococcal vaccination. In agreement with this possibility, in the present study, a significant positive correlation was detected between changes in the number of DN iNKT cells, though not of $CD4^+$ iNKT cells, and increases in Ab levels against serotype 14 antigen. Moreover, the increase in DN iNKT cells was more marked in responders than in low responders, and this difference was statistically significant for serotype 19F. However, the positive correlation between DN iNKT cells and Ab levels and the difference in DN iNKT cells between responders and low responders were not significantly detected in other serotypes, although there were such tendencies with lower p values. The increase of study subjects would help in making these differences statistically significant. In addition, there is a possibility that the increase of DN iNKT cell number in responders may be due to overall immune activation of these individuals in response to vaccine, rather than selective effect on NKT cells. This may not apply to our case, because there was no tendency of difference between low responders and responders in other NKT cell subsets (data not shown).

$CD4^+$ and DN iNKT cells are major subsets in humans, both of which secrete large amounts of IFN-γ upon stimulation [21]. Yet these subsets differ in their secretion of such Th2 cytokines as IL-4, IL-5 and IL-13, and in their expression of chemokine receptors, integrins and NK receptors [21,30–32]. Galli and co-workers have demonstrated that iNKT cells promote immunoglobulin production by B cells, an activity that is more potent in $CD4^+$ iNKT cells than in DN iNKT cells [33]. The same group has also reported that activated human iNKT cells directly support the proliferation of and immunoglobulin production by naive and memory B cells. All these experiments were conducted *in vitro*, however, and frequent stimulation of iNKT cells during culture has been reported to cause a shift in their cytokine profile toward a Th2-dominant condition [34], raising the possibility that cultured NKT cells are not always equivalent to those in circulation *in vivo*. In the present clinical study of individuals receiving PPV, the relationship between iNKT cells and Ab production does not seem to be identical between $CD4^+$ and DN iNKT cells. Taken together, the data suggest that these subsets play distinct roles in Ab production by B cells after PPV administration. Further investigation is necessary to define the precise mechanism by which this occurs.

On the other hand, only a limited subset of NKT cells expressing NK cell markers, such as CD56 or CD161, is reactive to α-GalCer-loaded CD1d tetramer [31]. Therefore, $CD3^+CD56^+$ NKT cells, described as NKT-like cells, are distinguished from iNKT cells by certain characteristics, including the differences in their cytokine production profiles and their TCR αβ chains [18]. Our results suggest that iNKT cells rather than NKT-like cells may be particularly involved in IgG production caused by pneumococcal capsular polysaccharides, because no correlation was observed between $CD3^+CD56^+$ NKT cell count and Ab response.

To the best of our knowledge, the current study is the first report presenting clinical data that suggests a possible relationship between the activation of iNKT cells and Ab responses after PPV administration. The increase in DN iNKT cell count seems to be particularly correlated with serotype-specific IgG production, suggesting a higher contribution from DN iNKT cells than from other subsets. The population size in this study was limited, and the

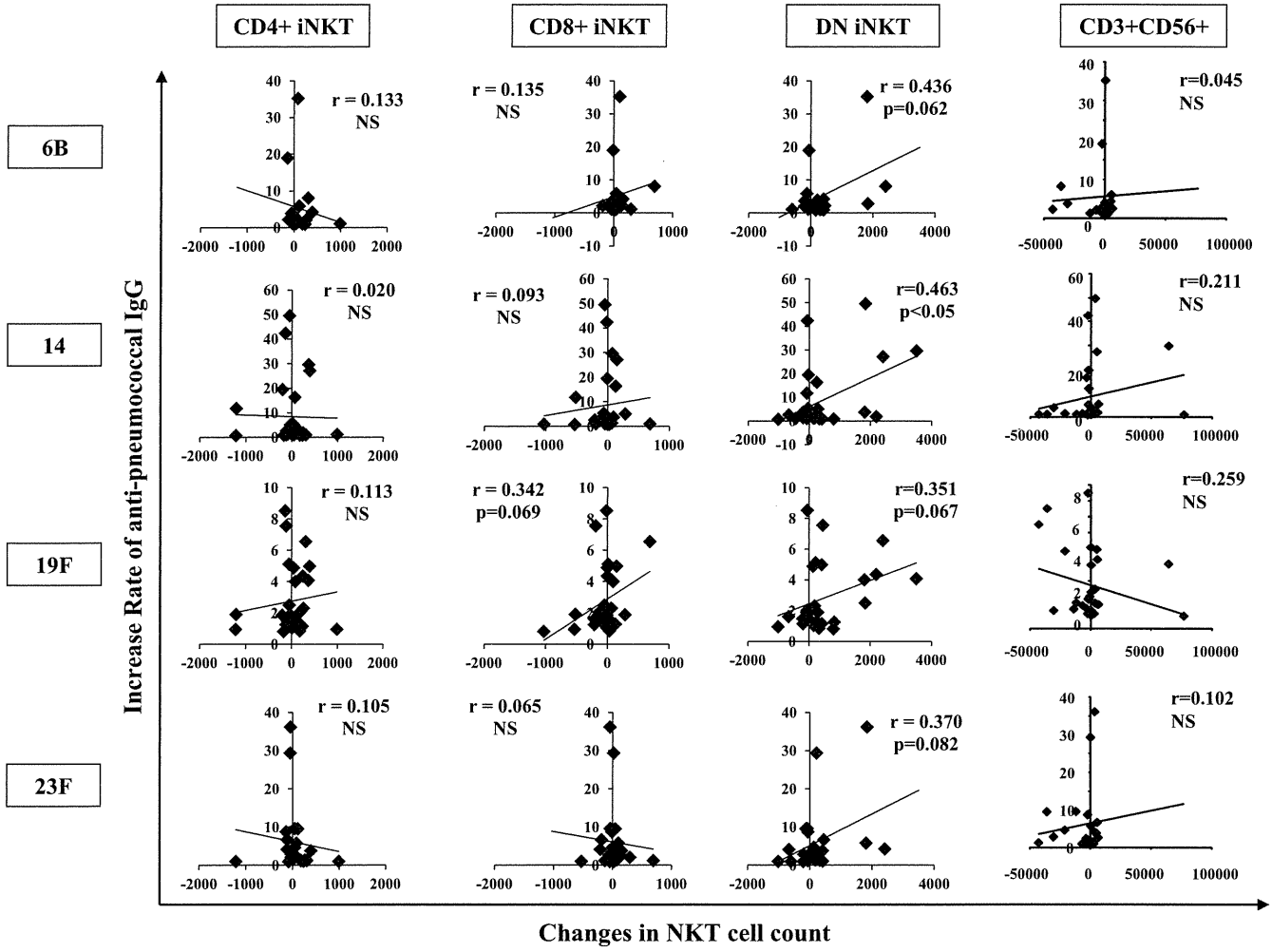

Fig. 3. Relationship between NKT cell counts and anti-pneumococcal IgG. Relationship between changes in NKT cell counts during the first 2 weeks post-vaccination and degree of change in serum anti-pneumococcal IgG levels from pre-vaccination to peak. Each symbol indicates the relationship for one subject. *R* and *P* values and number of subjects in each analysis are shown.

