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IV　研究成果の刊行物・別刷

Prognostic Factors in Influenza-associated Encephalopathy

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Background: Recently, reports of influenza-associated encephalopathy have increased worldwide. Given the high mortality and morbidity rates attributable to this severe neurologic complication of influenza, we conducted a nationwide study in Japan to identify the prognostic factors.

Methods: We retrospectively evaluated 442 cases of influenza-associated encephalopathy that were reported to the Collaborative Study Group on Influenza-Associated Encephalopathy, which was organized by the Japanese Ministry of Health, Labor, and Welfare in collaboration with hospitals, clinics, and local pediatric practices in Japan between 1998 and 2002. The outcome for each patient was classified as either survival or death. Predictors of death were identified using logistic regression analysis.

Results: Four major prognostic factors for death were found to be significant by multivariate analysis ($P < 0.05$) in the 184 patients for whom we had complete data: elevation of aspartate aminotransferase, hyperglycemia, the presence of hematuria or proteinuria, and use of diclofenac sodium.

Conclusions: We identified patients who had factors associated with a poor prognosis, and these findings might be clinically useful for the management of this illness.

Key Words: influenza-associated encephalopathy, hypercytokinemia, diclofenac, IL-6, TNF-α

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Influenza-associated encephalopathy is a severe neurologic complication of influenza characterized by an abrupt onset of seizures and coma within a few days of developing a high fever.[1] The number of patients with influenza-associated encephalopathy in Japan has increased in recent years, with more than 100 children younger than 6 years of age dying annually from this severe disease.[1] Recently, reports of influenza-associated encephalopathy have also increased worldwide.[2,3]

Blood abnormalities, such as thrombocytopenia and elevated serum aspartate aminotransferase (AST), and brain computed tomography (CT) abnormalities are associated with poor outcome.[1] Cyclooxygenase inhibitors, particularly aspirin, are known to cause Reye syndrome.[4,5] In Japan, cyclooxygenase inhibitors such as diclofenac sodium and mefenamic acid, but not aspirin, are widely used as antipyretic drugs in children. We found that some nonsalicylate antipyretic drugs, including diclofenac sodium and mefenamic acid, may be associated with the development of influenza-associated encephalopathy or may affect the severity of the disease.[1] However, we were unable to thoroughly assess the relationship between the use of these medicines and prognosis.

The mortality rate of this disease is as high as 30% without treatment.[1] Therefore, for the administration of intensive care, it is important to identify the factors that affect its prognosis. We investigated 442 cases of influenza-associated encephalopathy reported from 1998 to 2002 in the Collaborative Study Group on Influenza-Associated Encephalopathy organized by the Japanese Ministry of Health, Labor, and Welfare, and analyzed the prognostic factors using multivariate logistic regression analysis. We report several factors related to the poor prognosis of influenza-associated encephalopathy. To our knowledge, this is the first nationwide study of the prognostic factors of influenza-associated encephalopathy.

METHODS

Study Design. Questionnaires were developed by the Collaborative Study Group on Influenza-Associated Encephalopathy to assess the number of cases in all hospitals, clinics, and local pediatric practices (total of 3500 sites) between 1998 and 2002. Subsequently, a second questionnaire was sent to each applicable facility. The second questionnaire requested information on age, sex, virus type, history, flu vaccination record, peak body temperature, symptoms, laboratory data, CT findings, medication, diagnostic methods of influenza virus infection, and disease outcome. The age, peak body temperature, and laboratory data were provided directly by participants, whereas the other data were gathered using a multiple-choice questionnaire. The flu vaccination record covered the season during which patients suffered from influenza-associated encephalopathy. The possible responses to the questions regarding vaccination history were "unknown," "no," "once," and "twice." Vaccination histories were not con-

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firmed by other methods. The laboratory data were obtained at admission. Recently, it has been reported that prognosis could be improved by therapies such as methylprednisolone pulse and hypothermia therapy.[6,7] However, it was not possible to obtain information regarding these therapies for this study.

The study protocol was approved by the Institutional Review Board of Nagoya University Hospital.

