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Additive effect of pneumococcal vaccine and influenza vaccine on acute exacerbation in patients with chronic lung disease

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ABSTRACT

To determine the clinical efficacy of combined vaccination with 23-valent pneumococcal vaccine (PV) and influenza vaccine (IV) against pneumonia and acute exacerbation of chronic lung diseases (CLD), we conducted an open-label, randomized, controlled study among 167 adults with CLD over a 2-year period. Subjects were randomly assigned to a PV + IV group ($n = 87$) or an IV group ($n = 80$). The number of patients with CLD experiencing infectious acute exacerbation ($P = 0.022$), but not pneumonia ($P = 0.284$), was significantly lower in the PV + IV group compared with the IV group. When these subjects were divided into subgroups, an additive effect of PV with IV in preventing infectious acute exacerbation was significant only in patients with chronic obstructive pulmonary diseases ($P = 0.037$). In patients with CLD, the Kaplan–Meier survival curves demonstrated a significant difference for infectious acute exacerbation ($P = 0.016$) between the two groups. An additive effect of PV with IV on infectious acute exacerbation was found during the first year after vaccination ($P = 0.019$), but not during the second year ($P = 0.342$), and was associated with serotype-specific immune response in sera of these patients who used PV during the same period.

1. Introduction

Acute exacerbation frequently occurs among patients with chronic lung diseases (CLD), such as chronic obstructive pulmonary disease (COPD) and sequelae of pulmonary tuberculosis (SPTB) [1,2]. Morbidity, mortality and health-care costs of these patients largely result from on acute exacerbations [3]. Acute exacerbations are primarily triggered by bacterial or viral pathogens in COPD and SPTB. While *Streptococcus pneumoniae* (*S. pneumoniae*) is the most commonly identified cause of community-acquired pneumonia (CAP) by accounting for 16.5–38.9% of CAP among adults [4,5], this pathogen is also responsible for 8–25% of acute exacerbation in patients with CLD or COPD, which makes it a major bacterial pathogen [2,6,7]. Viral pathogens are also capable of inducing acute exacerbation of COPD, and the influenza virus was frequently detected in 5–29% of exacerbation of COPD [8,9].

Since antibodies to pneumococcal capsular polysaccharide (PPS) and complement provide protection against *S. pneumoniae* with homologous or cross-reactive capsular serotypes [10], pnemococcal polysaccharide vaccine (PV) is effective for preventing invasive pneumococcal diseases in patients with chronic illness, such as CLD. PV is, therefore, recommended for these patients [11–13]. Although the previous studies reported that PV is not effective in preventing pneumonia or acute exacerbation in patients with COPD [14–16], a recent, prospective study demonstrated an effect of PV in preventing pneumonia in such patients with less than 65 years of age with severe airflow obstruction [17]. In addition, a retrospective study previously reported the additive effects of PV with influenza vaccine (IV) in the reduction of hospitalization

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stays and death among elderly persons with CLD [18,19]. Although a large-scale prospective study demonstrated the additive effects of PV and IV in reducing hospital mortality due to pneumonia among elderly persons [20], no prospective study has been conducted to find the additive effects of PV combined IV for preventing pneumonia or acute exacerbation in patients with CLD. This open-label, randomized, controlled study was designed to determine whether PV and IV combined are superior to IV alone in preventing pneumonia or acute exacerbation among patients with CLD.

2. Materials and methods

2.1. Study design

For this study, 191 patients with CLD in a stable condition were enrolled after providing written informed consent at the respiratory clinic of 13 hospitals in the district of Kyushu and Okinawa, Japan between November 2001 and April 2002. All potentially eligible subjects (at least twice as many as the enrolled cases) were contacted by the members of the Pneumococcal Vaccine Trialist Group, belonging to one of these hospitals. As the study investigators, these doctors had a role in selecting the subjects for the study enrollment. Inclusion criteria were patients with CLD who previously experienced acute exacerbations, were able to comply with a schedule of monthly clinical visits and were between 40 and 80 years of age. Patients who were pregnant or had immunocompromised conditions such as active malignant diseases, renal insufficiency in dialysis or HIV infection, hypogammaglobulinemia or anatomical or functional asplenia and who had previously received 23-valent PV (Pneumovax, Banyu, Japan) were excluded. The enrollees were randomly assigned in equal proportion to either the group receiving PV and IV (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) or IV alone. By sealed envelope, each study site was randomly allocated either 10 or 20 cases. Equal numbers of sealed envelopes each containing a card indicating either the PV + IV group or the IV group were prepared, and each study participant chose an envelope. This vaccine allocation was done by the study investigators. In addition, the doctors who screened the subjects also conducted the exclusions and enrollments.

While the participants in the PV + IV group were separately immunized with 0.5 ml of PV and 0.5 ml of IV on separate occasions in 1 month intervals, the participants in the IV group were immunized with 0.5 ml of IV alone. All participants received IV once in both the 2001/2002 and 2002/2003 seasons. For this study, our group used a trivalent, split-virion, influenza vaccine, containing A/NewCaledonia/20/99H1N1, A/Panama/2007/99H3N2, and B/Johannesburg/5/99 for the 2001/2002 season; and for the 2002/2003 season, the study was conducted using vaccine containing A/NewCaledonia/20/99H1N1, A/Panama/2007/99H3N2 and B/Guangdong/7/97.

Demographic data were obtained from each participant at the time of enrollment. All participants were examined, typically once a month, at each hospital by physicians who were the members of the Pneumococcal Vaccine Trialist Group at each study hospital in the Kyushu and Okinawa districts. Patients were also asked to visit each study hospital for examination by a study physician, if they developed a fever, cough and sputum, or experienced breathlessness during the 2-year follow-up period.

To monitor the concentrations of anti-PPS IgG, serum samples were collected from the patients of the PV + IV group immediately before and at 1 month, 6 months, 1 year, and 2 years after the initial pneumococcal vaccination. Separated sera were stored frozen at −80 °C until used. All studies described herein were approved by the institutional review board of each institution.

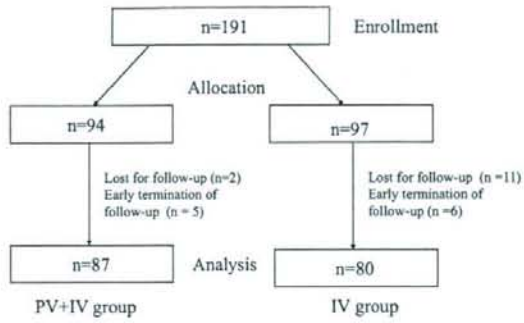

Fig. 1. Flow diagram of study patients with chronic lung disease.

2.2. Study population

One hundred and ninety-one patients with CLD were assigned to either the PV + IV group ($n = 94$) or to the IV group ($n = 97$) (Fig. 1). During 2-year follow-up period, 2 and 11 subjects were lost from the PV + IV group and IV groups, respectively. In addition, an early termination of follow-up occurred for 5 subjects for the PV + IV group and for 6 subjects in the IV group because they wanted to withdraw from the study. Subsequently, 87 subjects in the PV + IV group and 80 subjects in the IV group completed the analysis.

2.3. Outcome measures

The outcome measured was recorded as either the time to the first episode of pneumonia or to acute exacerbation after the enrollment in this study. A pneumonia diagnosis was based on either clinical symptoms (cough, sputum or fever) plus increased white blood cell counts or serum C-reactive protein, and the appearance of a new infiltration on a chest radiograph [21]. Acute exacerbation in CLD was defined by criteria as previously described [22]: (1) increased dyspnea, (2) increased sputum volume and (3) increased sputum purulence, and (4) absence of newly appeared infiltration on a chest radiograph. Acute exacerbation was diagnosed when two of the three respiratory symptoms existed or when one of these and one of additional symptoms, such as a fever without any other causes or increased cough was present [23]. When the laboratory examinations revealed an increase in their white blood cell counts or serum C-reactive protein, in addition to the clinical symptoms of acute exacerbation, patients were diagnosed as infectious acute exacerbation, and were therefore classified into one of two categories: either infectious or non-infectious acute exacerbation. Furthermore, when *S. pneumoniae* was isolated from purulent sputum in cases of acute exacerbation, patients were diagnosed as pneumococcal acute exacerbation as a subcategory of infectious acute exacerbation.

2.4. Measurement of anti-PPS IgG

Serum samples for serotype-specific IgG were available from only 35 of 87 patients with CLD in the PV + IV group for all intervals—1 month, 6 months, 1 year, and 2 years. 7 subjects died during the 2-year period, and 45 of the remaining subjects lacked at least one serum sample from the time interval at either 6 months, 1 year or 2 years. The concentrations of serotype-specific IgG were measured as previously described elsewhere [24]. The US reference

pneumococcal antiserum (89-SF), courtesy of Dr. Carl Frasch, was adsorbed to CWPS, but all other samples were adsorbed to CWPS (5 μg/ml) and 22F PPS (10 μg/ml) [25]. Serotype-specific IgG was determined for the four serotypes (6B, 14, 19F and 23F) that are the most prevalent among adult patients with pneumococcal infections in the US and Japan [5,26].

2.5. Statistical analysis

The case numbers of patients with COPD, SPTB, and other CLDs, and the case number of patients experiencing pneumonia, infectious acute exacerbation, pneumococcal acute exacerbation, and non-infectious acute exacerbation and the case numbers of death were compared using a χ^2-test. A Kaplan–Meier estimator was used to calculate the survival curve for subjects who developed neither pneumonia nor infectious or non-infectious acute exacerbation during the 2-year study period. The Cox's proportional hazard model was used to evaluate the effect of PV on the incidence of pneumonia and infectious or non-infectious exacerbation both in the first and second 1-year. The effects of age (age older than 55 years, 65 years, and older than 75 years) and female sex were considered in multivariate analysis. Differences in geometric mean concentrations (GMCs) of serotype-specific IgG over time were assessed using the Wilcoxon signed-ranks test. An interim target sample size of 82 was chosen to ensure that there would be at least an 80% chance to detect a difference of 0.2 (0.2 vs. 0.4) episodes per person per year, with a one-sided alpha level of 0.05, in the frequency of admission due to pneumonia between groups of PV + IV and IV. Data was considered to be statistically significant, if the P values were less than 0.05.

