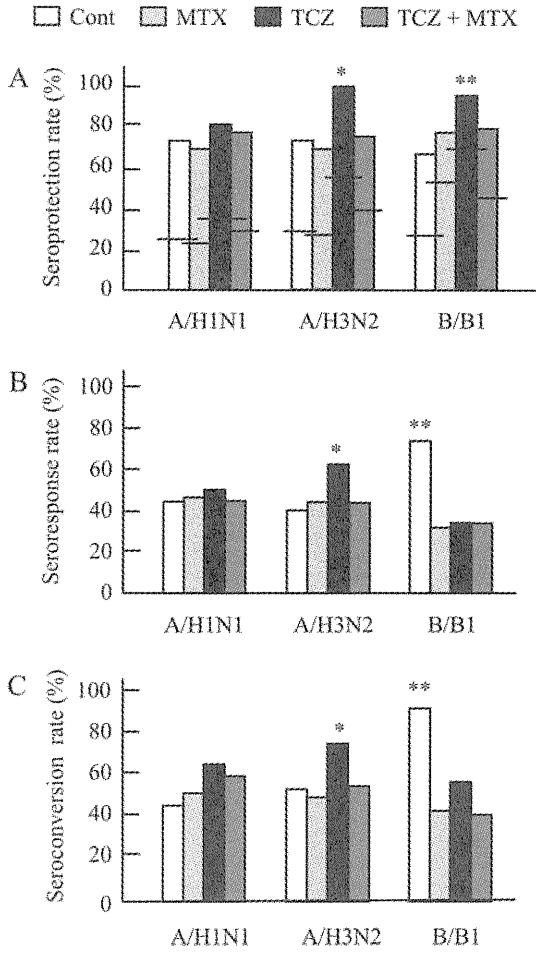

Figure 1 (A) Seroprotection rates for three influenza strains in the RA treatment groups prior to and after influenza vaccination. Horizontal bars represent levels of prevaccination protection rates for each influenza strain. *p=0.006 (TCZ vs Cont), p<0.0005 (TCZ vs MTX) and p=0.001 (TCZ vs TCZ+MTX). **p=0.007 (TCZ vs Cont, TCZ vs MTX) and p=0.023 (TCZ vs TCZ+MTX). (B) Seroresponse rates for three influenza strains in the RA treatment groups. *p=0.04 (TCZ vs MTX). **p=0.0009 (Cont vs MTX), p=0.002 (Cont vs TCZ) and p=0.022 (Cont vs TCZ+MTX). (C) Seroconversion rates for three influenza strains in the RA treatment groups. Seroconversion rates are expressed as percentages of patients with seroconversion out of seronegative patients before vaccination (antibody titres<10). *p=0.032 (TCZ vs MTX). **p=0.003 (Cont vs MTX) and p=0.002 (Cont vs TCZ+MTX). Data were compared using the χ^2 test or Fisher's exact probability test. Cont, RA control group; MTX, methotrexate group; RA, rheumatoid arthritis; TCZ, tocilizumab group; TCZ +MTX, combination therapy group.

TCZ. Uchiyama *et al*[17] reported that anti-TCZ antibodies are induced in monkeys receiving 30 mg/kg of TCZ weekly, suggesting that IL-6 does not play a crucial role in antibody production.

Most previous studies have shown that the use of MTX is unlikely to affect antibody response to influenza vaccine.[2–4 7 18] However, Gabay *et al*[19] have indicated that MTX significantly reduced responsiveness to AS03-adjuvanted pandemic H1N1 2009 (A/H1N1/2009) vaccine in patients with rheumatic diseases. The mechanism by which MTX impairs antibody response following vaccination is unknown, but several studies have proposed that MTX prevents proliferation of T cells and induces apoptosis in these cells.[20]

In conclusion, despite TCZ therapy, the immunogenicity of influenza vaccination appears to be conserved and sufficient in RA patients. MTX had a negative impact on vaccination efficacy, but adequate immune responses for protection were achieved by RA patients in the MTX and MTX+TCZ groups. Neither severe adverse effects nor RA flares were observed following vaccination. RA patients, even those receiving TCZ as monotherapy or in a combination therapy with MTX, should therefore be encouraged to receive influenza vaccination.