Case Definition. Influenza infection was defined on the basis of a positive result on viral culture, viral antigen testing, or viral ribonucleic acid polymerase chain reaction, or a 4-fold or greater rise in paired serum antibody titer test (hemagglutination inhibition or complement fixation test). Patients who did not meet all the criteria were excluded from the study. In viral antigen testing, the influenza type could not be determined in several cases because the diagnosis was made using a viral antigen test that could not distinguish between types A and B influenza. Patients were defined as having influenza-associated encephalopathy if they showed clinical symptoms and signs compatible with acute encephalopathy, such as altered consciousness (ie, delirium, confusion, and cognitive impairment) or loss of consciousness (ie, deep coma, coma, semicoma, stupor, and somnolence), and if these symptoms persisted for more than 24 hours. Patients with meningitis, myelitis, and febrile convulsions without prolonged unconsciousness were excluded. Cases of postictal unconsciousness with prompt recovery were classified as febrile convulsion. All of the cases reported as influenza-associated encephalopathy were reviewed thoroughly by members of the study group to confirm whether the diagnosis was appropriate. Doubtful cases were excluded from further analysis. The outcomes of influenza-associated encephalopathy were defined as survival or death. A survival outcome included all patients who were alive regardless of whether they had sequelae. In total, 442 influenza-associated encephalopathy cases in patients younger than 15 years of age were deemed appropriate for the study. Study participants provided informed consent or assented with parental consent.

Statistical Analysis. The data were analyzed using the Dr. SPSS software package version 2 (SPSS Inc. Tokyo, Japan). Twenty variables were analyzed to formulate a predictive model for death caused by influenza-associated encephalopathy. The variables identified as significant at $P < 0.05$ using univariate logistic regression analysis were entered into a multivariate logistic regression model, and the least significant variables were sequentially removed. In the multivariate logistic regression analysis, the model was adjusted by age, sex, virus type, history of allergy, record of flu vaccination, and use of acetaminophen. In the multivariate logistic regression analysis, a value of $P < 0.05$ was considered statistically significant. A P value between 0.05 and 0.20 was considered to show a tendency toward being a factor for poor prognosis because a risk existed of eliminating important prognostic factors in the logistic regression if the P values were restricted to <0.05. Odds ratios with 95% confidence intervals were also estimated.

RESULTS

Between 1998 and 2002, a total of 2624 sites contributed information and 693 patients were reported as potentially having influenza-associated encephalopathy, according to the primary questionnaire. In response to the second questionnaire, 585 cases from a total of 340 sites were reported and 442 cases in patients younger than 15 years of age deemed appropriate for further study. These included 97 patients who died and 345 patients who survived.

Of the 442 patients, 331 (74.9%) were between 1 and 6 years old, 232 were male, and 210 were female. No significant differences were observed in incidence or mortality between the sexes; 45 males and 52 females died. The death rates were 32.1%, 22.5%, 13.7%, and 16.4% in 1998–1999, 1999–2000, 2000–2001, and 2001–2002, respectively. We found 372 (84.2%) and 42 (9.5%) cases of type A and type B influenza, respectively. In the other 28 (6.3%) cases, the influenza type could not be determined. Fifty-four cases (22.1%) had a history of febrile convulsions.

Table 1 shows the numbers, percentages, odds ratios, and 95% confidence intervals from the univariate logistic regression analyses for the 20 variables divided by survival and death. In the univariate analyses, 14 variables had statistical significance ($P < 0.05$): peak body temperature of 40–41°C and ≥41°C; diarrhea; AST level of 100–500 IU/L and ≥500 IU/L; creatinine phosphokinase level of 200–1000 IU/L and ≥1000 IU/L; platelet count of $<10 \times 10^4/\mu L$; blood glucose level of <50 and ≥150 mg/dL; hematuria or proteinuria; CT showing edema, low-density areas, or hemorrhage; and use of diclofenac sodium and mefenamic acid for fever during influenza virus infection. These variables were retained for multivariate analysis.

The following variables related to patient background were also used in the multivariate analyses, although they were not significantly related to prognosis: age, sex, virus types, allergy history, flu vaccination record, and acetaminophen use. A history of febrile convulsion was excluded because of missing data.

We could not analyze all 442 patients in multivariate analysis because many of the factors with statistical significance in univariate analysis were missing data. A total of 13 of the 16 variables used in multivariate analysis had several missing data points. Thus, a total of 184 patients with complete data were included in multivariate analysis (Fig. 1). Reducing the number of cases from 442 to184 did not significantly alter the percentage of survival and death.