3. Results

Ages (mean ± S.D. years) and male sex (%) were 69.0 ± 9.0 and 63.5 for total subjects, 67.8 ± 9.5 and 69.0 for the PV + IV group, and 70.1 ± 7.4 and 57.5 for the IV group. The numbers of patients with three subcategories of CLD (COPD, SPTB and other CLDs) in the PV + IV and the IV groups are shown in Table 1. Other CLDs were bronchiectasis (n = 20; 10 for the PV + IV group and 10 for the IV group), bronchial asthma (n = 13, 6 for the PV + IV group and 7 for the IV group), pneumoconiosis (n = 14, 7 for the PV + IV group and 7 for the IV group), interstitial pneumonia (n = 9, 3 for the PV + IV group and 6 for the IV group), diffuse panbronchiolitis (n = 5, 4 for the PV + IV group and 1 for the IV group), and sarcoidosis (n = 1 for the IV group). Fifty-nine subjects received home oxygen therapy (HOT) (27 for the PV + IV and 32 for the IV), and 11 subjects (6 for the PV + IV and 5 for the IV) were treated with noninvasive positive pressure ventilation (NPPV). While a significant difference was found in the number of patients with SPTB between the two groups, no significant difference was found in the number of patients with COPD or other CLDs, nor in the number of patients receiving either HOT or NPPV.

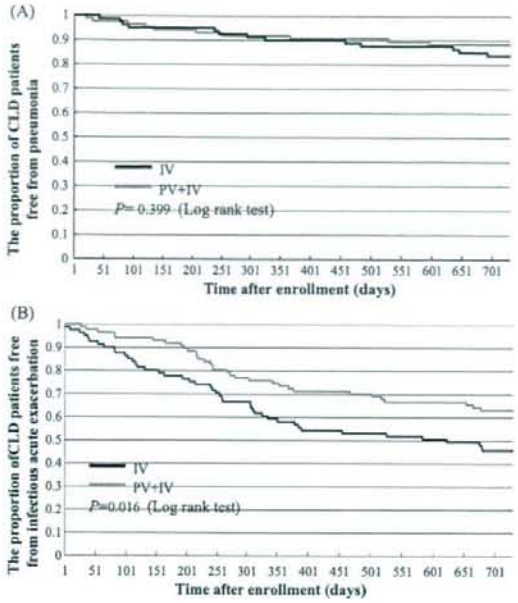

Fig. 2. Kaplan–Meier survival curves for patients with chronic lung diseases (CLD) showing the proportion of subjects free from pneumonia (A) and infectious acute exacerbation (B) between PV + IV group and IV group during the follow-up period.

The incidence of pneumonia (0.096 episodes/(patient year)) with CLD in this study was twice as higher as those of previous cohort studies of COPD patients (0.047–0.055 episodes/(patient year)) [14,17]. No significant difference was found in the number of patients developing pneumonia between the PV + IV group and the IV group (Table 1). Similarly, no significant difference was found in the Kaplan–Meier survival curves for pneumonia between the two groups (P = 0.399, Fig. 2A).

Table 1
Demographic features and outcome of patients with chronic lung diseases (CLD)

Characteristics	No. of patients (%)			P-value
	All subjects (n = 167)	PV + IV group (n = 87)	IV group (n = 80)	
Subcategories of CLD				
COPD	55(32.9)	24(27.6)	31(38.8)	0.125
Sequelae of pulmonary tuberculosis	50(30.0)	33(37.9)	17(21.3)	0.018
Other CLDs	62(37.1)	30(34.5)	32(40.0)	0.461
Outcome				
Pneumonia	25(32.9)	13(27.6)	12(38.8)	0.284
Acute exacerbation				
Infectious	76(45.5)	32(36.8)	44(55.0)	0.022
Pneumococcal	6(3.6)	1(1.1)	5(6.3)	0.106
Non-infectious	15(9.0)	9(10.3)	6(7.5)	0.557
Death	14(8.4)	7(8.0)	7(8.8)	0.870

PV: pneumococcal polysaccharide vaccine, IV: influenza vaccine, COPD: chronic obstructive pulmonary disease. Acute exacerbations are classified into two categories: infectious and non-infectious acute exacerbation. Infectious acute exacerbation involves pneumococcal acute exacerbation.

Table 2
Outcome of patients with COPD, sequelae of pulmonary tuberculosis (SPTB) and other chronic lung diseases (CLDs) **by vaccine group** during 2 years after vaccination

Subcategories of CLD/outcome	No. of patients (%)		*P*-value
	PV + IV group (n = 87)	IV group (n = 80)	
COPD			
Pneumonia	6 (**6.9**)	5 (**6.3**)	0.615
Acute exacerbation			
Infectious	9 (**10.3**)	21 (**26.3**)	0.037
Non-infectious	5 (**5.7**)	2 (**2.5**)	0.315
SPTB			
Pneumonia	3 (**3.4**)	2 (**2.5**)	0.218
Acute exacerbation			
Infectious	10 (**11.5**)	7 (**8.7**)	0.442
Non-infectious	2 (**2.3**)	3 (**3.8**)	0.594
Other CLDs			
Pneumonia	4 (4.6)	5 (6.3)	0.379
Acute exacerbation			
Infectious	13 (**14.9**)	16 (**20.0**)	0.599
Non-infectious	2 (2.3)	1 (1.3)	0.616

PV: pneumococcal polysaccharide vaccine, IV: influenza vaccine, COPD: chronic obstructive pulmonary diseases. Acute exacerbations are classified into two categories: infectious and non-infectious acute exacerbation.

The incidence of acute exacerbation (0.53 episodes/(patient year)) in this study was slightly lower than those of previous studies of COPD patients (0.85–1.08 episodes/(patient year)) [27,28]. No significant difference was found in the number of CLD patients with pneumococcal acute exacerbation (P = 0.106) or non-infectious acute exacerbation (P = 0.557) between the two groups (Table 1). In contrast, a significant difference was found in the number of CLD patients with infectious acute exacerbation (P = 0.022) between the two groups (Table 1). The Kaplan–Meier survival curves for CLD patients with infectious acute exacerbation (P = 0.016, Fig. 2B) also demonstrated a significant difference between the two groups. However, no significant difference was found in the mortality during the 2-year period after vaccination in both groups (Table 1).

Furthermore, we examined the number of patients experiencing pneumonia and infectious or non-infectious acute exacerbation in each subcategory (Table 2). No significant difference was found in the number of patients with COPD, SPTB and other CLDs experiencing either pneumonia or non-infectious acute exacerbation. In contrast, a significant difference was found in the number of patients with COPD experiencing infectious acute exacerbation (P = 0.037), but not in the number of patients with SPTB (P = 0.442) or other CLDs (P = 0.599). In COPD patients, the

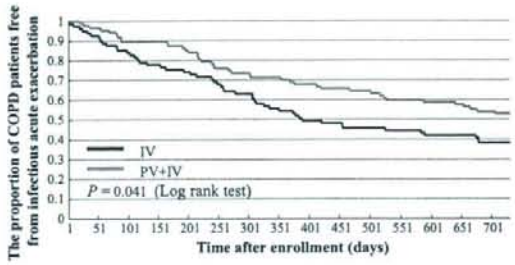

Fig. 3. Kaplan–Meier survival curve for patients with chronic obstructive pulmonary diseases (COPD) showing the proportion of subjects free from infectious acute exacerbation between PV + IV group and IV group during the follow-up period.

Kaplan–Meier survival curves for infectious acute exacerbation demonstrated a significant difference between the two groups (P = 0.041, Fig. 3), while no significant difference was found in the Kaplan–Meier survival curves for pneumonia (P = 0.543) or non-infectious acute exacerbation (P = 0.426) in COPD patients (data not shown).

While neither a univariate nor multivariate analysis demonstrated a significant association between any variables and the probability of either pneumonia or non-infectious acute exacerbation in either the first or second year, a significant association between the receipt of PV and a low probability of infectious acute exacerbation was found in patients with CLD in the first year, but not in the second year by a single variable analysis (P = 0.019) or multivariate analyses (P = 0.016, Table 3). No significant association was demonstrated between any variable, such as age older than 55 years, age older than 65 years, age older than 75 years or the female sex and the probability for infectious acute exacerbation, in either the first or second year.

The GMCs of serotype-specific IgG for four serotypes in sera from the 35 patients with CLD during the 2 years after their vaccinations are shown in Table 4. Before vaccination, the GMCs ranged from 2.6 μg/ml for serotype 23F to 5.69 μg/ml for serotype 14. One month after vaccination, significant increases in the GMCs of serotype-specific IgG were found for all serotypes in all subjects, compared to those before vaccination (P < 0.01, Table 4). The GMCs of serotype-specific IgG declined below pre-vaccination levels at 6 months post-vaccination for types 6B and 19F. In contrast, the GMCs of serotype-specific IgG remained above the pre-vaccination levels for type 23F and type 14 at 2 years post-vaccination, although they had declined from the immediate post-vaccine levels. Serotype-

Table 3
Estimated result of Cox's proportional hazard model for pneumonia, infectious acute exacerbation, and non-infectious acute exacerbation in patients with chronic lung diseases during the first and the second year

Period	Variables	Pneumonia		Acute exacerbation, infectious		Acute exacerbation, non-infectious	
		Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
First year	PV	0.70 (0.31–1.60)	0.402	0.58 (0.37–0.91)	0.019	1.40 (0.50–3.94)	0.522
	PV	0.72 (0.32–1.68)	0.452	0.56 (0.35–0.90)	0.016	1.56 (0.54–4.52)	0.411
	Age older than 55 years old	N.A.	N.A.	1.02 (0.33–3.12)	0.973	1.97 (0.22–17.4)	0.543
	Age older than 65 years old	1.05 (0.13–8.52)	0.963	1.15 (0.40–3.27)	0.798	0.33 (0.03–3.76)	0.375
	Age older than 75 years old	2.63(0.33–20.8)	0.359	0.92 (0.31–2.75)	0.878	1.97 (0.23–17.1)	0.534
	Female	0.41(0.14–1.23)	0.113	1.01 (0.63–1.61)	0.972	1.01 (0.33–3.08)	0.982
Second year	PV	0.54 (0.13–2.25)	0.397	0.65 (0.26–1.59)	0.342	0.99 (0.28–3.51)	0.987
	PV	0.47 (0.11–2.05)	0.314	0.64 (0.25–1.64)	0.351	1.03 (0.26–3.98)	0.969
	Age older than 55 years old	N.A.	N.A.	1.11 (0.11–10.76)	0.93	1.36 (0.12–14.97)	0.800
	Age older than 65 years old	0.31 (0.31–3.05)	0.315	1.46 (0.18–12.1)	0.725	0.36 (0.03–4.29)	0.418
	Age older than 75 years old	0.60 (0.61–5.77)	0.655	0.81 (0.09–7.73)	0.857	1.09 (0.11–10.78)	0.939
	Female	0.22 (0.27–1.84)	0.163	1.18 (0.47–2.99)	0.72	1.18 (0.32–4.37)	0.797

N.A.: not applicable, PV: pneumococcal polysaccharide vaccine, CI: confidence interval.