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.

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Competing interests None.

Patient consent Obtained.

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

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Impact of tocilizumab therapy on antibody response to influenza vaccine in patients with rheumatoid arthritis

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CONCISE REPORT

Pneumococcal polysaccharide vaccination in rheumatoid arthritis patients receiving tocilizumab therapy

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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

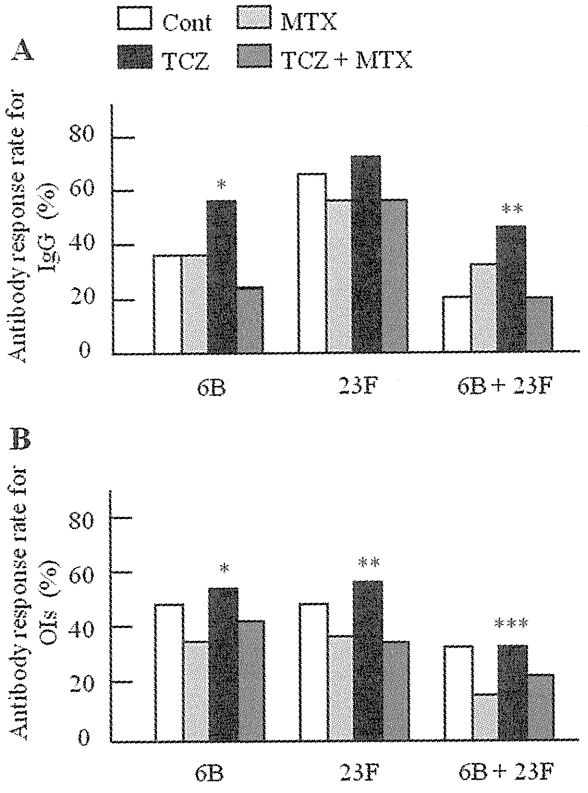

Figure 1 (A) Percentages of patients with twofold or more increases in serotype-specific IgG concentrations for serotypes 6B and 23F in the rheumatoid arthritis (RA) treatment groups. *p=0.046 (TCZ vs MTX) and p=0.0009 (TCZ vs TCZ+MTX). **p=0.005 (TCZ vs TCZ+MTX) and p=0.044 (TCZ vs Cont). (B) Percentages 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.

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Pneumococcal polysaccharide vaccination in rheumatoid arthritis patients receiving tocilizumab therapy

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Low opsonic activity to the infecting serotype in pediatric patients with invasive pneumococcal disease

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ABSTRACT

Serotype-specific protective immunity in pediatric patients with invasive pneumococcal disease (IPD) has not been fully investigated. To determine the protective immunity to the infecting serotype, the serotype-specific immunoglobulin G (IgG) levels and opsonization indices (OIs) were examined in 24 Japanese pediatric patients whose serum was collected within one month of an IPD episode between May 2008 and June 2011. The median age (range) of IPD patients was 17 (10–108) months and 63% were boys. In all 17 patients tested, the levels of serotype-specific IgG to the infecting serotype were higher than 0.2 μg/ml, but the OIs to the infecting serotype were <8. The avidities of 19F- or 6B-specific IgG in patients with levels higher than 5.0 μg/ml, but with undetectable OIs, were confirmed to be lower than those in patients with high OIs. Our data demonstrated that although the levels of serotype-specific IgG to the infecting serotype were higher than 0.2 μg/ml in sera of pediatric patients with IPD, the OIs were low one month after the IPD episode. Low opsonic activities in these patients may, in part, be explained by the low avidity of serotype-specific IgG.