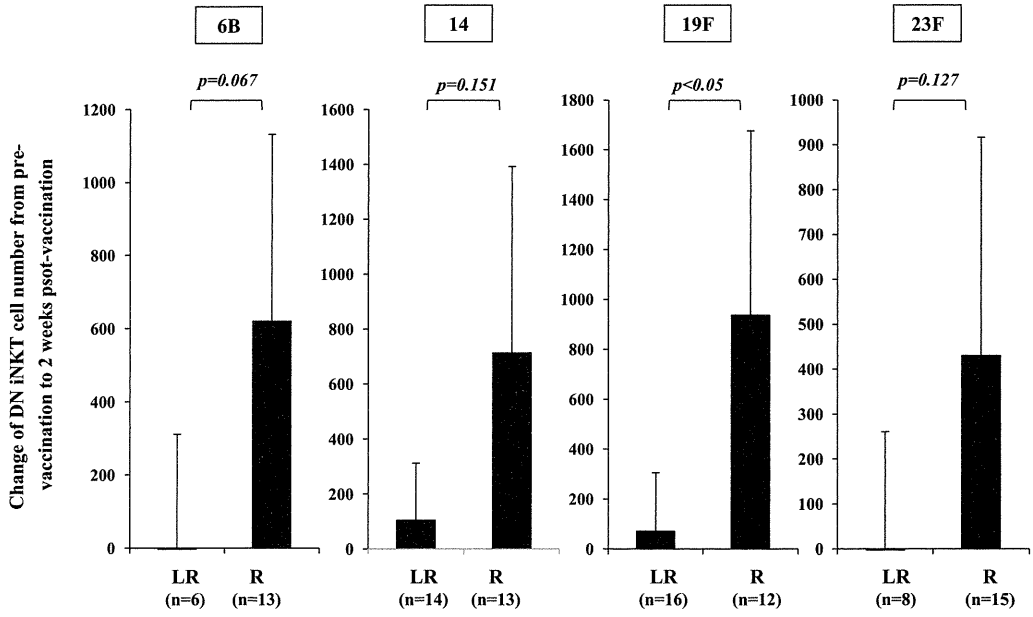

Fig. 4. Changes in DN iNKT cell counts in responders and low responders. Degree of change in DN iNKT cell count during the first 2 weeks after vaccination was compared between responders and low responders for each serotype. Data are expressed as the arithmetic means and 95% confidence intervals of indicated number of subjects. LR, low responders; R, responders.

enrolled subjects were aged (74.4 ± 6.6 years) and had underlying diseases that affected their immune condition. In these respects, there are some limitations in interpreting the results. At present, it remains to be elucidated how iNKT cells are involved in humoral immune responses to pneumococcal capsular polysaccharides in the clinical setting, but further investigations are already under way in our laboratory to define the precise mechanism underlying the relationship between iNKT cells and Ab responses.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Grant from the Ministry of Health, Labor and Welfare of Japan (Research on Emerging and Re-emerging Infectious Diseases), a Grant from the Ministry of Health, Labor and Welfare of Japan (H22-seisakusouyaku-ippan-012) and aid funding from Ohyama Health Foundation, Inc.

Conflict of interest statement: The authors have no financial conflict of interest.

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ARD Online First, published on January 23, 2013 as 10.1136/annrheumdis-2012-202658

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CONCISE REPORT

Pneumococcal polysaccharide vaccination in rheumatoid arthritis patients receiving tocilizumab therapy

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▶ Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2012-202658).

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Received 12 September 2012
Revised 21 November 2012
Accepted 28 December 2012

To cite: Mori S, Ueki Y, Akeda Y, *et al. Ann Rheum Dis* Published Online First: [*please include* Day Month Year] doi:10.1136/annrheumdis-2012-202658

ABSTRACT

Objectives We assessed the impact of tocilizumab (TCZ), a humanised monoclonal anti-interleukin-6 receptor antibody, on antibody response following administration of the 23-valent pneumococcal polysaccharide vaccine (PPV23).

Methods A total of 190 patients with rheumatoid arthritis (RA) received PPV23. Patients were classified into TCZ (n=50), TCZ + methotrexate (MTX) (n=54), MTX (n=62) and RA control (n=24) groups. We measured serotype-specific IgG concentrations of pneumococcal serotypes 6B and 23F using ELISA and functional antibody activity using a multiplexed opsonophagocytic killing assay, reported as the opsonisation indices (OIs), before and 4–6 weeks after vaccination. Positive antibody response was defined as a 2-fold or more increase in the IgG concentration or as a ≥10-fold or more increase in the OI.

Results IgG concentrations and OIs were significantly increased in all treatment groups in response to vaccination. The TCZ group antibody response rates were comparable with those of the RA control group for each serotype. MTX had a negative impact on vaccine efficacy. Multivariate logistic analysis confirmed that TCZ is not associated with an inadequate antibody response to either serotype. No severe adverse effect was observed in any treatment group.

Conclusions TCZ does not impair PPV23 immunogenicity in RA patients, whereas antibody responses may be reduced when TCZ is used as a combination therapy with MTX.

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) infection is responsible for substantial mortality and morbidity among adults aged ≥65 years or those with underlying chronic or immunosuppressive conditions. The CDC Advisory Committee on Immunization Practice has recommended the use of the 23-valent pneumococcal polysaccharide vaccine (PPV23) for prevention of invasive pneumococcal disease in at-risk populations.[1] Patients with rheumatoid arthritis (RA) are at an increased risk of contracting infectious diseases because of immunological changes that are intrinsic to RA and that result from immunosuppressive agents, and thus it is likely that pneumococcal vaccination can benefit this patient population.

Tocilizumab (TCZ), a humanised monoclonal antibody against the interleukin-6 (IL-6) receptor, is effective and generally well tolerated when administered either as monotherapy or in combination with methotrexate (MTX) in patients with moderate to severe RA. IL-6 was originally identified as a factor essential for B cell differentiation into antibody-producing plasma cells,[2] and IL-6-deficient mice had reduced antigen-specific IgG following immunisation with a T-cell-dependent antigen.[3] PPV23 induces serotype-specific IgG in a T-cell-independent polysaccharide antigen pathway, which can enhance pneumococcal opsonisation, phagocytosis and killing by phagocytic cells.[4] PPV23 immunogenicity is often impaired in certain groups of immunocompromised patients,[1] but evidence of PPV23 efficacy and safety is lacking in RA patients receiving TCZ.