Table 4
Serotype-specific IgG in sera from 35 patients with chronic lung diseases before 1 month, 6 months, 1 year and 2 years after vaccination

Serotype	Time point	GMC of IgG (μg/ml) (95% CI)
6B	Pre	3.46(2.51–4.76)
	1 month	5.26(3.78–7.54)*
	6 months	3.51(2.48–5.1)
	1 year	3.41 (2.48–4.85)
	2 year	2.68(1.86–3.97)
14	Pre	5.69(4.44–7.03)
	1 month	12.63(8.76–16.61)*
	6 months	10.35(7.17–13.6)*
	1 year	8.97(6.36–11.47)*
	2 year	7.76(5.53–10.07)*
19F	Pre	4.91 (3.67–6.55)
	1 month	7.05 (5.37–9.3)*
	6 months	4.81 (3.56–6.49)
	1 year	4.60(3.56–5.93)
	2 year	4.58 (3.46–6.05)
23F	Pre	2.60(1.97–3.42)
	1 month	5.82(3.71–9.12)*
	6 months	4.06 (2.72–6.05)*
	1 year	3.56(2.48–5.1)*
	2 year	2.8(1.9–4.1)

GMC: geometric mean concentration, CI: confidence interval.
* $P < 0.01$ (for comparison with pre-vaccination value).

specific IgG responses to PV, therefore, were found primarily in sera during the first year, but not during the second year, following vaccination.

4. Discussion

This study demonstrated an additive effect of PV with IV in preventing infectious acute exacerbation, but not pneumonia or non-infectious acute exacerbation, when compared to IV alone in patients with CLD. When theses subjects were divided into three subgroups according to their type of CLD, an additive effect of PV with IV in preventing infectious acute exacerbation was found only in COPD patients, but not in patients with SPTB or other pulmonary diseases. Since no attempt was made to blind the clinical assessors to the vaccine allocation in this study, the possibility of the bias on the clinical assessment obtained by the investigators can not be dismissed.

Although this was an open-label, randomized controlled study on a small scale, the enrolled patients with CLD in this study were at a high risk for pneumonia or acute exacerbation. Since pneumococci has a major role in the development of pneumonia associated with respiratory viruses such as influenza virus, these viruses contribute to the pathogenesis of bacterial pneumonia among children [29]. Other investigators have also demonstrated that influenza neuraminidase facilitated bacterial adherence of *S. pneumoniae*, and resulted in secondary bacterial pneumonia in mice [30,31]. Based on the interaction between influenza virus and pneumococci on the pathogenesis of bacterial pneumonia, an additive or synergistic effect of the combined vaccination with PV and IV in preventing pneumococcal pneumonia can be expected.

A large prospective study in Sweden reported the additive effects of PV with IV in reducing pneumococcal pneumonia as well as invasive pneumococcal diseases, compared to no vaccination, among adults aged 65 years or older during the first 6 months after vaccination [32]. The authors demonstrated the additive effect of PV with IV only in reducing the hospital mortality due to pneumonia, compared to a vaccination with IV alone or PV alone, at 1-year after vaccination [20]. Another investigator, however, did not find an additive effect of PV with IV in preventing pneumococcal pneumonia as well as pneumonia, compared to IV alone, among the same population during a 2-year period after vaccination [33].

A double-blind, randomized placebo-controlled trial in Thailand recently reported that IV was found highly effective in preventing the influenza-related acute respiratory illness, but not acute exacerbation or pneumonia, in COPD patients [23]. Since no effect of IV was found for preventing acute exacerbation among patients with COPD, our data suggests not only the importance of PV in addition to IV among patients with CLD, but also the role of pneumococcal infection in the pathogenesis of acute exacerbation in such patients.

Although no significant difference was found in the number of patients with pneumococcal acute exacerbation between the two groups in this study, a reduced number of pneumococcal infections in the PV + IV group may suggest the contribution of protective immunity raised by PV in such patients. More importantly, the additive effect of PV with IV on infectious acute exacerbation was significant during the first year, but not the second year after vaccination. This effect was associated with an immune response of serotype-specific IgG to PV, which was prominent in all serotypes during the first year.

Although the protective concentrations of serotype-specific IgG are not known, most of CLD patients showed a level of IgG much higher than the threshold (0.35 μg/ml) that predicts protection in infants against invasive disease at a population level after immunization with pneumococcal conjugate vaccine in this study [34]. A recent study also reported that higher levels of antipneumococcal antibodies did not correlate with protection from pneumococcal colonization in patients with COPD [35]. Therefore, the question arises as to why an additional increase of IgG in sera is required for preventing infectious acute exacerbation in COPD patients. A recent report demonstrated that the levels of anti-serotype-1 IgG in bronchoalveolar lavage (BAL) fluid were less than 10 ng/ml in 25 of 49 HIV-uninfected adults, while the mean level of serotype-1 specific IgG in sera of such subjects was 1608 ng/ml [36]. Thus, the level of specific IgG in the neat BAL fluid is at least 161 times lower than that in sera. According to this information and the data of levels of specific IgG in sera of patients with CLD before (2.60–5.69 μg/ml) and after vaccination (5.26–12.63 μg/ml) in this study, the levels of specific IgG in BAL fluid are estimated to be less than 35.3 ng/ml before vaccination and less than 78.5 ng/ml after vaccination, respectively. Since we found a significant effect of PV + IV on infectious acute exacerbation, compared with IV alone, the levels of serotype-specific IgG that rise higher than approximately 35 ng/ml in the lower airway fluid may be critical for preventing infectious acute exacerbation in patients with COPD.

Furthermore, the cost of acute exacerbation on patients with COPD creates a significant economic impact [3]. The literatures reports that the estimated costs for hospitalization due to acute exacerbation will range from $5655 to $7413 in developed countries [37–39]. The reduced frequency (27.2%) of acute exacerbation in the PV + IV group in this study, compared to the IV group, has significant economic implications for patients with COPD.

In conclusion, this pilot study demonstrated an additive effect of PV in combination with IV on infectious acute exacerbation of patients with COPD. This effect was closely associated with serotype-specific immune response to PV that was primarily found during the first year after vaccination. Further studies on a larger scale are required to investigate the additive effects of PV and IV on the incidence of acute exacerbation in COPD patients with various levels of airflow limitations.

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Induction of opsonophagocytic killing activity with pneumococcal conjugate vaccine in human immunodeficiency virus-infected Ugandan adults

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ABSTRACT

The levels of IgG determined by ELISA may have limited relevance in human immunodeficiency virus (HIV)-infected adults because of non-functional antibodies. 58 HIV-1-infected and 29 HIV-uninfected Ugandan adults were immunized with conjugate vaccine (CV) followed by polysaccharide vaccine (PV) after a 2-month interval, and the opsonophagocytic killing (OPK) titers against serotype 4 or 14 pneumococcal strains as well as the levels of serotype-specific IgG in sera were determined. Significant increases were found in the OPK titers and IgG levels for both serotypes after CV vaccination irrespective of HIV status. Increases in IgG levels and OPK titers were largely dependent on the $CD4^+$ cell counts, except for increases in the IgG levels for serotype 4. The proportions with serum OPK titer equal to or greater than 8 were 0–4.3% for serotype 4 and 26.7–42.9% for serotype 14 before vaccination, but the proportions increased up to 43.3–86.2% for serotype 4 and 63.3–96.6% for serotype 14 in all three groups 2 months after CV vaccination. The serum OPK titers remained at levels higher than the pre-vaccination level for at least 8 months after CV vaccination. A single dose of CV could afford some protective immunity in HIV-infected African adults before the introduction of antiretroviral therapy.

1. Introduction

More than 60% of the 40 million people living with HIV in the world are in sub-Saharan Africa [1]. HIV infection is, therefore, a major health concern facing adults in this area. The authors' previous study of community-acquired pneumonia (CAP) among adults in Uganda revealed a high prevalence of HIV-1 infection among these patients [2]. The majority of HIV-1-infected patients with CAP have peripheral CD4 counts below 400 μl^{-1}. *Streptococcus pneumoniae* (*S. pneumoniae*) was found to be a common pathogen, which was isolated in approximately 40% of bacterial CAP. The host defense against *S. pneumoniae* depends largely on opsonophagocytic killing antibodies to capsular polysaccharide (CPS) [3,4], although an antibody-independent, $CD4^+$ T cell-dependent mechanism of protection has been reported in mouse model [5,6]. Low opsonic activity in the sera of HIV-infected African adults, therefore, predisposes them to a serious risk of invasive pneumococcal infections [7–9].

Approaches to prevent bacterial pneumonia in adults with HIV infection involve antimicrobial prophylaxis, highly active antiretroviral therapy (ART) and vaccination [10]. Although a significant decrease in the rates of bacterial pneumonia in HIV-infected adults after antimicrobial prophylaxis with co-trimoxazole has been reported in Cote, d'Ivoire [11], further studies are required to confirm its effects in preventing bacterial pneumonia even in areas where penicillin-resistant pneumococci are highly prevalent in the population [12]. ART can prevent bacterial pneumonia in HIV-infected adults in developed countries, but this has not been confirmed in developing countries [13]. Although the WHO recommends that HIV-infected adults and adolescents, who are either in WHO clinical staging 4 or have a CD4 cell count below 200 μl^{-1} start

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ART [1], an access to ART is limited in sub-Saharan Africa including Uganda [14]. The WHO estimates that only 20% of the estimated 6.5 million people in low and middle-income countries who are in urgent need of ART were receiving ART at the end of 2005 [15].

Previous studies reported the effects of pneumococcal polysaccharide vaccine (PV) in HIV-infected African adults [16,17]. A clinical study of PV in HIV-infected Ugandans, however, has demonstrated an increased risk of invasive pneumococcal infection and no protection against all-cause pneumonia [18], and the same group subsequently reported a 16% reduction in all-cause mortality after PV vaccination [19]. The use of PV in HIV-infected African adults is therefore inconclusive. On the other hand, previous studies in the Netherlands, US and Uganda reported promising effects of pneumococcal conjugate vaccine (CV), which induced high levels of serotype-specific IgG among HIV-infected adults [20–22]. Furthermore, an enhanced antibody response to certain serotypes has been shown in HIV-infected adults following immunization with PV after prior immunization with CV [20,23]. The concentration of serotype-specific IgG determined by ELISA, however, may have limited relevance in HIV-infected adults and children because of non-functional anti-CPS IgG [6,7,24,25]. Analysis of serum opsonophagocytic killing (OPK) activity is, therefore, required to study the immunogenicity of CV in HIV-infected adults. This study was designed to determine whether immunization with CV followed by PV could increase the levels of serotype-specific IgG and OPK titers against *S. pneumoniae* in sera of HIV-infected African adults before the introduction of ART.