1. Introduction

Streptococcus pneumoniae is a leading human pathogen that causes a wide variety of diseases, ranging from otitis media to pneumonia, bacteremia, and meningitis in both children and adults [1]. Antibodies to pneumococcal capsular polysaccharide (CPS) and complement provide protection against pneumococcal strains with homologous or cross-reactive capsular serotypes [2]. Seven-valent pneumococcal conjugate vaccine (PCV7; Prevnar®, Pfizer) has been used for children in the USA since 2000 [3], and the incidence of invasive pneumococcal disease (IPD) caused by the seven vaccine serotypes (VTs) has declined markedly, although the incidence of non-VT infection has not declined [4–6]. A recent study reported that the incidence rate of IPD in children less than 5 years old was 12.6–13.8 per 100,000 in Chiba prefecture, Japan, before the introduction of PCV7 [7]. However, no information is available regarding a possible high-risk population for IPD in Japan, as was reported for Navajo children in the United States [8].

PCV7 was licensed in Japan in October 2009, and a 3 + 1 schedule (three doses for the primary series and one booster) was approved and implemented (http://idsc.nih.go.jp/vaccine/dschedule.html). Further, the Japanese government decided in November 2010 to subsidize PCV7 for children below 5 years of age.

Vaccine-induced protective immunity is currently estimated by measuring the concentrations of serotype-specific immunoglobulin G (IgG) using enzyme-linked immunosorbent assay (ELISA) [9] and the opsonization index (OI) using a multiplex-opsonophagocytic assay (MOPA) [10]. The World Health Organization working group suggested a serotype-specific IgG of

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concentration of 0.35 μg/ml as a putative measure of protection at a population level against invasive disease in infants after immunization with pneumococcal conjugate vaccine [11]. This working group also reported that antibody concentrations of 0.2–0.35 μg/ml measured with the ELISA using serum without serum absorption with 22F polysaccharide correlated best with an OI of 8, which in turn correlates best with protective efficacy. Henckaerts et al. proposed a protective threshold concentration of 0.20 μg/ml assessed with ELISA using serum absorption with 22F polysaccharide as a measure of the serotype-specific IPD efficacy for the pneumococcal conjugate vaccine [12], with the exception of serotype 19F [13]. A recent study also reported that the serological response rate following a three-dose PCV7 primary vaccination as determined using a threshold of ≥0.2 μg/ml IgG and an OI ≥ 8 corresponded well with overall effectiveness against IPD [14]. Although this threshold may not be necessarily applicable to individual patients, it is of interest to determine the protective immunity to the infecting serotype in sera collected during the acute phase in pediatric patients with IPD.

In this study, we therefore examined the IgG levels and OIs to the infecting serotype in sera of pediatric patients within one month of an IPD infectious episode. We report that the opsonic activity to the infecting serotype is low in sera obtained within one month of an episode of IPD.

2. Materials and methods

2.1. Patients

Thirty-two pediatric patients, whose cultures from sterile sites, such as blood or cerebrospinal fluid, were positive for *S. pneumoniae* between May 2008 and January 2012 at 22 hospitals in Japan, were investigated in this study. All patients were enrolled in this study when their attending doctors requested the measurement of the antipneumococcal antibodies in their sera. Sera were obtained from these 32 patients after the episode of IPD. All of the pneumococcal isolates were serotyped using coagglutination tests with rabbit antisera (Statens Serum Institute, Copenhagen, Denmark) at the Department of Bacteriology I, National Institute of Infectious Diseases. Serotype 6C was confirmed by an in-house factor antiserum [15]. All eight patients were excluded from our studies of the protective immunity to the infecting serotype: six patients for whom sera were collected more than one month after the onset of the IPD, one patient who received intravenous immunoglobulin as a treatment of IPD, and one patient with an underlying hypogammaglobulinemia. Consequently, we evaluated antipneumococcal IgG and the OIs to the infecting serotype in 24 pediatric patients with IPD. This study was reviewed and approved by the Ethics Committee of the RIMD, Osaka University, and conducted according to the principles expressed in the Declaration of Helsinki.