Table 2 summarizes the numbers and percentages of the 184 patients with complete data for these 16 variables and shows the adjusted odds ratios and 95% confidence intervals from multivariate logistic regression analysis. Four significant prognostic factors were used: AST ≥500 IU/L ($P = 0.04$), blood glucose ≥150 mg/dL ($P = 0.04$), hematuria or proteinuria ($P = 0.01$), and the use of diclofenac sodium ($P = 0.03$). The following variables showed a tendency toward being factors for poor prognosis: peak body temperature of 39–40°C ($P = 0.18$), 40–41°C ($P = 0.14$), and ≥41°C ($P = 0.05$); platelets $<10 \times 10^4/\mu L$ ($P = 0.18$); blood glucose <50 mg/dL ($P = 0.14$); CT showing edema, low-density areas, or hemorrhage ($P = 0.17$); and the use of mefenamic acid ($P = 0.09$).

Allergy history provided a nearly significant P value of 0.08 in the univariate analysis. The allergies reported included asthma, atopic dermatitis, and allergic rhinitis. We

TABLE 1. Characteristics on Admission and Univariate Analysis of Prognostic Factors in the 442 Patients Between 1998 and 2002

Variable	No. Patients		Odds Ratio (95% CI)	*P*
	Survival (n = 345)	Death (n = 97)		
Age group, yr				
<1	22	6	1	—
1–6	257	74	1.06 (0.41–2.70)	0.91
6–15	66	17	0.94 (0.33–2.69)	0.92
Sex				
Male	187	45	0.73 (0.47–1.15)	0.17
Female	158	52	1	—
Virus types				
Type A	294	78	0.59 (0.29–1.19)	0.14
Type B	29	13	1	—
Unclassified	22	6	—	—
Past history				
Allergy				
Yes	21	1	0.16 (0.02–1.21)	0.08
No	324	96	1	—
Febrile convulsion				
Yes	46	8	0.89 (0.38–2.07)	0.79
No	159	31	1	—
Flu vaccination record				
Yes	12	1	0.31 (0.04–2.37)	0.26
No	320	88	1	—
Peak body temperature, °C				
<39	45	4	1	—
39–40	107	21	2.21 (0.72–6.80)	0.17
40–41	125	35	**3.15 (1.06–9.36)**	**0.04**
≥41	18	18	**11.25 (3.34–37.86)**	**<0.001**
Symptoms				
Convulsion				
Yes	266	76	1.19 (0.66–2.15)	0.56
No	71	17	1	—
Abnormal behavior				
Yes	32	4	0.43 (0.15–1.25)	0.12
No	305	89	1	—
Arthralgia				
Yes	8	1	0.45 (0.06–3.62)	0.45
No	329	92	1	—
Diarrhea				
Yes	21	12	**2.23 (1.05–4.72)**	**0.04**
No	316	81	1	—
Blood examination AST, IU/L				
<100	227	22	1	—
100–500	53	28	**5.45 (2.89–10.27)**	**<0.001**
≥500	19	32	**17.38 (8.49–35.59)**	**<0.001**
Creatine phosphokinase, IU/L				
<200	189	33	1	—
200–1000	65	22	**1.94 (1.06–3.56)**	**0.03**
≥1000	16	20	**7.16 (3.37–15.22)**	**<0.001**
Platelet, $\times 10^4/\mu L$				
<10	25	39	**10.79 (5.87–19.85)**	**<0.001**
≥10	256	37	1	—
Blood glucose, mg/dL				
<50	2	6	**34.71 (6.40–188.29)**	**<0.001**
50–150	162	14	1	—
≥150	105	57	**6.28 (3.33–11.84)**	**<0.001**
Urine test				
Normal	192	14	1	—
Hematuria or proteinuria	54	38	**9.65 (4.87–19.10)**	**<0.001**
Brain CT findings				
Normal	151	16	1	—
Edema, low density area, hemorrhage	138	62	**4.24 (2.34–7.70)**	**<0.001**
Medication				
Acetaminophen				
Yes	179	44	0.96 (0.59–1.58)	0.88
No	137	35	1	—
Diclofenac sodium				
Yes	19	15	**3.66 (1.77–7.59)**	**<0.001**
No	297	64	1	—
Mefenamic acid				
Yes	15	9	**2.58 (1.09–6.14)**	**0.03**
No	301	70	1	—

Total numbers for most variables are less than 442 because of incomplete answers to the questionnaire. Values in bold indicate statistically significant results. A logistic regression analysis was used to determine the significant predictors of death.

CI indicates confidence interval; AST, aspartate aminotransferase; CT, computed tomography.