2. Materials and methods

2.1. Subjects

After providing written informed consent, HIV-1-infected and HIV-uninfected asymptomatic adults were enrolled by two physicians at the Joint Clinical Research Centre (JCRC), Kampala between February and June 2005. HIV-1 infection was determined by a screening EIA (HIV1&2 Rapid Serotest, Healthcare Technologies Ltd., Ashdod, Israel) and a second EIA for confirmation (Vironostika HIV Uni-FormII Ag/Ab, bioMétrieux, Marcy l'Etoile, France). Study subjects were classified into three subgroups: group I (HIV-1-infected adults with peripheral CD4$^+$ cell count: 200–499 μl^{-1}; $n=30$), group II (HIV-1-infected adults with peripheral CD4$^+$ cell count: $\geq$500 μl^{-1}; $n=28$) and group III (HIV-uninfected adults; $n=29$) (Table 1). Plasma HIV-1 RNA loads were quantified as described elsewhere for the total of 58 HIV-infected adults [26]. Enrolling 90 subjects in three study groups would have given the study 70% power at an alpha level of 0.05 to detect a significant increase in the percentage of persons responding with a $\geq$2-fold rise in the serum levels of serotype-specific IgG from 42% to 74% between groups of HIV-infected and HIV-uninfected adults [27].

2.2. Immunization of study subjects with pneumococcal vaccines

Seven-valent CV (Prevenar®, Wyeth-Lederle), containing 2 μg of six serotypes (4, 9V, 14, 18C, 19F and 23F) and 4 μg of CPS from 6B covalently linked to a CRM_{197}, non-toxic mutant diphtheria toxin, was given as an intradeltoid injection to all of the study subjects. Two months after CV vaccination, 23-valent PV (Pneumovax, Merck-Banyu), which contains 25 μg of each of 23 serotypes was given as an intradeltoid injection to all of the study subjects. All of the study subjects were requested to visit the outpatient department of JCRC, and be examined by two physicians immediately before and 2, 3 and 8 months after CV vaccination. Serum samples were also obtained at the time of the scheduled visits throughout the study, and stored at −80 °C until use. When they had new symptoms, the participants were asked to visit the outpatient department of the JCRC for medical examination by the physicians. At 8 months post-CV vaccination, the peripheral CD4$^+$ cell count was reexamined in HIV-infected adults. While all of HIV-infected subjects were successfully followed for the study period, six of the HIV-uninfected healthy control subjects were lost by the time of follow-up. Sera were subsequently collected from 23 HIV-uninfected subjects 8 months after CV vaccination.

2.3. Determination of serotype-specific IgG levels

Since preabsorption of serum with both cell wall polysaccharide (PS) and 22F PS is necessary to remove non-specific antibodies in HIV-infected adults and HIV-uninfected adults, the concentrations of serotype-specific IgG were measured using the US reference pneumococcal antiserum (89 SF-3), courtesy of Dr. Carl Frasch, as previously described [28,29]. Among 29 pneumococcal isolates known as etiologic agents of CAP in Ugandan adults [2], 5 strains of serotype 4 and 3 strains of serotype 14 were identified (unpublished data). Therefore, we decided to use ELISA to determine the IgG levels in sera for serotypes 4 and 14 CPS, which are major serotypes in Uganda.

2.4. Differentiation of HL-60 cells and OPK assay

Undifferentiated HL-60 cells (Cell number; JCRB0085, Health Science Research Resources Bank, Japan) were cultured in RPMI-1640 medium with 10% fetal calf serum (FCS) (Hyclone, South Logan, UT). HL-60 cells were differentiated into granulocytes in the presence of 1 μM all-*trans*-retinoic acid (Sigma Chemical Co., St. Louis, MO) in RPMI-1640 medium with 20% FCS for 3 days [30,31]. Cells were washed twice by centrifugation, and the supernatant was removed. 20 μl of PE-conjugated mouse anti-human CD11b monoclonal IgG_1 (BD PharMingen, San Diego, CA) or PE-conjugated mouse monoclonal IgG_1 isotype control (Dakocytomation AS, Glostrup, Denmark) were added to each tube and incubated at 4 °C for 30 min. Samples were washed twice and resuspended in PBS containing 1% paraformaldehyde, and analyzed by flowcytometry

Table 1
Clinical characteristics and laboratory data for 58 HIV-1-infected and 29 HIV-uninfected adults

Characteristic	Time point	Group I ($n=30$) HIV-1$^+$, CD4: 200–499 μl^{-1}	Group II ($n=28$) HIV-1$^+$, CD4: $\geq$500 μl^{-1}	Group III ($n=29$) HIV-1$^-$
Age, mean years ± S.D.	Pre	36.77 ± 7.45[a]	37.11 ± 7.63[a]	26.83 ± 7.70
Peripheral CD4$^+$ cell count, mean cell/μl ± S.D.	Pre	352.00 ± 80.71[a,b]	720.25 ± 170.69[c]	882.45 ± 257.45
log_{10} plasma HIV RNA load copies/ml ± S.D.	Pre	4.81 ± 0.85[b]	3.84 ± 0.83	ND
Peripheral CD4$^+$ cell count, mean cell/μl ± S.D.	8 m post-CV	362.41 ± 155.82[b]	707.39 ± 213.79	ND

ND: Not done.
[a] $P<0.001$ (vs. group III).
[b] $P<0.001$ (vs. group II).
[c] $P<0.01$ (vs. group III).

(FACSCalibur, BD Biosciences, San Jose, CA). While no expression of CD11b was found in the undifferentiated cells, increased expression of CD11b was found in the differentiated cells by retinoic acid (data not shown). The expression of CD11b was specific because no increase was found in the fluorescent intensity of the differentiated cells stained with PE-conjugated control antibody.

OPK titer against *S. pneumoniae* was measured as described elsewhere with some modification [32]. Briefly, the differentiated HL-60 cells were used at an effector/target cell ratio of 400/1. 10 μl of serially diluted serum sample was aliquoted into each well of 96-well microtiter plate. *S. pneumoniae* serotype 4 (strain P-03-106) or serotype 14 (strain P-03-170), both isolated from patients with CAP, were used for this assay. In addition, we also employed two reference strains (DS2382-94 for serotype 4 and DS2214-94 for serotype 14) as standard quality control strains for this assay [32]. These reference strains were gifts from Dr. Moon H. Nahm, University of Alabama at Birmingham. 20 μl of bacterial suspension (~10^3 cfu) was added to each well. Then the plate was allowed to incubate at 37 °C in a 5% CO_2 atmosphere for 15 min. Following this incubation period, 10 μl of rabbit complement (Dynal Biotech Inc., Lake Success, NY) was added to each well. Then, 40 μl of washed cells (4×10^5 cells) was immediately added to each well. The assay plate was incubated at 37 °C for 45 min with horizontal shaking (220 rpm). A 5 μl aliquot from each well was plated onto an agar medium plate. Plates were incubated overnight and viable colonies were counted for each well. Typically, 10–60 colonies were counted. The OPK titers were expressed as the reciprocal of the serum dilution with ≥50% killing compared with the growth in the complement control wells. Serum samples with titers of <8 were reported as a titer of 4 for analysis of the levels of serotype-specific IgG, but excluded for analysis of the correlation between the levels of IgG or HIV-1 viral load and the serum OPK titers. Quality control sera (sera with a known titer) were added to each plate and the blinded test samples for OPK titer were examined only when the titers of quality control sera were identical. Functional activity of serotype-specific IgG was expressed as a concentration of IgG required for 50% killing of pneumococcal strain by dividing the IgG concentration of a test sample by OPK titer.

2.5. Statistical analysis

The subjects' ages, $CD4^+$ cell count and levels of HIV-1 viral load were compared by one-way analysis of variance and by multiple comparison methods. Differences in geometric mean concentrations (GMCs) of serotype-specific IgG, OPK titers and the ratios of OPK titer to serotype-specific IgG were assessed using the Friedman and Steel-Dwass tests [33]. The significance of the correlation was estimated using Spearman's rank correlation. Data were considered significant when $P < 0.05$.

3. Results

No difference was found in age between groups I and II, but differences in age were significant between either of group I or II and the group III ($P < 0.001$, Table 1). Differences in $CD4^+$ cell count and the level of HIV-1 viral load were significant between groups I and II ($P < 0.001$), which is in agreement with our previous report [26]. No significant decrease in the $CD4^+$ cell count was found in either group I or II during the 8 months post-CV vaccination. ART was initiated in one case (32 years old, male) in the group I at 7 months post-CV vaccination because of recurrent pneumonia. The $CD4^+$ cell count and plasma $\log_{10}$ HIV viral load of this case were 212 μl^{-1} and 5.78, respectively, at the time of enrollment.

No significant difference was found in the GMCs of IgG specific to either serotype 4 or 14 in sera among the three groups before vaccination (Table 2). Two months after CV vaccination, significant increases in the GMCs of IgG specific to both serotypes 4 and 14 were found for all three groups, compared to those before vaccination ($P < 0.01$).

No significant difference was found in the GMCs of IgG specific to serotype 4 among the three groups 2 months after CV vaccination ($P = 0.23$). In contrast, the GMCs of IgG specific for serotype 14 were highest in group III, followed by group II, with group I having the lowest GMC 2 months after CV vaccination, and the difference between groups I and III was significant at this time point ($P < 0.05$). One month post-vaccination with PV (3 months after CV vaccination), however, no significant increase was found in the GMCs of IgG specific to either serotype 4 or 14 compared to those at 2 months after vaccination with CV in each group. Although the GMCs of IgG specific to serotype 14 largely declined at 8 months post-vaccination with CV, no significant decrease in the GMCs of IgG specific to serotypes 4 was found in any group between 2 months after CV vaccination and 8 months after CV vaccination. The GMCs of IgG specific to both serotypes 4 and 14 were still higher 8 months after CV vaccination than before vaccination. In addition, no correlation was found between the fold increase of specific IgG to either serotype 4 or 14 after CV vaccination and the levels of HIV-1 viral load in HIV-infected adults.