2.2. ELISA

Antipneumococcal IgG antibodies were measured with the WHO approved ELISA using a standard reference serum (89-SF) and C-polysaccharide and 22F polysaccharide absorptions as previously described [9,16]. The levels of serotype-specific IgG for the infecting serotypes including 6B, 9V, 14, 19F and 23F were determined according to the WHO protocol [a detailed protocol is available at www.vaccine.uab.edu/ELISAProtocol (89SF)].

2.3. MOPA

A multiplexed opsonophagocytic killing assay (MOPA) for the infecting serotype based on antibiotic-resistant target bacteria was performed at the Research Institute for Microbial Diseases, Osaka University, as previously described [10]. The quality control serum was prepared from pooled sera of adults vaccinated with the 23-valent pneumococcal polysaccharide vaccine (PPV23; Pneumovax®, MSD), and this was used in each assay. The OI was defined as the serum dilution that killed 50% of bacteria, and the OIs were determined using opsotiter3 software according to the WHO protocol (at www.vaccine.uab.edu/UAB-MOPA). Only the OI results for the infecting serotypes including 6B, 6C, 14, 19A, 19F and 23F were used in this study.

2.4. Measurement of protective immunity

Neither the serotype-specific IgG nor the OI was available in one patient with serotype 15B and another with serotype 24F infection. Only the OI was available in three patients with serotype 19A and two patients with serotype 6C infection. The OIs were not determined in another five patients because their sera contained antibiotics. Consequently, the level of serotype-specific IgG or OI to the infecting serotype was measured in 17 patients, and both the levels of serotype-specific IgG and OIs were measured in only 14 patients.

2.5. Avidity of serotype-specific IgG

The avidity of the serotype-specific IgG in sera was evaluated using ELISA by the previously published method with a minor modification [17]. Serum samples that had been preadsorbed C-polysaccharide and 22F CPS were added to the coated microtiter plates, and the plates were incubated for 1 h at 37 °C. After washing the plates, sodium thiocyanate (NaSCN) at concentrations from 0 to 1.0 M was added to each well and the plates were incubated for 15 min at room temperature. After washing of the plates, diluted goat anti-human IgG HRP-conjugate was added to each well. After incubation for 1 h at room temperature, the substrate solution was added to the plates, followed by incubation for 20 min at room temperature. The optical density at 405 nm was measured. The avidity of serotype-specific IgG was expressed as the percentage of absorbance remaining after treatment with different concentrations of NaSCN.

3. Results

The clinical characteristics of the 24 pediatric patients with IPD are shown in Table 1. The diagnosis of these patients included meningitis ($n = 11$), bacteremia ($n = 10$), and bacteremic pneumonia ($n = 2$) and septic arthritis ($n = 1$). The median age (range) was 17 (10–108) months, and 63% were boys. Four patients (17%) had associated comorbid conditions including immune thrombocytopenia and splenectomy, meningoencephalocele, asplenia and single ventricle, and hydrocephalus (V-P shunt). In the 24 examined, the most common infecting serotype was 6B (9 isolates, 38%), followed by 19F (4 isolates, 17%), 19A (3 isolates, 13%), 6C and 14 (2 isolates each 8%) and one isolate each of 9V, 15B, 23F and 24F (4%). The median (range) period from the onset of IPD to the time of serum collection was two (0–23) days.

Three patients received PPV23 due to pre-existing medical conditions (Table 1). Before their episode of IPD, two patients infected with serotype 19F and one patient infected with serotype 9V received PPV23. Because PPV23 contains serotypes 19F and 9V, all three cases were considered PPV23 vaccine failure (VF). Ten patients received one to three doses of PCV7 at various ages as shown in Table 1. Only one patient (Case 18) completed a course of three doses of PCV7 between 2 and 6 months of age. The other nine patients were immunized with PCV7 during the catch-up phase. PCV7 breakthrough infection (BTI) was defined where a patient who received at least one dose of PCV7 had an episode

Table 1
Clinical characteristics of 24 pediatric patients with invasive pneumococcal disease (IPD).