The objective of the present study was to evaluate the influence of TCZ therapy on antibody response to PPV23 in RA patients. We determined the serum concentrations of serotype-specific IgG using ELISAs and the functional antibody activity using multiplexed opsonophagocytic killing assays (OPAs) in RA patients being treated with TCZ, MTX or TCZ and MTX, and in control RA patients who received neither drug.

METHODS

Patients

RA patients who were receiving TCZ therapy (at least the first dose of an intravenous infusion of 8 mg/kg every 4 weeks) and/or MTX (4–18 mg per week) for ≥12 weeks at our rheumatology outpatient clinics were invited to participate in this open-label study. RA patients who had been treated with bucillamine or salazosulfapyridine were also included as RA controls. All participants fulfilled the 1987 American College of Rheumatology criteria for RA diagnosis. Exclusion criteria were current prednisolone use (≥10 mg/day), current use of immunosuppressive antirheumatic drugs other than MTX (such as tacrolimus, cyclosporine, leflunomide, cyclophosphamide and azathioprine), a recent history (within 6 months) of pneumococcal infection and a history of pneumococcal vaccination. Patients who had changed treatments during the follow-up period or those who had received biological agents other than TCZ were also excluded from this study.

Vaccine

We used commercially available PPV23 (Pneumovax NP, Merck Sharp & Dohme Corp., Tokyo, Japan) containing 25 μg each of 23 capsular polysaccharide

types. From October 2011 to March 2012, each patient received a single dose of vaccine (0.5 ml) subcutaneously in the upper arm. For RA patients receiving TCZ, the vaccination was performed on the same day as the TCZ infusion.

ELISAs for serotype-specific IgG and multiplexed OPAs

Sera were collected immediately before and 4–6 weeks after vaccination and stored at −30°C until tested. To measure serotype-specific IgG concentrations and functional antibody activity against pneumococcus serotypes 6B and 23F, we performed ELISAs and multiplexed OPAs, respectively. For detailed protocols, see online supplementary text.

Antibody response

Fold increases relative to pre-vaccination values (post-vaccination value to pre-vaccination value ratios) were determined. Positive antibody response was defined as a 2-fold or more increase in IgG concentrations or as a 10-fold or more increase in opsonisation indices (OIs).[5]

Monitoring adverse effects

Adverse events that occurred during a follow-up period of 4–6 weeks after vaccination were recorded. Systemic adverse effects included fever, headache, myalgia, asthenia and fatigue. Local adverse events included pain/tenderness, swelling/induration and erythema at the injection sites.

Statistical analysis

To access the PPV23 immunogenicity in patients in each treatment group, IgG concentrations and OIs before and after vaccination were transformed into logarithmic values. IgG geometric mean concentrations (GMCs) and geometric mean OIs (GM-OIs) were calculated as the exponential of an arithmetic mean of log-transformed values. For details regarding statistical analysis, see online supplementary text.

RESULTS

Clinical and demographic characteristics

A total of 190 RA patients were divided into four groups according to their ongoing anti-RA therapy. There was one group of 50 patients treated with TCZ as monotherapy (TCZ group), 62 patients treated with MTX alone (MTX group), 54 patients who received a combination therapy consisting of TCZ and MTX (TCZ+MTX group) and 24 patients who did not receive either drug (RA control group). Prior to participating in this study, no patients had received a pneumococcal vaccination. Patients' clinical and demographic characteristics are shown in table 1.

Serotype-specific IgG concentrations

After vaccination, serotype-specific IgG GMCs to pneumococcal serotypes 6B and 23F in all four groups were increased significantly ($p<0.0005$; table 2). For serotype 6B, a significantly higher post-GMC was obtained in the TCZ group compared with that in the TCZ+MTX group ($p=0.004$). The TCZ group also showed a significantly greater fold increase than did the TCZ+MTX group ($p=0.036$). For serotype 23F, the TCZ group also showed a significantly higher post-GMC than did the MTX group ($p=0.027$). Increases were twofold or more in all treatment groups, and there were no statistically significant differences.

Opsonophagocytic killing assays

After vaccination, GM-OIs for the 6B and the 23F serotypes were increased significantly in all four groups ($p<0.0005$; table 2). For serotype 6B, the post-vaccination GM-OI was significantly higher in the TCZ group compared with that in the MTX group ($p=0.001$). The TCZ group also showed a significantly higher post-vaccination GM-OI for serotype 23F compared with the MTX group ($p=0.001$) or with the TCZ+MTX group ($p=0.042$). For either serotype, there were no significant differences in fold increases among the four treatment groups.

Table 1 Clinical and demographic characteristics of RA patients prior to pneumococcal vaccination

	MTX group (n=62)	TCZ+MTX group (n=54)	TCZ group (n=50)	RA control (n=24)	p Values between treatment groups
Male/female	11/51	4/50	7/43	5/19	NS
Age, mean (95% CI) (years)	68.3 (66.6 to 70.1)	65.1 (63.1 to 67.0)	68.3 (65.8 to 70.8)	69.2 (65.3 to 73.1)	NS
RA duration, mean (95% CI) (years)	10.0 (7.8 to 12.1)	9.1 (7.3 to 10.8)	12.5 (9.6 to 15.3)	11.3 (6.0 to 16.6)	NS
MTX dose, median (IQR) (mg/week)	8 (6 to 8)	8 (6 to 8)	–	–	NS
MTX duration, median (IQR) (months)	48 (14.3 to 86.3)	48.5 (26 to 81)	–	–	NS
TCZ duration, median (IQR) (weeks)	–	56 (16 to 95)	58 (15 to 98)	–	NS
Use of prednisolone, number of patients (%)	17 (27.4)	14 (25.9)	12 (24)	1 (4.2)	0.018 (M vs C) 0.029 (T/M vs C) 0.049 (T vs C)
Prednisolone dose, median (IQR) (mg/day)	0 (0 to 2)	0 (0 to 1)	0 (0 to 1)	0 (0 to 1)	NS
Positive RF, number of patients (%)	35 (56.5)	39 (72.2)	31 (62)	8 (33.3)	0.001 (T/M vs C) 0.021 (T vs C)
Positive anti-CCP Abs, number of patients (%)	44 (71.0)	46 (85.2)	41 (82)	11 (45.8)	0.029 (M vs C) 0.0003 (T/M vs C) 0.001 (T vs C)
Lymphocytes, mean (95% CI) (/μl)	1374 (1230 to 1517)	1651 (1420 to 1881)	1717 (1545 to 1890)	1600 (1358 to 1842)	NS
Serum IgG, mean (95% CI) (mg/dl)	1286 (1194 to 1377)	1172 (1075 to 1269)	1196 (1121 to 1271)	1394 (1258 to 1530)	NS

Data were obtained immediately before pneumococcal vaccination. p Values between treatment groups were determined using the Mann–Whitney U test, ANOVA (analysis of variance) with a Tukey's HSD (honesty significant difference) post hoc test, the Kruskal–Wallis test with a Scheffe post hoc test, the χ^2 test or Fisher's exact probability test.
anti-CCP Abs, anti-cyclic citrullinated peptide antibodies; M, MTX group; MTX, methotrexate; NS, not significant; RA, rheumatoid arthritis; RF, rheumatoid factor; T, TCZ group; T/M, TCZ +MTX group; C, RA control; TCZ, tocilizumab.