The concentration of 0.35 μg/ml for serotype-specific IgG has been suggested by world health organization (WHO) working group as a putative measure of protection against invasive disease in infants at a population level after immunization with pneumococcal conjugate vaccine [34]. This working group reported that antibody concentrations of 0.2–0.35 μg/ml correlated best with an

Table 2
Comparison of geometric mean concentrations of serotype-specific IgG in sera from HIV-1-infected patients and HIV-uninfected adults before vaccination and after vaccination

Serotype	Time point	GMC of IgG (μg/ml) (95% CI) in sera from		
		Group I ($n = 30$) HIV-1$^+$, CD4: 200–499 μl^{-1}	Group II ($n = 28$) HIV-1$^+$, CD4: ≥500 μl^{-1}	Group III ($n = 29$) HIV-1$^-$
4	Pre	1.55 (1.15–2.08)	0.82 (0.58–1.18)	0.83 (0.59–1.16)
	2 m post-CV	4.17 (3.03–5.73)[a]	4 (2.78–5.74)[a]	6.75 (4.78–9.52)[a]
	3 m post-CV (1 m post-PV)	4.56 (3.51–5.92)[a]	3.88 (2.73–5.52)[a]	6.87 (4.89–9.66)[a]
	8 m post-CV	5.52 (4.41–6.9)[a]	4.07 (3.19–5.19)[a]	4.64 (3.60–5.98)[a,d]
14	Pre	5.27 (3.76–7.37)	5.1 (3.40–7.66)	4.67 (2.75–7.94)
	2 m post-CV	25.31 (16.79–38.14)[a,b]	42.44 (23.41–76.95)[a]	52.11 (31.41–86.45)[a]
	3 m post-CV (1 m post-PV)	23.14 (15.96–33.55)[a,c]	42.76 (23.98–76.24)[a]	53.86 (34.11–85.05)[a]
	8 m post-CV	17.73 (12.68–24.79)[a,c]	28.66 (17.18–47.81)[a]	37.8 (24.99–57.18)[a,d]

CI: Confidence interval; Pre: pre-vaccination; CV: pneumococcal conjugate vaccine; PV: pneumococcal polysaccharide vaccine.
[a] $P < 0.01$ (vs. pre-vaccination).
[b] $P < 0.05$ (vs. group III).
[c] $P < 0.01$ (vs. group III).
[d] $n = 23$ in group III.

OPK titer of 8 [34], which in turn correlated best with protective efficacy in infants. The proportions of subjects with IgG specific to serotype 4 of ≧0.35 μg/ml were 100% in group I, 85.7% in group II and 79.3% in group III before vaccination and 100% for all groups 2 months after CV vaccination in this study. The proportions of subjects with IgG specific to serotype 14 of ≧0.35 μg/ml were 100% in groups I and II and 96.4% in the group III, before vaccination and 100% for all groups 2 months after CV vaccination.

The geometric means of serum OPK titers for the serotypes 4 and 14 were similarly low in sera of all three groups before vaccination (Table 3). The differences among the three groups were not statistically significant. Two months after CV vaccination, significant increases in the serum OPK titers for both serotype strains 4 and 14 compared to pre-vaccination levels were found in all three groups ($P<0.01$). The serum OPK titers for both serotypes 4 and 14 were highest in group III followed by group II, with group I having the lowest titers 2 months after CV vaccination. Serum OPK titers for both serotypes 4 and 14 differed significantly between groups I and III at 2, 3 and 8 months after vaccination with CV ($P<0.01$). Group II had significantly higher OPK titers than group I for serotype 4 at 3 and 8 months post-vaccination with CV, and for serotype 14 at 8 months post-vaccination with CV ($P<0.05$). In additional experiments, the OPK titers of nine serum samples (three from each group) were determined using strains of DS2382-94 and DS2214-94 which are widely used as target strains for serotypes 4 and 14, respectively. The values of serum OPK titer were identical or close to those determined using the non-reference strains (data not shown).

The proportions of subjects with a serum OPK titer of ≧8 were very low (0–4.3%) for serotype 4 in all three groups before vaccination (Fig. 1). On the other hand, the proportion was lower in group I (26.7%) than those in group II (42.9%) or group III (41.4%) for serotype 14 before vaccination. In contrast, marked increases in the proportion with a serum OPK titer of ≧8 were found for both serotypes in all three groups two months after CV vaccination. The proportion of the serum OPK titer of ≧8 was highest in group III, followed by group II and group I for both serotypes at 2 and 3 months post-CV vaccination. Eight months after CV vaccination, the proportions of subjects with a serum OPK titer of ≧8 decreased in groups I and II for both serotypes.

Since few serum samples contained detectable OPK titers for serotype 4 before vaccination (one in group I, none in group II and three in group III), correlations were not assessed between the levels of serotype-specific IgG and the serum OPK titers. On the other hand, the concentrations of serotype-specific IgG significantly correlated with OPK titers for serotype 14 before vaccination in groups II ($r=0.61$, $P<0.05$, $n=14$) and III ($r=0.61$, $P<0.05$), but not in group I ($r=0.56$, $P=0.08$, $n=11$). Two months after CV vaccination, significant correlations between two parameters were found for serotype 4 in groups I ($r=0.74$, $P<0.01$, $n=15$) and III ($r=0.67$, $P<0.001$, $n=26$), but not in group II ($r=0.27$, $P=0.2$, $n=23$) and for serotype 14 in groups II ($r=0.85$, $P<0.001$, $n=23$) and III ($r=0.38$, $P=0.05$, $n=28$), but not in group I ($r=0.44$, $P=0.06$, $n=11$).

The GMCs of serotype-specific IgG required for 50% killing ranged 136–601 ng/ml in the group I, 79–377 ng/ml in the group II and 72–316 ng/ml in the group III, although few data are available for serotype 4-specific IgG before vaccination (Table 4). No

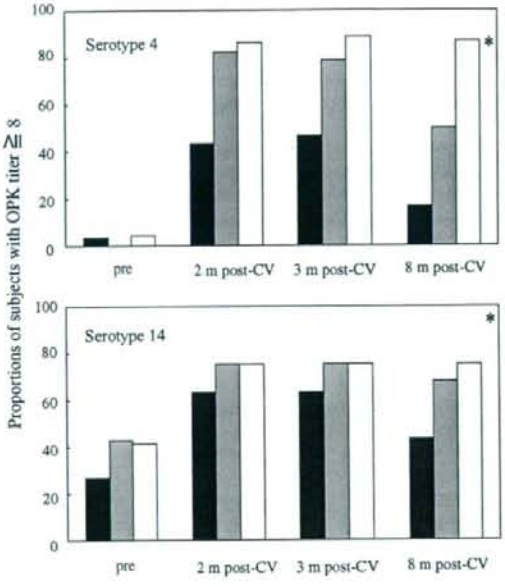

Fig. 1. The proportion of subjects with opsonophagocytic killing (OPK) titers equal to or greater than 8 for serotype 4 (upper panel) and serotype 14 (lower panel) in groups I ($n=30$, closed bar), II ($n=28$, grey bar), and III ($n=29$, open bar) before and 2, 3 and 8 months after vaccination with pneumococcal conjugate vaccine (CV). *$n=23$ in group III at 8 months after CV vaccination.

Table 3
Comparison of geometric mean opsonophagocytic killing (OPK) titers of sera from HIV-1-infected and HIV-uninfected adults before vaccination and after vaccination

Serotype	Time point	GM OPK titer (95% CI) of sera from		
		Group I ($n=30$) HIV-1$^+$, CD4: 200–499 μl^{-1}	Group II ($n=28$) HIV-1$^+$, CD4: ≧500 μl^{-1}	Group III ($n=29$) HIV-1$^-$
4	Pre	4.29 (3.72–4.94)	4 (4–4)	4.62 (3.76–5.66)
	2 m post-CV	13.93 (7.78–24.94)[a,b]	32 (19.08–53.68)[a,c]	75.66 (45.15–126.77)[a]
	3 m post-CV (1 m post-PV)	13.61 (7.91–23.41)[a,b,e]	37.12 (22.21–62.07)[a]	73.87 (45.71–119.36)[a]
	8 m post-CV	6.81 (4.61–10.04)[b,d,e]	18.11 (10.18–32.22)[a]	44.58 (26.55–74.84)[a,g]
14	Pre	7.29 (5.10–10.43)	12.8 (7.52–21.82)	12.01 (6.94–20.78)
	2 m post-CV	41.26 (19.57–87.01)[a,c]	107.63 (51.49–225.02)[a]	275.03 (173.88–435.03)[a]
	3 m post-CV (1 m post-PV)	43.21 (20.42–91.46)[a,b]	118.84 (57.32–246.38)[a]	281.68 (181.69–436.72)[a]
	8 m post-CV	13.61 (7.62–24.3)[b,e]	47.55 (22.52–100.42)[d]	116.94 (69.96–195.45)[a,f,g]

CI: Confidence interval; Pre: pre-vaccination; CV: pneumococcal conjugate vaccine; PV: pneumococcal polysaccharide vaccine.
[a] $P<0.01$ (vs. pre-vaccination).
[b] $P<0.01$ (vs. group III).
[c] $P<0.05$ (vs. group III).
[d] $P<0.05$ (vs. pre-vaccination).
[e] $P<0.05$ (vs. group II).
[f] $P<0.05$ (vs. 2 and 3 months post-CV).
[g] $n=23$ in group III.