No.	Age (months)	Sex	Diagnosis	Comorbid condition	Infecting serotype	Serum obtained days after IPD	Antibody to the infecting serotype		Vaccination before IPD (doses)	Age at each dose (month)	Category of IPD after PPV23	Category of IPD after PCV7	Outcome
							IgG (μg/ml)	OI					
1	108	M	Meningitis	ITP, splenectomy	19F	10	6.53	2	PPV23(1)	62	Vaccine failure	NA	Alive
2	50	M	Meningitis	Meningoencephalocele	19F	17	5.1	2	PPV23(1)	42	Vaccine failure	NA	Alive
3	75	M	Bacteremia	Asplenia, single ventricle	9V	1	0.57	NT	PPV23(1)	24	Vaccine failure	NA	Dead
4	14	M	Bacteremia	None	6B	11	0.34	2	None	–	NA	NA	Alive
5	38	M	Meningitis	None	19F	4	1.08	2	None	–	NA	NA	Alive
6	14	M	Bacteremia	None	14	5	2.1	5	None	–	NA	NA	Alive
7	13	M	Bacteremia	None	6B	4	2.25	NT	None	–	NA	NA	Alive
8	12	M	Meningitis	None	6B	20	1.81	7	PCV7(1)	10	NA	Breakthrough infection	Alive
9	10	M	Meningitis	None	19F	0	0.85	NT	None	–	NA	NA	Alive
10	17	M	Bacteremic pneumonia	None	19A	2	NA	NT	None	–	NA	NA	Alive
11	30	M	Bacteremic pneumonia	None	6B	0	0.53	2	PCV7(1)	28	NA	Vaccine failure	Alive
12	17	F	Meningitis	None	24F	1	NA	NA	PCV7(1)	16	NA	Non-VT infection	Alive
13	12	F	Meningitis	None	6B	12	0.78	2	None	–	NA	NA	Alive
14	10	M	Meningitis	None	15B	2	NA	NA	None	–	NA	NA	Alive
15	30	F	Bacteremia	None	6B	0	1.18	2	PCV7(1)	26	NA	Vaccine failure	Alive
16	26	F	Bacteremia	None	19A	1	NA	2	None	–	NA	NA	Alive
17	15	F	Bacteremia	None	14	0	1.75	2	None	–	NA	NA	Alive
18	10	M	Bacteremia	None	19A	0	NA	2	PCV7(3)	4, 5, 6	NA	Non-VT infection	Alive
19	30	F	Meningitis	Hydrocephalus (V-P shunt)	6B	23	0.92	2	PCV7(1)	28	NA	Vaccine failure	Alive
20	17	F	Meningitis	None	6B	0	1.38	2	PCV7(2)	9, 11	NA	Breakthrough infection	Alive
21	11	F	Septic arthritis	None	23F	0	0.55	2	PCV7(3)	7, 8, 9	NA	Breakthrough infection	Alive
22	16	F	Bacteremia	None	6B	0	5.62	2	None	–	NA	NA	Alive
23	49	M	Meningitis	None	6C	1	NA	2	PCV7(1)	36	NA	Non-VT infection	Alive
24	14	M	Bacteremia	None	6C	7	NA	NT	PCV7(2)	9, 10	NA	Non-VT infection	Alive

OI, opsonization index; ITP, immune thrombocytopenia; PPV23, 23-valent pneumococcal polysaccharide vaccine; PCV7, 7-valent pneumococcal conjugate vaccine; NA, not applicable; NT, not tested because of antibiotic use; VT, vaccine type.