Table 2 Concentrations of pneumococcal polysaccharide antigen serotype-specific IgG antibodies and opsonisation indices in the RA treatment groups before and after 23-valent pneumococcal polysaccharide vaccination

Serotype	MTX group (n=62)	TCZ+MTX group (n=54)	TCZ group (n=50)	RA control group (n=24)	p Values between treatment groups
IgG GMCs (μg/ml)					
6B					
Before	1.2 (1.0 to 1.5)	1.1 (0.9 to 1.3)	1.3 (1.0 to 1.7)	1.1 (0.8 to 1.6)	NS
After	2.2 (1.7 to 2.7)*	1.7 (1.3 to 2.3)*	6.1 (2.6 to 4.9)*	2.5 (1.5 to 4.4)*	0.004 (T/M vs T)
Fold increase	1.5 (1.1 to 3.0)	1.6 (1.2 to 1.9)	2.8 (1.4 to 4.4)	1.8 (1.3 to 3.7)	0.036 (T/M vs T)
23F					
Before	1.0 (0.8 to 1.2)	0.9 (0.7 to 1.2)	1.3 (1.0 to 1.7)	1.0 (0.6 to 1.5)	NS
After	2.4 (1.8 to 3.3)*	2.5 (1.8 to 3.5)*	4.6 (3.4 to 6.4)*	3.6 (1.8 to 5.7)*	0.027 (M vs T)
Fold increase	2.6 (1.4 to 4.1)	2.9 (1.0 to 6.9)	3.4 (1.5 to 6.8)	3.5 (1.7 to 5.6)	NS
GM-OIs					
6B					
Before	18.8 (18.7 to 32.1)	24.5 (14.7 to 42.1)	43.8 (22.4 to 85.6)	20.70 (7.0 to 61.0)	NS
After	115.6 (64.1 to 206.4)*	232.8 (124.0 to 437.0)*	692.3 (265.1 to 1366)*	262.4 (74.4 to 916.0)*	0.001 (M vs T)
Fold increase	4.5 (1 to 12.5)	6.8 (1.7 to 35.5)	12 (3.5 to 62.4)	8.5 (2.2 to 52.0)	NS
23F					
Before	10.1 (6.6 to 15.3)	15.5 (10.3 to 23.6)	27.9 (15.2 to 51.4)	17.6 (7.5 to 42.1)	0.018 (M vs T)
After	72.2 (39.3 to 133.0)*	124.0 (62.2 to 244.7)*	437.0 (221.4 to 862.6)*	219.2 (82.3 to 578.2)*	0.001 (M vs T) 0.042 (M/T vs T)
Fold increase	7 .0 (2.7 to 15.8)	5.0 (1 to 40)	18.8 (2.7 to 75.1)	11.0 (3.1 to 30.6)	NS

IgG GMCs and GM-OIs are expressed as the mean (95% CI). Fold increases are expressed as the median (IQR). Differences between pre- and post-vaccination GMCs of serotype-specific IgG and those between pre- and post-vaccination GM-OIs were assessed using a paired-sample t test. The four treatment groups were compared using ANOVA (analysis of variance) with a Tukey's HSD (honestly significant difference) post hoc test or the Kruskal–Wallis test with a Scheffe post hoc test.
*p<0.0005 compared with pre-vaccination IgG GMCs or GM-OIs.
GMC, geometric mean concentration; GM-OI, geometric mean opsonisation index; M, MTX group; MTX, methotrexate; NS, not significant; RA, rheumatoid arthritis; T, TCZ group; T/M, TCZ+MTX group; TCZ, tocilizumab.

There was a moderate correlation between IgG concentrations and OIs for the 6B and the 23F serotypes (serotype 6B: r=0.623, p<0.0005; serotype 23F: r=0.601, p<0.0005).

Antibody response rates (percentages of patients with positive antibody response)

The TCZ group antibody response rates were comparable with those of the RA control group for serotypes 6B and 23F (figure 1).

For the IgG concentration specific to serotype 6B, the antibody response rate was significantly higher in the TCZ group (56%) compared with that in the MTX group (37%) and the TCZ+MTX group (24%, p=0.046 and p=0.0009, respectively; figure 1A). For serotype 23F, there was no significant difference in the antibody response rate among the four treatment groups (Control: 67%; MTX: 57%; TCZ+MTX: 56%; TCZ: 72%). The percentage of patients with positive antibody response for both strains were significantly greater in the TCZ group (46%) compared with the TCZ+MTX group (20%, p=0.005) and the RA control group (21%, p=0.044).

For OIs specific to serotype 6B, the TCZ group showed a significantly higher antibody response rate than did the MTX group (56% vs 34%, p=0.019; figure 1B). For serotype 23F, the antibody response rates were significantly higher in the TCZ group (58%) compared with those in the MTX group (37%, p=0.027) and the TCZ+MTX group (35%, p=0.020). For both strains, a higher proportion of patients in the TCZ group responded to pneumococcal vaccination compared with the patients being treated with MTX alone (34% vs 16%, p=0.028).

Predictive factors for antibody response to PPV23

In a multivariate logistic regression analysis, TCZ use was not identified as the predictive factor for antibody response to pneumococcal vaccination for either IgG concentrations or OIs. The negative association of current MTX use with antibody response was confirmed for IgG concentrations specific to serotypes 6B and 23F (for serotype 6B: OR 0.45, 95% CI 0.25 to 0.82, p=0.009; for serotype 23F: OR 0.56, 95% CI 0.31 to 1.04, p=0.007) and OIs for serotype 23F (OR 0.54, 95% CI 0.29 to 0.99, p=0.046).

Vaccination safety

Two patients in the TCZ+MTX group had a fever. Local adverse events were observed in 12 patients (2 in the MTX group, 7 in the TCZ+MTX group and 3 in the TCZ group). All adverse effects were mild.

DISCUSSION

Following immunisation with PPV23, IgG concentrations and OIs for the 6B and the 23F serotypes were significantly increased in all treatment groups. Antibody response rates in the TCZ group were comparable with those of the RA control group for each serotype. Ongoing use of MTX is likely to have affected the antibody response to PPV23.