Table 4
Comparison of geometric mean concentration (GMC) of serotype-specific IgG required for 50% killing (OPK) titers of sera from HIV-1-infected and HIV-uninfected adults before vaccination and after vaccination

Serotype	Time point	GMC of IgG (ng/ml) required for 50% killing (95% CI) [no. of subjects evaluated] in sera from		
		Group I (n = 30) HIV-1$^+$, CD4: 200–499 μl^{-1}	Group II (n = 28) HIV-1$^+$, CD4: ≥500 μl^{-1}	Group III (n = 29) HIV-1
4	Pre	284 (NA) [1]	NA (NA)	179 (9–3492) [3]
	2 m post-CV	136 (80–234) [14]	102 (68–155) [23]	72 (52–100) [26]
	3 m post-CV (1 m post-PV)	137 (80–234) [15]	79 (56–111) [23]	76 (55–106) [26]
	8 m post-CV	201 (57–706) [7]	86 (46–161) [16]	96 (56–163) [22][a]
14	Pre	441 (236–825) [11]	248 (139–441) [14]	307 (149–631) [15]
	2 m post-CV	302 (182–501) [19]	279 (204–382) [23]	176 (101–305) [28]
	3 m post-CV (1 m post-PV)	229 (142–372) [19]	246 (163–369) [23]	174 (106–288) [28]
	8 m post-CV	601 (364–992) [14][b]	377 (269–529) [18]	316 (207–481) [22][a]

CI: Confidence interval; Pre: pre-vaccination; CV: pneumococcal conjugate vaccine; PV: pneumococcal polysaccharide vaccine; NA: not available.
[a] n = 23 in group III.
[b] P < 0.05 (vs. 3 m post-CV).

significant difference was found in the GMCs of serotype-specific IgG required for 50% killing in sera among the three groups before and after vaccination. No significant difference was also found in the GMCs of serotype-specific IgG required for 50% killing of each group among different time points, with a exception of a significant difference in those of group I between 3 months after CV vaccination and 8 months after CV vaccination (P < 0.05). No significant effect of vaccination with PV following CV was found in the serum OPK titers for either serotype 4 or 14 in any group. Eight months after CV vaccination, the serum OPK titers for serotypes 4 and 14 largely declined compared to those 2 months after CV vaccination. The OPK titers for serotypes 4 and 14 in sera of the three groups, however, remained higher than pre-vaccination levels at 8 months post-vaccination with CV. In addition, no correlation was found between the increase in serum OPK titers for both serotypes 4 and 14 after CV vaccination and the levels of HIV-1 viral load in HIV-infected adults.

4. Discussion

In this study, it has been demonstrated that a single dose of CV can increase serum OPK titers against pneumococcal strains as well as the levels of serotype-specific IgG, in HIV-uninfected and HIV-infected Ugandan adults with peripheral CD4$^+$ cell counts of ≥200 μl^{-1} for at least 8 months after CV vaccination. HIV-infected adults with peripheral CD4$^+$ cell counts less than 200 μl^{-1} who were at risk for pneumococcal infections were not involved in this study, because they are recommended to receive ART [1] and are known to be poorly responsive to CV [20,27]. Serotype 14-specific IgG response to a single dose of CV was dependent on CD 4$^+$ cell counts in HIV-infected adults, which was in agreement with previous reports [20,22], but the magnitude of IgG response to CV in our study was higher than that shown in a previous study [22]. In contrast, serotype 4-specific IgG response to a single dose of CV was independent of CD4$^+$ cell counts in our study: this finding was also consistent with a previous study [22].

The major limitation in this study may be the lack of an unvaccinated control group. Pneumococcal pneumonia or nasopharyngeal colonization may increase the levels of serotype-specific IgG in sera from subjects, in addition to the immunological effects by vaccination, during the study period [35]. Since only five episodes of pneumonia with unknown etiology were identified in the HIV-infected and HIV-uninfected groups (two episodes in group I, one episode in group II and two episodes in group III) during the 8 months (data not shown), we speculate that the possibility of active immunization by pneumococcal pneumonia among study participants was low in this study. Since the nasopharyngeal carriage of pneumococcal strains was not examined in this study, the influence of nasopharyngeal carriage on the levels of IgG and OPK titers in serum cannot be dismissed.

Although a previous study reported an immunological effect of a CV primed PV booster with a 4-week interval in HIV-infected adults [23], no effects of PV following CV vaccination were found in the levels of serotype 4 or 14-specific IgG in our study. The lack of additional immune response after PV following CV could be explained by the differences in races and ages of the participants and in the timing of the PV boost 2 months following CV in this study [23]. Another reason for the lack of additional immune response by PV might be the increased levels of serotype 4 or 14 specific IgG achieved by CV, because a recent study similarly demonstrated that elderly subjects with a serotype-specific IgG higher than 5 μg/ml in sera before vaccination tended to respond to PV at a lower magnitude [36]. The undiminished persistence of the levels of OPK titers and of IgG shown in this study up to 8 months after CV vaccination may, in part, be explained by the boosting effect of nasopharyngeal carriage of pneumococcal strains [37].

The general consensus on measuring the serum OPK titers encouraged the authors to develop this assay for the evaluation of immunogenicity in this study [24]. Although an OPK assay for the measurement of functional antibody activity has been available [32], there were difficulties in achieving differentiation of HL-60 cells into granulocytes in the presence of dimethylformamide as a previous study indicated [38]. In this study, it has been shown that differentiated HL-60 cells by the use of retinoic acid can be applicable for an OPK assay against *S. pneumoniae* for evaluation of the immunogenicity of CV.

While the proportions of subjects with serotype-specific IgG of ≥0.35 μg/ml were higher than 80% and 95% for serotypes 4 and 14 among HIV-infected and HIV-uninfected Ugandan adults in this study, a previous study demonstrated that the proportions of subjects with serotype-specific IgG were less than 10% for serotype 4 and less than 30% for serotype 14 among unvaccinated HIV-infected African children [25]. A relatively low proportion of subjects, however, exhibited OPK titers of ≥8 for serotypes 4 and 14 in both HIV-infected and -uninfected Ugandan adults before vaccination in this study. Although WHO working group reported that 0.2–0.35 μg/ml for the levels of serotype-specific IgG correlate with an OPK titer of 8 in infants [34], this threshold may not be applicable for unvaccinated adults.

In this study, we determined the levels of serotype-specific IgG, but not IgM or IgA. While a poor correlation was found between the opsonic activity and the levels of either CPS-specific IgM or IgA, the serum opsonic activity correlated best with the levels of CPS-specific IgG in healthy adults following CV vaccination [39,40]. The levels of IgG significantly correlated with the OPK titers for serotype 4, except for group II, and for serotype 14, except for group I, 2

months after CV vaccination in this study. Furthermore, the GMCs of serotype-specific IgG required for 50% killing ranged 79–601 ng/ml in HIV-infected adults and 72–315 ng/ml in HIV-uninfected adults after a single dose of CV vaccination in this study. In contrast, the GMCs of serotype-specific IgG required for 50% killing ranged 2–119 ng/ml in infant populations after CV vaccination [41–43]. Collectively, our present data suggest that opsonic function of serotype-specific IgG after CV vaccination is much decreased in Ugandan adults, irrespective of HIV infection, than those in infants.

In conclusion, this study document the immunogenicity of a single dose of CV on the OPK titer and the levels of serotype-specific IgG in sera of HIV-infected Ugandan adults with $CD4^+$ cell count higher than 200 μl^{-1}. A single dose of CV increased the serum OPK activity as well as the levels of IgG, and maintained them above their pre-vaccination levels for at least 8 months after CV vaccination. A single dose of CV could increase protective immunity in HIV-infected African adults with $CD4^+$ cell count higher than 200 μl^{-1} before the introduction of ART. A clinical trial of the efficacy of CV in this population is needed in sub-Saharan Africa.

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Mumps Virus Reinfection is Not a Rare Event Confirmed by Reverse Transcription Loop-Mediated Isothermal Amplification

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Clinically apparent mumps reinfection is considered extremely rare, but several cases have been suspected of reinfection in an out-patient clinic. In this study, virological examination, virus isolation, the reverse transcription loop-mediated isothermal amplification (RT-LAMP), and IgG and IgM EIA antibodies, were examined in order to identify mumps reinfection. Patients were divided into three categories; the reinfection group comprised 29 patients with a history of natural infection, the vaccine-failure group consisted of 37 patients with an immunization history, and two patients had histories of both immunization and mumps infection. Another 25 patients were enrolled as a primary infection group. Mumps virus was isolated in 5 (17%) and the genome was detected in 12 (41%) of 29 in the reinfection group. Reinfection was confirmed in 21/28, demonstrating high avidity of IgG EIA. Mumps virus was isolated in 15 (41%) and there was a higher positivity of genome amplification in 25 (68%) of 37 patients in the vaccine-failure group. Among these, 23 were confirmed as secondary vaccine failure by high avidity IgG EIA serology. In the primary infection group, the isolation rate and genome detection rate was higher in 16 (64%) and in 18 (72%) of 25 patients, respectively. There was no significant difference in virus load among the three groups but high mumps virus load was suspected in the IgM EIA-positive group based on the shorter amplification time on RT-LAMP. Mumps virus reinfection was confirmed by RT-LAMP and an IgG avidity test and was not a rare event. ***J. Med. Virol. 80:517–523, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: reinfection; vaccine failure; IgM EIA; IgG EIA; avidity tests

INTRODUCTION

Mumps virus is a single-stranded negative sense RNA virus in the Paramyxovirus family. The genome structure of mumps virus is arranged from a leader sequence and followed by N, P, M, F, SH, HN, L genes and a trailer sequence. Mumps virus has two surface glycoproteins, fusion (F) and hemagglutinin-neuraminidase (HN) proteins, and infects only humans or primates. Major targets for neutralizing antibodies are HN and F proteins. Membrane (M) protein is located at the inner layer of the particle to stabilize the spherical structure of the particle. Genome RNA is surrounded by the polymerized nucleocapsid (N) protein and composed as ribonucleocapsid (RNP) together with phospho (P) and large (L) proteins [Carbone and Wolinsky, 2001]. These are essential for genome transcription and replication. The small hydrophobic (SH) protein is not present in the virus particle but detected in the infected cell [Takeuchi

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et al., 1996]. The SH gene is a hyper variable region and used as the target of molecular epidemiological study. Mumps virus is now classified into 12 genotypes (A–L) worldwide, based on sequence diversity in the SH region [Jin et al., 2005].

Mumps virus causes typical acute parotitis characterized by parotid swelling with tenderness, generalized illness with fever and has characteristic tissue tropism to neural and glandular tissues, causing aseptic meningitis, encephalitis, pancreatitis, and orchitis as complications. The most susceptible age group is 4–5 years old and most adults demonstrate serological evidence of past infection. Immunity induced by natural infection is considered life-long immunity and vaccine-acquired immunity is also considered to be maintained for a long period [Plotkin, 2004]. In the US and European countries, widespread acceptance of MMR vaccine has dramatically decreased the number of mumps infections [Peltola et al., 1994; CDC, 1998] but quite recently mumps outbreaks have been reported throughout the world. Especially, more than 56,390 patients were reported in the UK during 2004–2005 [CDC, 2006a], as well as multi-state outbreaks in the US [CDC, 2006b,c], and in several countries [Utz et al., 2004; Donaghy et al., 2005; Šantak et al., 2006]. In Japan, MMR vaccine was administered between 1989 and 1993, but discontinued because of an unexpectedly high incidence of aseptic meningitis [Ueda et al., 1995; Kimura et al., 1996]. Since 1993, a monovalent mumps vaccine has been used but vaccine coverage is estimated to be less than 30% [Nagai et al., 2007]. Mump virus is still circulating worldwide and some clinically apparent mumps patients had a clinical history of previous infection or immunization [Utz et al., 2004; Watson-Creed et al., 2006]. Although most cases of recurrent parotitis were considered due to a virus infection other than mumps virus, rare cases of mumps reinfection have been reported [McQuone, 1992; Davidkin et al., 2005].