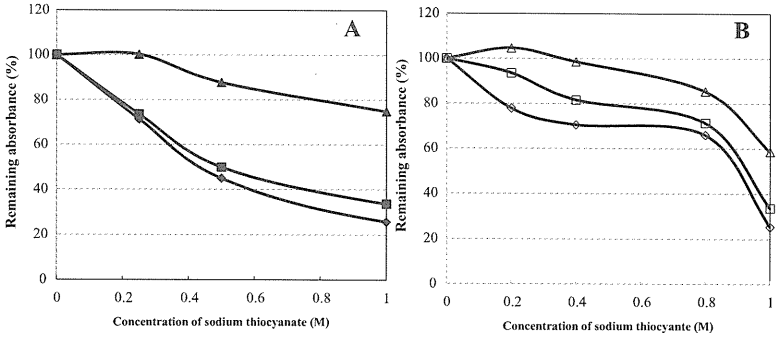

Fig. 1. Avidity of serotype 19F-specific IgG (A) and serotype 6B-specific IgG (B) in sera from pediatric patients with invasive pneumococcal diseases. Two serum samples from Case 1 (closed diamond) and Case 2 (closed square), and the positive control serum (closed triangle) from Case 6 (four months after the episode of IPD and one month after two doses of PCV7 vaccination) were examined for the avidity of serotype 19F-specific IgG. Two serum samples from Case 22 before (open diamond) and after (open square) two doses of PCV7 vaccination, and a positive control serum (open triangle) collected from Case 6 after two doses of PCV7 were used to test the avidity of serotype 6B-specific IgG.

of IPD for which the pneumococcal isolate was a PCV7 serotype, and PCV7 VF was defined as the subset of BTI in which the patient had completed the Advisory Committee on Immunization Practice (ACIP)-recommended PCV7 vaccine schedule at least two weeks before the IPD [18,19]. An instance of an IPD patient who had had at least one dose of PCV7 and for whom the pneumococcal isolate was not a PCV7 serotype was defined as PCV7 non-VT infection. Of 10 patients who received PCV7 previously, three cases (Cases 11, 15 and 19) were classified as PCV7 VF, and three cases (Cases 8, 20 and 21) were classified as PCV7 BTI. The other four cases (Cases 12, 18, 23 and 24) were classified as PCV7 non-VT infection.

The level of serotype-specific IgG or the OI for the infecting serotype was determined for 17 of 24 cases. The levels of specific IgG for the infecting serotype ranged widely from 0.34 to 6.53 μg/ml. In all 17 cases, the level of specific IgG for the infecting serotype was higher than 0.20 μg/ml, the putative threshold for preventing IPD [12,14]. The geometric mean concentration for the 17 cases was 1.35 μg/ml. In contrast, the OI for the infecting serotype was <8 in all of 17 cases. In particular, obvious discrepancies were found in two patients with serotype 19F (Cases 1 and 2) and one patient with serotype 6B (Case 22) who had serotype-specific IgG higher than 5 μg/ml and undetectable OI.

To investigate these discrepancies, we next examined the avidities of serotype 19F-specific IgG in sera from Cases 1 and 2, and the avidities of serotype-6B specific IgG in sera from Case 22. The percentages of remaining absorbance to 19F CPS of the positive control serum (IgG 7.25 μg/ml, OI 2336) collected from a patient (Case 6) after two doses of PCV7 vaccination were 100–75% at concentrations of 0.25–1.0 M of NaSCN (Fig. 1A). In contrast, the percentages of remaining absorbance to 19F CPS of sera from Cases 1 (IgG 6.53 μg/ml, OI 2) and 2 (IgG 5.10 μg/ml, OI 2) to 19F CPS were 74–44% and 71–26% at concentrations of 0.25–1.0 M of NaSCN, respectively.

The percentages of remaining absorbance to 6B CPS of the positive control serum (IgG 4.16 μg/ml, OI 4626) collected from Case 6 after two doses of PCV7 99–59% at concentrations of 0.4–1.0 M of NaSCN (Fig. 1B). In contrast, the percentages of remaining absorbance of serum from Case 22 before PCV7 vaccination (IgG 5.62 μg/ml, OI 2) and after two doses of PCV7 vaccination (IgG 2.37 μg/ml, OI 562) were 71–25% and 81–34% at concentrations of 0.4–1.0 M of NaSCN.