Results of the present study indicate that TCZ does not diminish T-cell-independent antibody production after PPV23 immunisation. In addition, we recently reported that RA patients receiving TCZ can produce an adequate antibody response to influenza vaccine, which are T-cell-dependent protein antigens.[6] These findings suggest that both T-cell-dependent and T-cell-independent antibody response pathways are conserved in RA patients who are treated with TCZ. There is an increasing awareness of lethal synergism between influenza virus and pneumococcus; influenza virus contributes to secondary pneumococcal pneumonia and can subsequently increase mortality.[7 8] In addition, a large-scale trial suggested that a significant

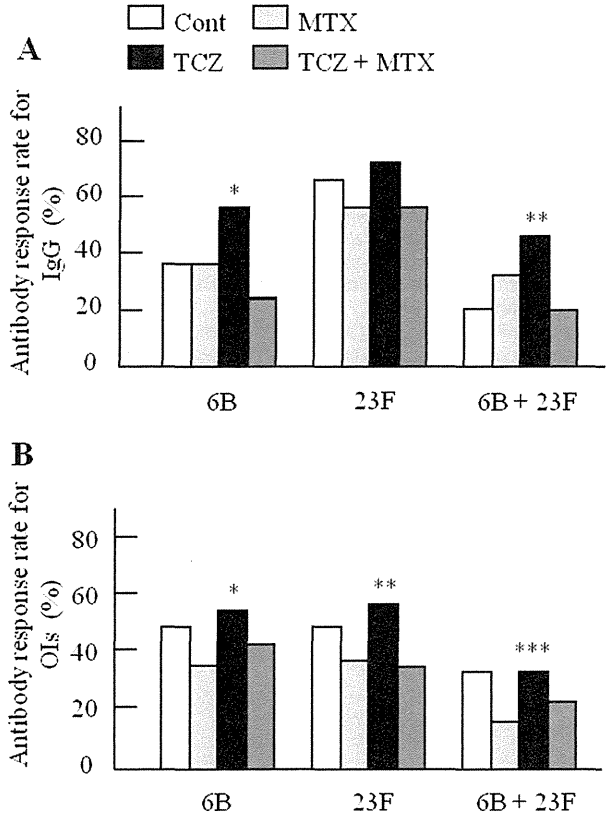

Figure 1 (A) Percentages of patients with twofold or more increases in serotype-specific IgG concentrations for serotypes 6B and 23F in the rheumatoid arthritis (RA) treatment groups. *p=0.046 (TCZ vs MTX) and p=0.0009 (TCZ vs TCZ+MTX). **p=0.005 (TCZ vs TCZ+MTX) and p=0.044 (TCZ vs Cont). (B) Percentages of patients with 10-fold or more increases in OIs for serotypes 6B and 23F in the RA treatment groups. *p=0.019 (TCZ vs MTX). **p=0.027 (TCZ vs MTX) and p=0.020 (TCZ vs TCZ+MTX). ***p=0.028 (TCZ vs MTX). Data were compared using the χ^2 test or Fisher's exact probability test. OIs, opsonisation indices; Cont, RA control group; MTX, methotrexate group; TCZ, tocilizumab group; TCZ+MTX, combination therapy group.

proportion of viral pneumonia, including influenza, is attributable to bacterial co-infection and that this co-infection may be preventable by bacterial vaccination.[9] Immunisation with both influenza and pneumococcal vaccines may, therefore, provide additive benefits for RA patients compared with a single vaccination, even if they are receiving TCZ therapy.

Previous studies have shown that MTX therapy reduced the antibody response to PPV23,[10–13] which is in agreement with the data obtained in the present study. Although T-cell-dependent protein antigens may be more immunogenic than polysaccharide antigens in immunocompromised patients,[14] MTX was also reported to be a strong predictive factor for an impaired antibody response to protein-conjugate pneumococcal vaccine.[15] Offering PPV23 vaccination before introduction of MTX therapy may be considered in RA patients.[11 16] In contrast, a study by Elkayam *et al*[17] did not demonstrate a detrimental effect of immunosuppressive drugs such as MTX on PPV23 immunogenicity in RA patients. Coulson *et al*[18] have also suggested that a single PPV23 administration offers up to 10 years of protection against the development of pneumococcal pneumonia in RA patients receiving MTX therapy. Determining serotype-specific IgG concentrations after PPV23 vaccination in patients receiving MTX therapy is recommended.[19]

In the present study, no patients were receiving high doses of prednisolone or antirheumatic agents with immunosuppressive effects other than MTX. In addition, there were no differences in the prednisolone dose among the four treatment groups, and the median dose of prednisolone was zero among all groups. The number of prednisolone users was significantly lower in the RA control group; however, there were no significant differences or trends in antibody response to each serotype compared with the other three groups. We can, therefore, say that the influence of such agents on PPV23-induced antibody response was minimal in the present study.

One limitation of this study is the relatively small number of patients in each group and the RA control group in particular. Since most RA patients had already received one or more immunosuppressive antirheumatic drugs, as recommended by the current therapeutic guidelines, it was difficult to recruit a sufficient number of patients who had never received such drugs. Another limitation is that we determined antibody response to only two pneumococcal serotypes. We chose serotypes 6B and 23F because these are the main causative serotypes of pneumococcal pneumonia in Japan and these are representative penicillin-resistant pneumococci.[20] However, the immune response to PPV23 may not be consistent among the 23 serotypes. Lastly, unlike influenza vaccines, antibody levels that are protective against invasive pneumococcal disease in adults have not been clearly defined. We used a 2-fold increase in the IgG concentration or a 10-fold increase in the OI as a measure of positive antibody response to PPV23 in this study, which was also used in previous studies;[5] however, how this threshold may best correlate with protection against invasive pneumococcal disease remains to be determined.

In conclusion, ongoing TCZ therapy does not preclude pneumococcal polysaccharide vaccination in RA patients; however, antibody responses may be reduced when TCZ is administered in combination with MTX.

Acknowledgements The authors are grateful to Michiyo Hayakawa and Yumi Hattori for technical assistance in measuring serotype-specific IgG concentrations and OIs.

Contributors *All authors contributed to study conception and design, acquisition* of data, analysis and interpretation of data, and drafting of the manuscript with regard to important intellectual content.

Funding The study was supported by research grants from the Ministry of Health, Labour and Welfare of Japan and research funds from the National Hospital Organization (NHO), Japan.

Competing interests TH has received lecture fees from Mitsubishi-Tanabe Pharmaceutical Co., Eisai Co. Ltd. and Abbott Japan Co. Ltd. The other authors have no financial relationships that could lead to a conflict of interest.

Patient consent Obtained.

Ethics approval The ethics committees of participating hospitals approved the protocol for this study.

Provenance and peer review Not commissioned; externally peer reviewed.

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Pneumococcal polysaccharide vaccination in rheumatoid arthritis patients receiving tocilizumab therapy

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Ann Rheum Dis published online January 23, 2013
doi: 10.1136/annrheumdis-2012-202658

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Notes

Population-Based Study of *Streptococcus suis* Infection in Humans in Phayao Province in Northern Thailand

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Abstract

Background: *Streptococcus suis* infection in humans has received increasing worldwide recognition.