The possibility of mumps reinfection causing clinical symptoms has been controversial for a long time and the key issue for mumps reinfection is to establish an accurate diagnosis [Gut et al., 1995; Crowley and Afzal, 2002]. Mumps virus infection sometimes causes non-symptomatic infection and acute parotitis may be caused by several viruses other than mumps virus such as parainfluenza viruses and enteroviruses [McQuone, 1992; Davidkin et al., 2005]. Since it is difficult to accurately diagnose mumps virus infection from clinical symptoms alone, serological examinations of IgG and IgM enzyme-linked immunoassay (EIA) antibodies are employed for diagnosis. The principal serological diagnosis of reinfection is made by the presence of high levels of IgG EIA antibodies without demonstrable IgM antibodies, but this method is problematic for the diagnosis of mumps reinfection. Some patients with reinfection have IgM antibodies. Virus isolation and/or detection of mumps virus genome should be performed to obtain an accurate diagnosis of mumps virus infection.

In this report, the clinical significance of mumps reinfection and vaccine failure were investigated. During mumps outbreak, patients with clinically apparent acute parotitis were enrolled and categorized by past history of mumps infection or vaccination. Virus isolation, the detection of mumps virus genome by reverse transcription loop-mediated isothermal amplification (RT-LAMP) in salivary swab and serum IgG and IgM EIA antibodies were examined. The RT-LAMP method is characterized by auto-cycling DNA synthesis with strand displacement at a fixed temperature, which is performed by *Bst* DNA polymerase with high sensitivity, specificity, and using a simple procedure [Okafuji et al., 2005]. Mumps virus reinfection and vaccine failure were confirmed after natural infection and immunization and the incidence was not a rare event as considered previously.

PATIENTS AND METHODS

Patients

Sixty-eight clinically apparent mumps patients with a past history of mumps infection or previous immunization history were enrolled between 2003 and 2006. Past history of mumps was confirmed by medical records and immunization records. Based on the mumps status, patients were divided into three categories; the group suspected of reinfection comprised 29 patients with a history of natural infection, the vaccine-failure group consisted of 37 patients with an immunization history, and two patients had histories of both immunization and mumps infection. Twenty-five patients were also enrolled in this study as the primary infection group who had no history of mumps infection or immunization.

IgG and IgM EIA Antibodies Against Mumps Virus

Acute phase sera were obtained to investigate IgG antibodies against mumps virus, using IgG Mump EIA (Denka Seiken, Tokyo, Japan). IgM EIA was also examined using IgM EIA kits (Denka Seiken). Serum sample was diluted at 1:200 for IgG EIA and at 1:100 for IgM EIA and assays were performed according to the respective instruction manuals.

Avidity Tests

IgG EIA avidity test was performed using IgG EIA kits. Serum was diluted at 1:200 by diluents supplied in the kits. After the diluted serum was added to antigen-coated wells, wells were washed with 6 M urea and the following steps were performed according to the manufacturer's instruction. Avidity index was calculated as the ratio of the absorbance (percentage) in wells treated with 6 M urea divided by the absorbance of untreated wells. Avidity index >32% was considered a secondary immune response [Narita et al., 1998].

Virus Isolation and Genotype Analysis

Vero cells were cultured in 24-well plates in minimum essential medium (MEM) supplemented with 5%

fetal calf serum (FCS) and 0.1 ml of salivary swab sample was inoculated into Vero cells. After contact for 1 hr at 37°C, samples were removed and MEM supplemented with 2% FCS was added. Within two passages, samples that induced mumps virus-specific cytopathic effect (CPE) were considered virus isolation positive. Samples that did not induce CPE through two passages in Vero cells were considered negative [Kashiwagi et al., 1997].

Genome RNA was extracted from 200 μl of culture fluids and converted to cDNA. Reveise transcription PCR (RT-PCR) was performed in the SH gene as previously reported and mumps virus genotypes were determined by sequencing the PCR products by ABI 3130 [Kashiwagi et al., 1997; Takahashi et al., 2000; Inou et al., 2004].

Detection of Mumps Virus Genome by RT-LAMP

Total RNA was extracted from 200 μl of salivary swab sample using magnetic beads RNA purification kit (Toyobo Co., Ltd, Osaka, Japan) and the RNA pellet was suspended in 25 μl of distilled water, as reported previously [Okafuji et al., 2005]. RT-LAMP primer set was synthesized; two outer primers (F3 and B3), two inner primers, a forward inner primer (FIP) and backward inner primer (BIP), and two loop primers (Loops F and B). For the reaction of RT-LAMP, 5 μl of sample RNA was added in 20 μl of RT-LAMP reaction mixture, as previously reported [Notomi et al., 2000; Okafuji et al., 2005]. The reaction mixture was subjected to real-time turbidimeter LA200 (Teramecs, Kyoto, Japan). As the LAMP reaction progresses, the reaction by-products pyrophosphate ions bind to magnesium ions, forming white precipitate of magnesium pyrophosphate. Thus, the measurement of turbidity is closely related to the amplification of DNA and turbidity >0.1 was considered LAMP positive [Mori et al., 2004].

The virus genome quantity in the samples was calculated by monitoring the reaction time in seconds until the threshold turbidity of 0.1 was reached [Okafuji et al., 2005].

Statistical Analysis

Virus isolation rate and RT-LAMP positive rate were compared among the groups by Chi-square test. RT-LAMP reaction time was compared by Student's *t*-test.

RESULTS

Virus Isolation and Genome Detection by RT-LAMP

Sixty-eight salivary swabs were obtained from patients suspected of having mump reinfection or vaccine failure based on clinical symptoms and past history; 29 in the reinfection group had a past history of natural infection, 37 in the vaccine-failure group had an immunization history and the remaining two had histories of both natural infection and immunization. Twenty-five salivary swabs were obtained from patients suspected of primary infection as a control. The results of virus isolation and genome amplification are shown in Table I. Of 25 patients considered to have primary infection, mumps virus was isolated in 16 (64%) and genome was detected in 18 (72%). Mumps virus was isolated in 15 (41%) of 37 patients in the vaccine-failure group, showing a significantly lower isolation rate compared to that in the primary infection group and the genome was detected in 25 (68%). In 29 patients suspected of reinfection based on medical history, virus isolation was positive in 5 (17%) and the genome was detected in 12 (41%), showing a significantly lower rate in comparison with the other two groups. Mumps virus genome was detected in 5 (14%) of 35 sera obtained from the vaccine-failure group, none in reinfection group, and two of 12 sera obtained from primary infection group. Although the positive rate was lower than those in the vaccine-failure and primary infection groups, mumps virus infection was identified by virological examinations in patients with acute parotitis who had a history of natural mumps infection and suspected of mumps reinfection. The interval between the last immunization and acute infection was 31.65 months and that between the last natural infection and acute parotitis was 39.81 months.

Virus isolation and genome amplification were analyzed based on the serological status and the results are shown in Table II. A salivary swab and serum samples were obtained within 2 days of illness. IgG EIA antibodies were positive in 70 samples and IgM EIA antibodies were positive in 37, negative in 33. Mumps virus was isolated in 21 (57%) and genome was detected in 28 (76%) salivary swabs and 5 (14%) sera of 37 IgM EIA-positive cases, whereas mumps virus was isolated in 5 (15%) and genome was amplified in 15 (45%) salivary swabs of 33 IgM EIA-negative cases. In seven

TABLE I. Virus Isolation and Detection of Mumps Virus Genome From Salivary Swab and Sera by LAMP According to the Histories of Vaccination and Natural Infection

Past history (number of patients)	Virus isolations+	Salivary swab LAMP+	Serum LAMP+
Vac +/natural infect. + (n = 2)	1	2	0/1
Vac +/natural infect. − (Vac. F) (n = 37)	15 (41%)	25 (68%)	5/35
Vac −/natural infect. + (reinfect) (n = 29)	5 (17%)	12 (41%)	0/28
Vac −/natural infect. − (primary) (n = 25)	16 (64%)	18 (72%)	2/12
Total (n = 93)	37	57	7/76

Vac. F., vaccine-failure group; reinfect, reinfection group; primary, primary infection group.

TABLE II. Virus Isolation and Detection of Mumps Virus Genome From Salivary Swab and Sera by LAMP According to the Serological Status of IgG and IgM EIA

Serological status of EIA (number of patients)	Virus isolation	Salivary swab LAMP+	Serum LAMP+
IgG +/IgM + (n = 37)	21 (57%)*	28 (76%)**	5 (14%)
IgG +/IgM − (n = 33)	5 (15%)*	15 (45%)**	1 (3%)
IgG −/IgM − (n = 7)	1 (14%)	1 (14%)	0

*$P < 0.01$.
**$P < 0.05$.

cases negative for both IgG and IgM EIA, one patient was positive for virus isolation and genome amplification. In the IgM EIA-positive cases, mumps virus was efficiently isolated ($P < 0.01$) and the genome was detected with a higher positivity rate than that in the IgM EIA-negative cases ($P < 0.05$).

IgG avidity was examined in 28 sera from the reinfection group and in 35 sera from the vaccine-failure group. Results of virus isolation and genome detection are shown in Table III. Avidity index less than 32% indicated a primary immune response, while sera with over 32% in avidity indicated secondary immune response [Narita et al., 1998]. Twenty-one sera showed high avidity among the reinfection group and 23 among the vaccine-failure group. When we used a high avidity criterion >40%, 15 in the reinfection group and 14 in the vaccine-failure group showed high avidity. Mumps virus was isolated in 4 and the genome was detected in 9 of 21 high avidity cases in the reinfection group. Mumps virus was isolated in 6 and the genome was detected in 13 of 23 high avidity cases in the vaccine-failure group.