4. Discussion

In pediatric patients with IPD, the serum OIs for the infecting serotype within one month after the infectious episode were <8 in all 17 patients tested for OI, although the levels of IgG for the infecting serotype were higher than 0.2 μg/ml in all 17 patients tested for serotype-specific IgG. Undetectable OIs suggest that the serotype-specific IgG in their sera are largely nonfunctional. Soininen et al. similarly reported that sera from unimmunized children without nasopharyngeal carriage contained serotype-specific IgG, but infrequently had serotype-specific opsonic activity [20].

Three patients received PPV23 before PCV7 was licensed in Japan in 2009 because they were at increased risk for pneumococcal disease. Although the current guideline of the ACIP recommends that children aged 2–18 years with underlying medical conditions should receive PPV23 after completing all recommended doses of PCV13 [21], pediatricians should be aware of the possible induction of nonfunctional IgG by PPV23 in high-risk children aged >2 years. Two patients with PCV7 BTI received one or two doses of PCV7 9–11 months after birth, and two patients with PCV7 VF received only one dose of PCV7 26–28 months after birth. All four of these patients comprised the catch-up cases for PCV7. Interestingly, all cases with BTI or VF were caused by serotype 6B. A recent study from the US reported that 155 of 753 (21%) pediatric IPD cases were PCV7 BTIs caused predominantly by serotypes 6B (32%) and 19F (29%) [18]. The PCV7 BTIs caused by serotype 6B were more likely to have occurred in children who received only one or two PCV7 doses (84%) compared with infections caused by other VTs (61%). Rennels et al. also reported a low immune response to 6B and other serotypes, including 9V and 18C in children who received fewer than three doses of PCV7 [22].

Our data demonstrated that sera collected from Cases 1, 2 and 22 containing 19F- or 6B-specific IgG levels higher than 5.0 μg/ml, but lacking opsonic activity, contained lower avidity of serotype-specific IgG than the positive control sera with high OIs. An improvement of the avidity of 6B-specific IgG was confirmed in the sera with a high OI from Case 22 by two doses of PCV7 vaccination. Two previous studies using sera from healthy adults with or without vaccination with PPV23 demonstrated that higher avidity antibodies were more effective than lower avidity antibodies in in vitro complement-dependent opsonophagocytosis and for in vivo protection against pneumococcal infection in mice [23,24]. These data are, partially, in agreement with our findings of high levels of serotype-specific IgG with low avidity in serum from pediatric patients within one month after IPD. The low avidity of serotype-specific IgG levels may explain the undetectable OIs in sera collected from Cases 1, 2 and 22 within one month of an IPD episode.

O'Brien et al. recently reported the pneumococcal antibody status in a child with of PCV7 vaccine failure caused by serotype 14

[25]. In this patient, the serotype-specific IgG and the OIs in serum were 4.98 μg/ml and 1024, respectively, 35 days after the administration of three doses of PCV7. However, this patient developed occult bacteremia at 9.6 months of age, 53 days after the third dose of PCV7. Because of a slightly decreased serotype-specific IgG (4.25 μg/ml) and a significantly decreased OI of 4 in the serum of this patient after this episode of IPD, the authors suggested that the functional antibodies existing during infection with consumed by binding to the serotype 14 antigen. This finding also suggests that the ELISA assay detected some nonspecific or nonfunctional IgG in the serum of this patient, and is in agreement with the findings in the sera of our pediatric patients with IPD.

The limitations of our study are the small number of IPD cases examined and the variable timing of serum collection, although the sera were all collected within one month after the IPD episode. These limitations meant that we were unable to compare the induction of opsonic activity to the infecting serotype between the acute phase and the convalescence phase in pediatric patients with IPD.

In conclusion, in all of 17 patients tested within one month of an IPD episode, the serum OIs to the infecting serotype were <8, whereas the levels of serotype-specific IgG were higher than 0.2 μg/ml. Low avidity of the serotype-specific IgG were confirmed in three patients associated with the serotype-specific IgG levels higher than 5 μg/ml, but with undetectable OIs.