Methods and Findings: A prospective study of *S. suis* infection in humans was conducted in Phayao Province in northern Thailand to determine the incidence and the risk behaviors of the disease in this region in 2010. Thirty-one cases were confirmed. The case fatality rate was 16.1%, and the estimated incidence rate was 6.2 per 100,000 in the general population. The peak incidence occurred in May. The median age of the patients was 53 years and 64.5% were men. Consumption of raw pork products was confirmed in 22 cases and the median incubation period (range) was 2 days (0–11) after consumption of raw pork products. Isolates from 31 patients were confirmed as serotype 2 in 23 patients (74.2%) and serotype 14 in eight patients (25.8%). The major sequence types (STs) were ST1 (n = 20) for serotype 2 and ST105 (n = 8) for serotype 14. The epidemiological analysis suggested three possible clusters, which included 17 cases. In the largest possible cluster of 10 cases in Chiang Kham and its neighboring districts in May, the source of infection in four cases was identified as a raw pork dish served at the same restaurant in this district. Microbiological analysis confirmed that three of four cases associated with consumption of raw pork at this restaurant were attributable to an identical strain of serotype 2 with ST1 and pulsotype A2.

Conclusions: Our data suggest a high incidence rate of *S. suis* infection in the general population in Phayao Province in 2010 and confirm a cluster of three cases in 31 human cases. Food safety control should be strengthened especially for raw pork products in northern Thailand.

Citation: Takeuchi D, Kerdsin A, Pienpringam A, Loetthong P, Samerchea S, et al. (2012) Population-Based Study of *Streptococcus suis* Infection in Humans in Phayao Province in Northern Thailand. PLoS ONE 7(2): e31265. doi:10.1371/journal.pone.0031265

Editor: Tara C. Smith, University of Iowa, United States of America

Received October 17, 2011; **Accepted** January 4, 2012; **Published** February 21, 2012

Funding: This work was supported by research grants from the Department of Medical Sciences, Ministry of Public Health of Thailand, and Grants-in-Aid for Scientific Research (B: 21406027), and the program of Research Centers for Emerging and Reemerging Infectious Diseases launched by a project commissioned by the Ministry of Education, Science and Culture, and the Ministry of Health, Labor and Welfare of Japan. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Streptococcus suis is a zoonotic pathogen that can cause invasive infection in humans who have close contact with infected pigs or contaminated pork-derived products. The numbers of reported human cases, especially in Southeast Asian countries, have increased dramatically in the past few years [1–3]. Although serotype 2 is the most prevalent in humans, human cases involving serotypes 1, 4, 14 and 16 have been reported [1–5]. In a retrospective study in 2006–2008 in Thailand, *S. suis* infection was confirmed in bacterial cultures of blood or cerebrospinal fluid (CSF) from 179 patients. These isolates were determined to be serotype 2 for 165 cases (92.2%), serotype 14 for 12 cases (6.7%), and one case each (0.6%) of serotypes 5 and 24 [4–6]. Human infection with serotype 2 was sporadic, with a case fatality rate of 9.5% in adults, and most of these cases were located primarily in northern Thailand [4].

The population of the 17 provinces in northern Thailand was 11,788,684 in 2010 (Figure 1A) [7]. Some local residents have a traditional custom of consuming raw pork dishes such as "Loo" (raw pork meat and blood), "Lap" (raw pork meat), and fermented raw pork in this region. An outbreak of *S. suis* infection including 29 laboratory-confirmed cases occurred in the Phu Sang district, Phayao Province, in northern Thailand in April and May of 2007 (Figure 1B) [8] A major route of transmission during this outbreak was the consumption of raw pig blood. This province is located

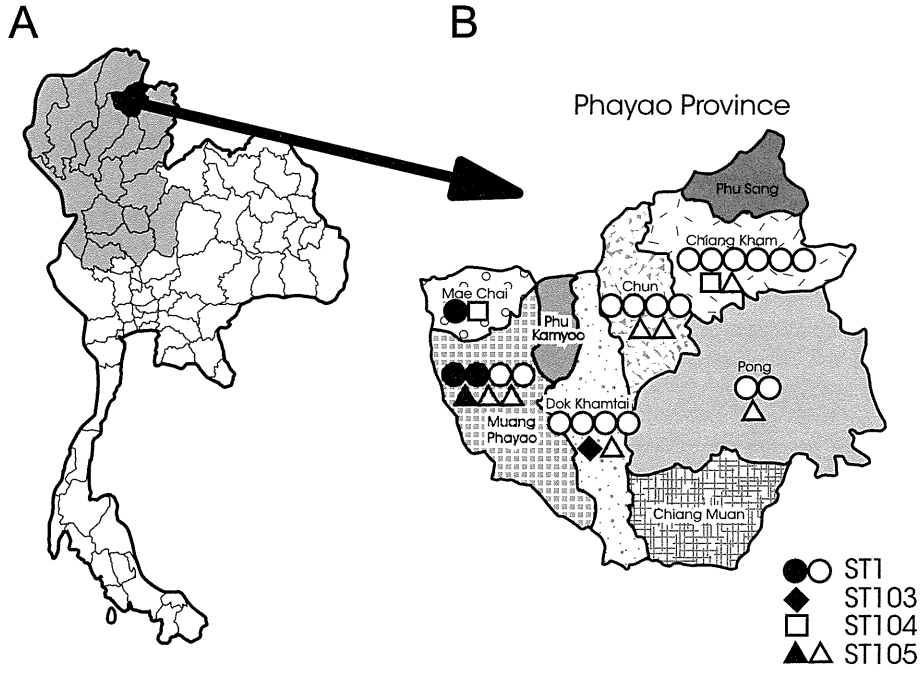

Figure 1. Location of the study site and distribution of human isolates. (**A**) Location of Phayao Province in northern Thailand. (**B**) Distribution and sequence typing of 31 human isolates of *Streptococcus suis* in Phayao Province in 2010 (B). One symbol is one case. Closed symbols denote fatal cases, and open symbols denote nonfatal cases.
doi:10.1371/journal.pone.0031265.g001

close to the border with the Lao People's Democratic Republic, and the population of this province was 486,304 in 2010 [7]. Although previous studies reported that human cases of *S. suis* infection are associated with the recent consumption of raw pork products in northern Thailand and Vietnam [2–6,8–11], the annual incidence rate of this disease in this region remains unknown.

In this study, we conducted a population-based study of *S. suis* infection in humans to determine the incidence rate of this disease in Phayao Province in 2010. We also investigated the risk behaviors of this disease and the possible clustering of cases in relation to the risk behaviors.