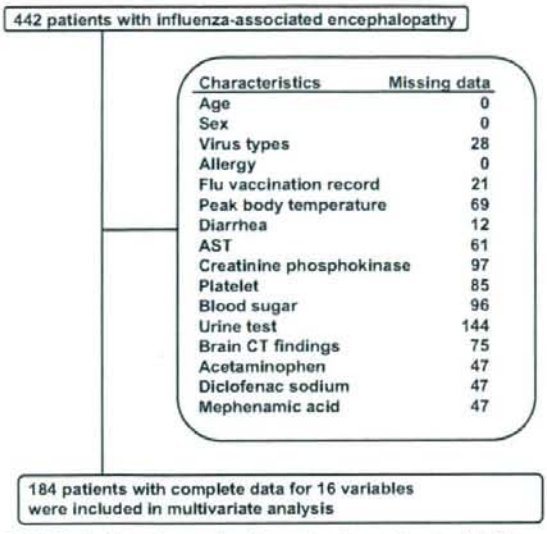

FIGURE 1. Flow chart of patient selection. A total of 442 patients with influenza-associated encephalopathy were reduced to 184 patients because of missing data points in the variables. Variables with missing data are shown with the number of missing data points. AST indicates aspartate aminotransferase; CT, computed tomography.

placed patients with allergies in the good prognosis group in univariate analysis. However, allergy history did not have a significant effect in multivariate analysis, possibly because of control of confounding.

DISCUSSION

An outbreak of encephalopathy suspected to have been caused by influenza infection prompted a national survey of influenza-associated encephalopathy at all hospitals and pediatric clinics in Japan, as well as this analysis of 442 cases. To our knowledge, this is the first study on the prognostic factors of influenza-associated encephalopathy. The mortality rate was as high as 30% without treatment.[1] Therefore, for the administration of intensive care, it is important to identify the factors that affect its prognosis. Using multivariate analysis, we identified several factors that were related to the poor prognosis of this disease.

A severely elevated transaminase level, thrombocytopenia, and hematuria or proteinuria were associated with an unfavorable outcome in influenza-associated encephalopathy. Although the pathogenesis of this disease is still unclear, several reports[8–10] have suggested that it involves cytokines, such as soluble tumor necrosis factor receptor-1, interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), IL-8, and IL-10. Nerve and liver cells may be induced to undergo apoptosis in influenza-associated encephalopathy patients as a consequence of hypercytokinemia, resulting in disseminated intravascular coagulopathy and multiple organ failure.[8,10–13] Studies have reported hemophagocytosis in influenza-associated encephalopathy patients, suggesting the activation of macrophages and microglia cells by hypercytokinemia.[14] A severely elevated transaminase level, thrombocytopenia, and hematuria or proteinuria may be associated with disseminated intravascular coagulopathy, multiple organ failure, and hemophagocytosis resulting from hypercytokinemia induced by this disease.[8,10,11]

We also showed that hyperglycemia is a factor leading to poor prognosis. IL-6 may lead to increased cortisol levels, followed by a pronounced dose-dependent increase in blood glucose.[15,16] Therefore, we postulated that the systemic hypercytokinemia in influenza-associated encephalopathy causes hyperglycemia and that the glucose levels reflect the degree of pathogenicity.

Hypoglycemia provided a significant P value of <0.05 in univariate analysis. However, this did not result in a significant difference in the multivariate analysis, which was probably because the number of patients with hypoglycemia in multivariate analysis was reduced from 8 to only 2 cases, or 1.1% of patients, because of missing data. Hypoglycemia is a symptom of Reye syndrome with a very poor prognosis.[5,17,18] Medium-chain acyl-CoA dehydrogenase deficiency is the most common disorder of fatty acid β-oxidation, and occurs acutely in Reye's-like syndrome, which is often provoked by infection.[19] Reye's-like syndrome is similar to influenza-associated encephalopathy in several of its symptoms, such as loss of consciousness, seizures, and increased aminotransferase levels.[1,20] Therefore, we postulated that influenza-associated encephalopathy may include a metabolic disorder, such as medium-chain acyl-CoA dehydrogenase deficiency.