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Appendix A. The Japanese IPD Study Group

In addition to TO, NI, KM, JN, BC, BC, KT, YA, KO, the members of the Japanese IPD Study Group are Kenji Okada (National Fukuoka Hospital), Takashi Nakano (Kawasaki Medical University), Hideki Akeda (Okinawa Prefectural Nanbu Medical Center), Masako Habu (Tokyo Metropolitan Bokutoh General Hospital), Eri Yamaguchi (Chidoribashi Hospital), Kei Komiya (Nihon University School of Medicine), Shinji Kido (Toyota Memorial Hospital), Takahiro Niizuma (Koshigaya Municipal Hospital), Masato Arao (Saitama Medical University), Fumie Ishiwada (Chiba Kaihin Municipal Hospital), Mai Kubota (Shizuoka Children's Hospital), Kenji Furuno (National Fukuoka-Higashi Medical Center), Yoshio Yamaguchi (National Hospital Organization Tochigi Hospital), Kaoru Obinata (Juntendo University Urayasu Hospital), Mikio Yoshioka (KKR Sapporo Medical Center), and Tomomi Naito (Saiseikai Kawaguchi General Hospital).

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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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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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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.

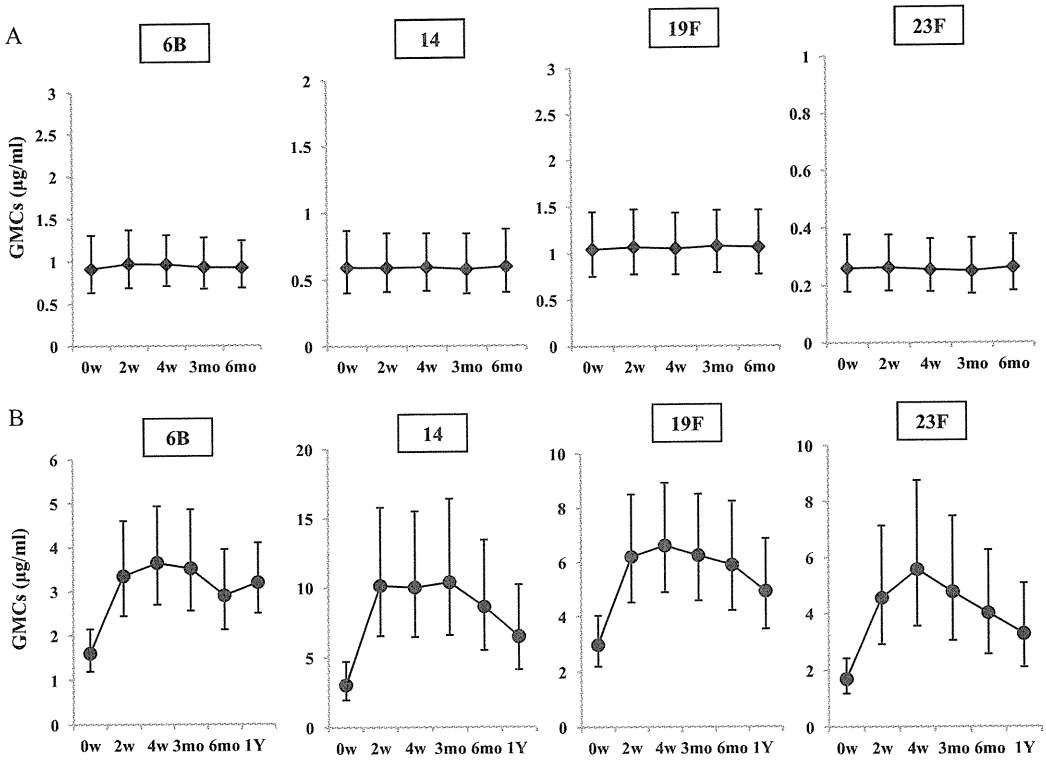

Fig. 1. Serum levels of anti-pneumococcal polysaccharide Abs after PPV injection. Concentrations of IgM (A: $n=15$) and IgG (B: $n=55$) Abs against each serotype of pneumococcal capsular polysaccharide in sera were measured at indicated time points after PPV administration. Data are shown as the geometric 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