Difference in Viral Load

The reaction time (sec) to reach the RT-LAMP reaction threshold of turbidity over 0.1 was monitored. Mean reaction time +1.0 SD (sec) is shown in Figure 1. There was no significant difference in reaction times of RT-LAMP among patients with reinfection, vaccine failure, and primary infection. The reaction times were compared in IgM EIA-positive and -negative cases. RT-LAMP reaction became positive in 1,853 sec on average in IgM EIA-positive cases, which was significantly earlier reaction time than 2,350 sec in IgM EIA-negative cases ($P < 0.05$). There was no significant difference in RT-LAMP reaction time between the high avidity and low avidity groups.

Mump Virus Genotypes

Thirty-seven strains of mumps virus were isolated and 30 strains were sequenced in two different districts, Tokyo and Takamatsu. Among 22 strains isolated in Tokyo, 21 were identified as genotype G and one as genotype B. In Takamatsu, four of seven isolates were genotype G and the remaining three were genotype B.

DISCUSSION

There are several difficulties and limitations in the clinical study of mumps reinfection: (i) clinical case definition of mumps illness, (ii) case ascertainment by virological examinations, (iii) determination of immunization history or history of natural mumps infection. Vinagre et al. [2003] examined 50 cases of chronic recurrent parotitis and one case of cytomegalovirus, two cases of enterovirus, one case of influenza virus, and three cases of mumps virus were diagnosed by indirect immunofluorescence assay and serological examinations. Davidkin et al. [2005] examined 848 patients with mumps-like illness who had been vaccinated with MMR. Among these patients, mumps virus infection was serologically confirmed in 17 (2%) and 84 cases (14%) were serologically diagnosed as other virus infections in 601 cases in which stored sera were available; Epstein–Barr infection (7%), parainfluenza virus types 1–3 (4%), adenovirus (3%), and HHV-6 infection. They examined serological tests only and did not employ sensitive methods for the detection of mumps virus genome by RT-PCR. Thus, the sensitivity of diagnostic examinations affected the finding that only 2% of cases were identified as demonstrating mumps virus infection in their report.

In this study, patients who attended the outpatient clinics, complaining of acute onset of bilateral or

TABLE III. Virus Isolation and Detection of Mumps Virus Genome From Salivary Swab and Sera by LAMP Among the Reinfection and Vaccine-Failure Groups

	IgG avidity	Virus isolation +	Salivary swab LAMP+
Reinfect. group (n = 28)	>40% (n = 15)	3	6
	>32% (n = 21)	4	9
Vac. F. group (n = 35)	>40% (n = 14)	3	5
	>32% (n = 23)	6	13

Reinfect. group, the patients who had a history of natural mumps infection; Vac. F. group, the patients who had been immunized with mumps vaccine.

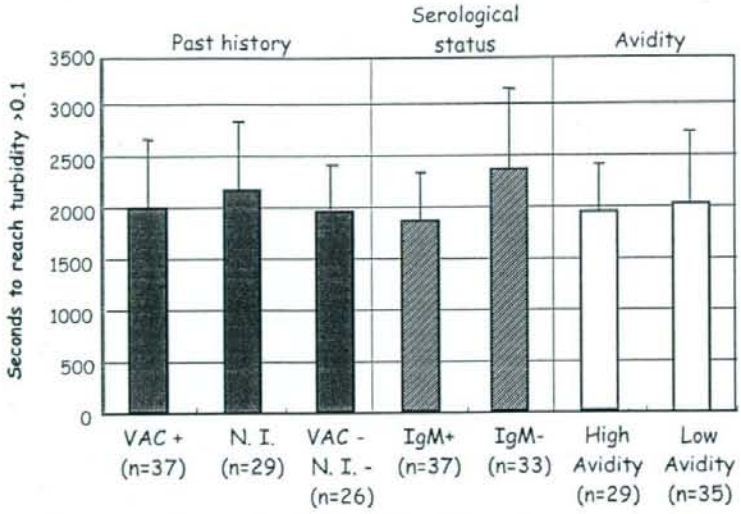

Fig. 1. Comparison of the RT-LAMP reaction time in seconds to reach threshold turbidity >0.1 in different mumps categories based on past history of immunization or natural infection, IgM EIA positivity, and IgG EIA avidity tests. Vac, immunization history; N.I., natural infection.

unilateral parotid gland swelling, were enrolled. Immunization history and history of natural mumps infection were confirmed by medical records. Mumps virus infection was confirmed by virus isolation and by RT-LAMP. Uncertainties and limitations of this study are whether the previous natural mumps infections were definitely caused by mumps virus and whether asymptomatic infection might have occurred among cases in the vaccine-failure and primary infection groups. Among 29 patients in the reinfection group, virological examinations were performed during the previous episode in five patients and all were positive for IgM antibodies or RT-PCR. Mumps virus was isolated in 15 (41%) of 37 patients in the vaccine-failure group with a significantly lower rate compared with that in the primary infection group, but the genome was detected in 25 (68%) which was similar to the detection rate in the primary infection group. In 29 patients suspected of reinfection based on medical history, virus isolation was positive in 5 (17%) and the genome was detected in 12 (41%), showing a significantly lower rate compared with those in the vaccine-failure and primary infection groups. In 25 patients considered as having primary infection without histories of vaccination or natural infection, mumps virus was isolated in 16 (64%), which was higher than that in the reinfection group ($P < 0.01$) and the genome was detected in 18 (72%), which was a significantly higher positive rate ($P < 0.05$) compared with that in the reinfection group. In cases showing reinfection or secondary vaccine failure, the serological definition was a high level of IgG with negative IgM, while IgM antibodies are thought to be positive in primary infection. However, in some reinfection cases, IgM antibodies are detected. In 37 IgM EIA positive patients irrespective of past histories, a higher virus isolation rate was demonstrated in 21 (57%), higher positive rate on RT-LAMP in 28 salivary swabs (76%), and in 5 sera (14%) compared with those in the IgM EIA negative group. Reaction time of RT-LAMP for genome amplification was shorter in IgM positive cases than in IgM negative cases, indicating that IgM positivity reflects higher virus replication.

Narita et al. [1998] reported that IgG antibodies with low avidity were detected in primary infection and high avidity antibodies in reinfection cases. In this study, avidity test was used to obtain serological evidence of secondary immune response. Twenty-one sera showed high avidity among 28 in the reinfection group and 23 sera of 35 in the vaccine-failure group. When the higher criterion on avidity index >40% was used, 15 in the reinfection group and 14 in the vaccine-failure group showed high avidity >40%. Reinfection and secondary vaccine failure were confirmed serologically.

In the UK outbreak in 2004, only 2.4% of confirmed mumps cases occurred in persons who would have been eligible routinely for two doses of MMR [CDC, 2006a]. However, several reports on mumps outbreaks demonstrated that a large proportion of patients had been immunized once or twice with MMR [Davidkin et al., 2005; Watson-Creed et al., 2006]. A mumps outbreak was reported at a summer camp in New York in 2005 and 31 cases were identified among vaccinated campers and vaccinated or unvaccinated staff members [CDC, 2006b]. There was no significant difference in clinical symptoms between vaccinated and unvaccinated individuals [CDC, 2006b]. The efficacy of live attenuated mumps vaccine was reported to be approximately 95% in phase III prelicensure serological responses [Plotkin, 2004]; however, protective efficacy was reported to range from 61 to 91% in several outbreaks [Briss et al.,

1994]. Vandermeulen et al. [2004] analyzed a mumps outbreak in several kindergarten and primary schools and overall vaccine efficacy was 64% in particular outbreaks. They also estimated that the proportion of vaccinated children with mumps increased progressively by increasing the interval since the last dose of mumps vaccination, indicating the secondary vaccine failure. The possibility of primary vaccine failure should be considered in the study. Several strains of live attenuated mumps vaccine have been licensed but the characteristics are different. Schlegel et al. [1999] reported that the infection rate was 67% (54–78%) in children vaccinated with the Rubini strain which was a similar rate of 63% (17–92%) in unvaccinated children, but significantly lower infection rate, 14% (3–31%), was reported in those vaccinated with the Jeryl-Lynn strain and 8% (1–22%) in those with Urabe strain. Similar results were reported elsewhere [Harling et al., 2005].

Currently, mumps virus is classified into 12 different genotypes based on the sequence diversity in the SH gene [Jin et al., 2005]. Mumps virus is thought to be serologically monotypic and the vaccine did prevent infection with different circulating wild genotypes [Nöjd et al., 2001]. In many countries, circulating wild-type genotypes drastically shifted to different genotypes from the parental vaccine strains [Utz et al., 2004]. Rubin et al. [2006] reported that 44% of 74 EIA-positive sera collected randomly showed significantly different NT titers against the Enders strain (genotype A) and Lo1/UK/88 (genotype D) with fourfold or greater differences. MMR vaccine contains the Jeryl-Lynn strain as the mumps component, which is classified as genotype A. Current circulating strains isolated in outbreaks in the UK and US were classified into genotype G and the genotypic difference can explain the recent outbreaks [Šantak et al., 2006; Watson-Creed et al., 2006]. Inou et al. [2004] reported that there was no significant difference in NT titers among major circulating strains of four genotypes B, G, J, L in Japan, using sera obtained from vaccine recipients of the Hoshino strain (genotype B) and that they showed a different antigenicity from the Enders strain (genotype A). They also reported that the major circulating genotype was genotype G with minor genotypes B and L showing geographically different genotype distributions in 2000–2001. In this study, there was no significant change in circulating genotypes during 2000–2005 in Japan. Four vaccine strains have been used in Japan and these were genotype B, antigenically similar to the current circulating strains.

Although long-lasting protective immunity after natural infection or vaccination has been accepted, recent studies have demonstrated that vaccine-acquired immunity waned and the long-tern efficacy is limited. Mumps cases have been reported in highly vaccinated populations but the incidence of reinfection was considered rare. Reinfection was confirmed by serological tests and or virus isolation [Gut et al., 1995; Crowley et al., 2002]. In this study, cases were diagnosed by the detection of mumps virus genome with RT-LAMP, sensitive genome amplification, and IgG EIA avidity test. Viral load can be calculated by monitoring the time to reach a threshold of >0.1 turbidity. We compared the reaction time (sec) in different background categories and the reaction time was significantly shorter in the IgM EIA-positive group than in the IgM EIA-negative group, suggesting a high virus load in the IgM positive group. In this report, mumps virus reinfection was confirmed using a sensitive genome amplification RT-LAMP and IgG avidity test. Furthermore, two patients had histories of both immunization and past natural infection. Thus, immunological memory appears to be frail in mumps virus infection in comparison with that of measles or rubella virus infections and the findings demonstrated that reinfection and secondary vaccine failure are not rare events.

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