High-grade fever, particularly $\geq 41°C$, showed a tendency toward being a prognostic factor in the multivariate analysis. Some patients with a poor outcome exhibit a mitochondrial β-oxidation disorder evoked by inactivated carnitine palmitoyltransferase II during high-grade fever in influenza-associated encephalopathy.[21] Analysis of the genotypes and allele compositions of carnitine palmitoyltransferase II have revealed a thermolabile phenotype that occurs more frequently in influenza-associated encephalopathy patients than in healthy subjects.[21] In addition, the use of the nonsalicylate antipyretic drug diclofenac to alleviate fever affected the prognosis of the disease, and the use of mefenamic acid tended to also influence the prognosis, whereas the use of acetaminophen was considered to have little effect. In May 2001, the Japanese Ministry of Health, Labor, and Welfare banned the use of these antipyretic drugs to alleviate fever in influenza infection based on the data of the Collaborative Study Group on Influenza-Associated Encephalopathy.[1] However, it is still unclear whether these drugs are related to the pathogenesis of influenza-associated encephalopathy. Shiga-like toxin II or Shiga-like toxin II-stimulated cytokines may change the brain penetration of diclofenac sodium and mefenamic acid, and consequently increase the risk of the drugs having central nervous system side effects.[22]

We did not find a significant correlation between the prognosis of influenza-associated encephalopathy and flu vaccination record. A more extensive study is required to reveal whether flu vaccination can improve the prognosis after developing the disease because only 13 patients, or 3.1% of the 442 cases, had been immunized against flu.

TABLE 2. Characteristics of Admission and Multivariate Analysis of Prognostic Factors in the 184 Patients Included in This Study Between 1998 and 2002

Variable	No. Patients		Odds Ratio (95% CI)	*P*
	Survival (n = 149)	Death (n = 35)		
Age, yr				
<1	11	1	1	—
1–6	115	31	1.12 (0.05–26.46)	0.94
6–15	23	3	0.09 (0.001–6.24)	0.26
Sex				
Male	76	12	0.49 (0.14–1.74)	0.27
Female	73	23	1	—
Virus types				
Type A	135	29	0.56 (0.06–5.78)	0.63
Type B	14	6	1	—
Past history				
Allergy				
Yes	8	1	1.19 (0.06–24.19)	0.91
No	141	34	1	—
Flu vaccination record				
Yes	7	1	0.29 (0.02–5.57)	0.41
No	142	34	1	—
Peak body temperature, °C				
<39	22	1	1	—
39–40	59	9	11.72 (0.31–442.39)	0.18
40–41	60	18	15.65 (0.42–577.88)	0.14
≥41	8	7	42.61 (0.98–1851.87)	0.05
Symptom				
Diarrhea				
Yes	9	5	2.28 (0.26–20.25)	0.46
No	140	30	1	—
Blood examination AST, IU/L				
<100	103	7	1	—
100–500	33	8	1.76 (0.40–7.64)	0.45
≥500	13	20	**7.88 (1.15–54.00)**	**0.04**
Creatine phosphokinase, IU/L				
<200	101	15	1	—
200–1000	37	9	1.03 (0.20–5.30)	0.97
≥1000	11	11	1.08 (0.13–9.16)	0.94
Platelet, $\times 10^4/\mu L$				
<10	16	20	3.79 (0.55–26.24)	0.18
≥10	133	15	1	—
Blood glucose, mg/dL				
<50	1	1	28.79 (0.35–2372.98)	0.14
50–150	83	5	1	—
≥150	65	29	**4.73 (1.10–20.30)**	**0.04**
Urine test				
Normal	108	10	1	—
Hematuria or proteinuria	41	25	**7.96 (1.76–35.92)**	**0.01**
Brain CT findings				
Normal	85	6	1	—
Edema, low density area, hemorrhage	64	29	2.59 (0.68–9.90)	0.17
Medication				
Acetaminophen				
Yes	94	21	1.46 (0.38–5.56)	0.58
No	55	14	1	—
Diclofenac sodium				
Yes	8	7	**16.34 (1.27–210.18)**	**0.03**
No	141	28	1	—
Mefenamic acid				
Yes	4	5	9.44 (0.70–127.73)	0.09
No	145	30	1	—

Values in bold indicate statistically significant results. A logistic regression analysis was used to determine the significant predictors of death.

CI indicates confidence interval; AST, aspartate aminotransferase; CT, computed tomography.

The survey of influenza-associated encephalopathy is continuing. However, some parts of the questionnaires have been changed and fewer sites are now included in the survey. These changes were made mainly because the "Private Information Protection Law" came into effect in Japan in 2003, making it very difficult to obtain individual information. We did not obtain information regarding the therapy for influenza-associated encephalopathy because there were no standardized therapeutic protocols between 1998 and 2002. Recently, it has been reported that certain therapeutic regimens can improve the prognosis of influenza-associated encephalopathy.[6,7] In 2005, therapies such as methylprednisolone pulse, plasma exchange,

and hypothermia therapy were proposed by the Collaborative Study Group on Influenza-Associated Encephalopathy, and further studies to improve influenza-associated encephalopathy prognosis via therapy are currently underway.