Methods

Human cases

We organized a network for the surveillance of *S. suis* infection in humans that includes the Phayao Public Health Office and two tertiary hospitals (Phayao Provincial Hospital and Chiang Kham General Hospital), and five district hospitals (Mae Chai Hospital, Chiang Muan Hospital, Dok Khamtai Hospital, Chun Hospital, and Pong Hospital); the districts within this province are shown in Figure 1B. We enrolled hospitalized patients with sepsis or bacterial meningitis when a biochemical test suggested the presence of *S. suis* in isolates from blood or CSF, and prospectively investigated the clinical and epidemiological features of the enrolled cases at these seven hospitals between January to December of 2010.

The clinical information of the enrolled case was recorded by attending physicians at a hospital in a network for the surveillance of *S. suis* infection in Phayao Province. The clinical information included the date of onset of illness and the hospital admission, and the risk behaviors, such as occupational exposures, the recent contact with pigs or raw pork products and the recent consumption of raw pork products. For the cases with the recent contact with pigs or raw pork products, the date and the location of exposure were recorded. For the cases with the recent consumption of raw pork products, the date and place of consumption of raw pork products and the type of dishes containing raw pork products were recorded. The clinical categories included meningitis and nonmeningitis based on the definition previously described [4]. The meningitis category involved confirmed meningitis, bacteremic meningitis, and probable meningitis. All patients in the meningitis category had typical meningeal signs, such as neck stiffness and an acute onset. Bacteremic meningitis was defined as a positive result in both the CSF and blood cultures, confirmed meningitis was defined as a positive culture in the CSF only, and probable meningitis was defined as a positive blood culture. The nonmeningitis category included the clinical manifestations of sepsis and sepsis with focal signs other than meningitis (septic arthritis or bacteremic pneumonia). Sepsis was defined as systemic inflammatory response syndrome with a positive blood culture.

The possible clustered cases were defined as human cases of laboratory-confirmed *S. suis* infection in combination with the recent close contact with pigs or raw pork products or with the recent consumption of raw pork products in the same or neighboring districts within 14 days of each onset of illness. This incubation period was based on a previous report of a human *S. suis* outbreak in Sichuan, China, which showed that the interval between exposure and onset is 1–14 days [12]. This population-based study of *S. suis* infection in humans was reviewed and approved by the Ethics Committees of the Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand. This study was conducted according to the principles expressed in the Declaration of Helsinki. The patient or guardian provided written informed consent for all cases. This study was registered at the UMIN Clinical Trial Registry (UMIN000006449).

Microbiological study

The isolates were subjected to the following biochemical tests: API Strep (bioMérieux, Durham, NC, USA) and *S. suis*-specific and *S. suis* serotype 2- or 1/2–specific polymerase chain reaction [4,13]. The final serotype of all strains was confirmed by coagglutination tests using rabbit antisera (Statens Serum Institute, Copenhagen, Denmark).

Multilocus sequence typing (MLST) was performed as described by King et al. [14], with a modification for *mutS* as described by Rehm et al. [15]. MLST alleles and the resulting sequence type (ST) were assigned using the *S. suis* MLST database, which can be accessed at http://ssuis.mlst.net. Pulsed-field gel electrophoresis (PFGE) was performed as described previously [16]. The pulsotypes were designated as previously described [4], and assigned to clusters of isolates with >80% similarity within the dendrogram.

Statistical analysis

The clinical characteristics including male sex, age, risk factor, the days from the consumption of raw pork products to the onset of illness, the days from the onset of illness to the admission between fatal and nonfatal cases were compared using Fisher's exact test or Mann–Whitney *U* test with SPSS version 15.0 software. Data were considered significant for p values<0.05.

Results

Patients

The locations of nine districts in Phayao Province and the distribution of the 31 cases in these districts are shown in Figure 1B. No case was found in the districts of Phu Sang, Phu Kamyoo, and Chiang Muan. Fatal cases were found in the districts of Muang Phayao, Dok Khantai, and Mae Chai. There was no relationship between geographical distribution of cases and the location of fatal cases. The monthly incidence of the 31 cases in each district is shown in Figure 2. The peak incidence occurred in May, and 23 cases (71.9%) were detected between April and June.

The clinical features of the 31 patients admitted with *S. suis* infection in Phayao Province between January and December 2010 are shown in Table 1. The median age (range) of these patients was 53 years (26–74) of which 64.5% were men and 35.5% were women. Five of the 31 cases (16.1%) were fatal. Recent exposure to pigs or raw pork products was noted in two cases (6.5%). One case occurred in a pork meat seller (Case 21 in Table 2) and was associated with the daily occupational exposure to the raw pork products at the wet market. Another case (Case 27 in Table 2) had the daily exposure to pigs bred at home.

Because 30 cases were confirmed in the general population of Phayao Province in 2010, the incidence rate of this disease in humans was 6.2 per 100,000 (30 in 486,304) of the general population in this province in 2010. Before the onset of illness, a recent history of consumption of raw pork products was confirmed for 22 (71.0%) of the 31 cases. A pig breeder (Case 27) also had a recent history of raw pork consumption. No information was available about the recent consumption of raw pork products or exposure to pigs or raw pork products in the remaining eight cases.

The clinical manifestations in the 31 patients included fever (n = 27; 87.1%), headache (n = 19; 61.3%), hearing loss (n = 12; 38.7%), altered consciousness (n = 9; 29.0%), and diarrhea (n = 6; 19.4%). The comorbid illnesses of these patients included alcoholic liver cirrhosis (n = 4; 12.9%), hypertension (n = 3, 9.7%), diabetes mellitus (n = 1; 3.2%), rheumatoid arthritis (n = 1; 3.2%), aplastic anemia (n = 1; 3.2%), and spinal canal stenosis (n = 1; 3.2%). No comorbid illness was found in 20 patients (64.5%). None of the demographic features, including the risk behavior of recent consumption of raw pork products and recent exposure to pigs or raw pork products, was significantly associated with a fatal outcome (Table 1). The median interval (range) between the consumption and onset of illness was 2 days (0–11) for 22 patients. The median period (range) from the onset of illness to admission was 2 days (0–14) for 31 patients. The interval from the onset of illness to admission was not associated significantly with a fatal outcome, although the interval tended to be longer in the fatal cases than in the nonfatal cases. The meningitis category (n = 20; 64.5%) included five cases of confirmed meningitis, nine cases of

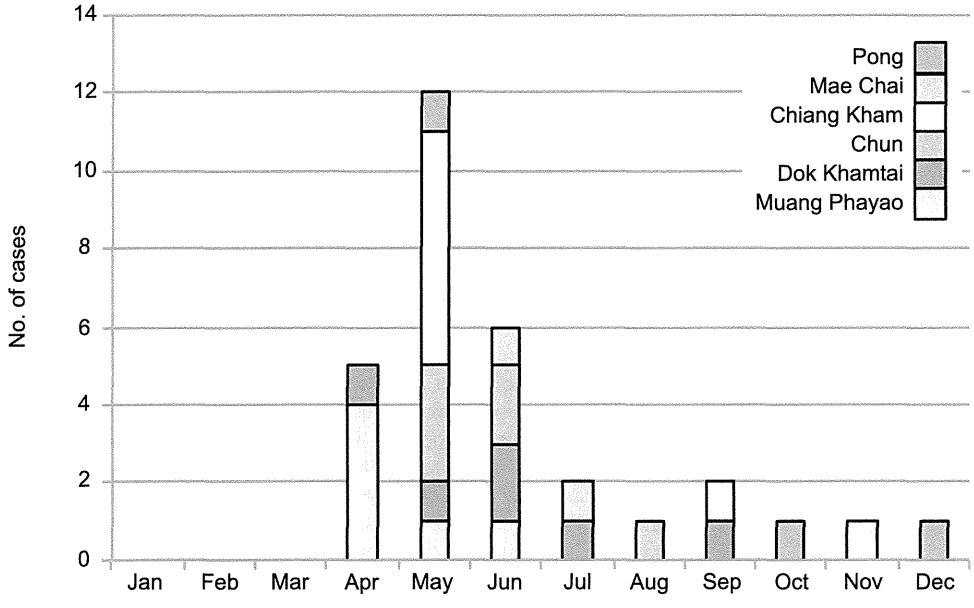

Figure 2. Monthly distribution of human cases of *Streptococcus suis* infection in each district in Phayao Province in 2010.
doi:10.1371/journal.pone.0031265.g002

Table 1. Clinical characteristics of 31 human cases of *Streptococcus suis* infection in Phayao Province, 2010.

Characteristics	All reported cases	Nonfatal case, n = 26; 83.9%	Fatal case, n = 5; 16.1%	*p*-value
Demographic				
Male, no. of cases (%), n = 31	20 (64.5)	18 (69.2)	2 (40)	0.317
Age, median (range), n = 31	53 (26–74)	52 (26–74)	64 (36–72)	0.115
Risk behavior, no. of cases (%)				
Recent consumption of raw pork products, n = 31	22 (71.0)	20 (76.9)	2 (40)	0.131
Recent contact with pigs or raw pork products, n = 31	2 (6.5)	2 (7.7)	0 (0)	1
Days from the consumption of raw pork products to the onset of illness median (range), n = 22	2 (0–11)	2 (0–11)*	1.5 (1–2)**	0.623
Days from the onset of illness to the admission median (range), n = 31	2 (0–14)	2 (0–7)	4 (0–14)	0.176

*n = 20,
**n = 2.
doi:10.1371/journal.pone.0031265.t001

bacteremic meningitis, and six cases of probable meningitis. The nonmeningitis category (n = 11; 35.5%) included five cases of septic arthritis and six cases of sepsis.

Clustered cases

We next examined whether the clustered cases that were linked epidemiologically and caused by an identical strain, were included in the 31 cases. The clinical, epidemiological and microbiological features of 31 human cases of *S. suis* infection is shown in Table 2. In 22 patients with a recent history of consumption of raw pork products, these products were consumed at home by 14 patients and at 5 different restaurants by eight patients. The most frequent dish (14/22 cases; 63.6%) was "Loo". Three possible clusters including 17 cases associated with recent consumption of raw pork products or the recent exposure to pigs or raw pork products were found based on the case definition in the 31 cases. A possible cluster in the Muang Phayao district found in April included three cases (shown as PC I). Another possible large cluster including 10 cases was found in the districts of Chiang Kham, Chun, and Pong in May (shown as PC II). The other possible cluster including four cases was found in the districts of Dok Khamtai and Chun between May and June (shown as PC III). Interestingly, four patients visited restaurant C and consumed "Loo" in Chiang Kham district between May 8 and 15, 2010. These four patients had febrile illness 1–4 days after consuming "Loo" at this restaurant. By contrast, no epidemiological linkage was found in the other 13 cases in three possible clusters.

Isolates of *S. suis*

S. suis was isolated from all 31 patients. Of the 31 isolates, 23 (74.2%) were serotype 2 and the other eight (25.8%) were serotype 14 (Table 2). The sequence typing of serotype 2 isolates were ST1 for 20 isolates (64.5%), ST104 for two isolates (6.5%), and ST103 for one isolate (3.2%). All eight serotype 14 isolates were ST105 (25.8%). In four patients in the possible large cluster (PC II in Table 2) in Chiang Kham and its neighboring district with a history of visiting restaurant C, serotype 2 strain with ST1 and pulsotype A2 was isolated from three cases, and serotype 2 strain with ST1 and pulsotype A was isolated from one case.

Discussion

In this study, we confirmed 31 human cases of *S. suis* infection with a case fatality rate of 16.1% in Phayao Province in 2010. This case fatality rate is equivalent to that recorded previously in Thailand [4,11,12]. To exclude the possibility that human cases of *S. suis* infection in residents of Phayao Province were detected in hospitals in the surrounding three provinces of Chiang Rai, Lampang, and Nan, we investigated all human cases in these provinces through the hospital network surveillance system for *S. suis* infection organized by the Thai NIH in 2010 [17]. Because no human cases from Phayao Province were found in these three provinces in this surveillance, our data represent a population-based study of *S. suis* infection in humans in this province.

The incidence rate (6.2 per 100,000) in the general population in Phayao Province in 2010 is 69 times higher than that (0.09 per 100,000) in Hong Kong [18], which is the sole available data for the general population in Southeast Asian countries. By contrast, the incidence rate of this disease is as low as 0.002/100,000 in the general population in a developed country such as The Netherlands [19]. Our present data suggest that the highest incidence rate of this disease among adults in the general population in this region is associated with the habitual behavior of consuming raw pork products. Given the incidence rate of this disease and the population in northern Thailand, the estimated number of human cases can be calculated as 730 per year in this region.

The disease incidence peaked during the rainy season (June to August) in a retrospective study between 2006 and 2008 in all 76 provinces of Thailand [4]. By contrast, the peak incidence was May 2010 in our current study. A previous outbreak in the Phu Sang district, Phayao Province, was also found during April and May in 2007 [8]. The shift of the peak incidence to April and May might be related to the Songkran Festival (a traditional new year festival in Thailand) in April and other harvesting festivals during this period in this region.

A recent case–control study in southern Vietnam reported that eating undercooked pig blood or intestine within 2 weeks of the appearance of infection was the most important risk factor [20]. In our study, we also confirmed that more than 70% of cases with *S. suis* infections were associated with the recent consumption of raw pork products. Importantly, the estimated incubation period for this disease after oral consumption of raw pork products was only 2 days. This finding strongly suggests that the oral consumption of raw pork products is the major transmission route. A previous study of an *S. suis* outbreak in Sichuan, China, similarly reported a median interval of 2.2 days between exposure and onset of infection, although the transmission route in this outbreak was direct contact with the blood or tissues of sick or dead pigs [12].