In conclusion, we identified several factors related to the poor prognosis of influenza-associated encephalopathy. Use of diclofenac sodium was the causal factor of poor prognosis. The other factors seem to reflect systemic hypercytokinemia, which is thought to play a role in the pathogenesis of the disease. However, all of these factors (with the exception of the use of diclofenac sodium) may be secondary to the disease process because they are seen in subjects who are moribund from a number of causes. Although these factors cannot be used to make an early diagnosis, our results have 2 major implications: the prognostic factors that we identified are easy to examine clinically, and these factors are important for the administration of intensive care in cases of influenza-associated encephalopathy.

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Increased Incidence of Adamantane-Resistant Influenza A(H1N1) and A(H3N2) Viruses During the 2006–2007 Influenza Season in Japan

To the Editor—Deyde et al. [1] described an increased incidence of adamantane-resistant influenza A (H1N1) and A(H3N2) viruses worldwide during the 2005–2006 influenza season. Following their observations on the spread of adamantane resistance among A(H3N2) viruses in Asia and North America from 2001 to 2005 [2], we reported a similarly increased incidence of drug-resistant A(H3N2) viruses in Japan, which accounted for 65.3% of circulating strains during the 2005–2006 season [3]. Dual mutations, S193F and D225N, in the hemagglutinin (HA) molecule were noted in all resistant strains, in addition to the S31N mutation in the M2 gene that has conferred resistance (named clade N) [3]. Deyde et al. [1] call this group subclade 2b.

Here, we describe a follow-up study performed in Japan to assess the incidence of adamantane-resistant A(H1N1) and A(H3N2) viruses during the 2006–2007 season and to analyze genetic changes in the HA molecule. Between 22 November, 2006, and 18 May, 2007, we conducted an influenza study in 14 medical facilities located in 4 areas in Japan: Niigata, Gunma, Kyoto, and Nagasaki Prefectures. In total, 1453 nasopharyngeal swab samples were collected from patients with influenza-like illness who visited the different medical facilities. From these samples, 1004 influenza viruses were isolated; of these viruses, 120 (12.0%) were A(H1N1), 632 (62.9%) were A(H3N2), and 252 (25.1%) were B viruses.

The incidence of adamantane-resistant A(H1N1) viruses increased dramatically, rising from 0 of 61 (0%) during the 2005–2006 season to 77 of 120 (64.2%) during the 2006–2007 season; when evaluated by area, the incidence ranged from 52.4% to 100.0%. All resistant strains had a S31N mutation in the M2 gene. The viruses were genetically similar to A/Solomon Islands/3/2006, a new vaccine component for the 2007–2008 season, which is equivalent to what Deyde et al. [1] call clade 2 (figure 1) [1, 4]. Resistant and sensitive strains were categorized in different groups by genetic sequencing of the HA gene. All resistant strains had the amino acid changes R192M, A193T, and T197K, whereas sensitive strains had E276K. These 3 amino acid changes in resistant strains were localized near the receptor-binding and antigenic sites [4, 5]. However, these changes were not observed in the clade 2a strains from the 2005–2006 influenza season described in the report by Deyde et al. [1].

With respect to A(H3N2), 566 (89.6%) of 632 isolates were adamantane resistant and were shown to have the S31N mutation in the M2 gene by genetic sequencing [6]. The incidence of adamantane-resistant viruses was higher, compared with the past season (65.3% of viruses were resistant during the 2005–2006 season), and the percentage of resistant viruses in the 4 areas ranged from 80.6% to 94.8%. Genetic sequencing of the HA gene revealed that resistant A(H3N2) strains analyzed during the 2006–2007 season had amino acid changes S193F and D225N, as did viruses in clade N (i.e., subclade 2b) during the 2005–2006 season. Furthermore, sensitive strains also grouped together with resistant strains as clade N. (figure 1).

We confirmed an increased incidence of adamantane-resistant A (H3N2) and A (H1N1) virus strains during the 2006–2007 influenza season in Japan. At present, resistant strains circulate without selection pressure from the drug, because none of our patients was known to have received amantadine, as in other reports [7, 8]. The assumption was made that certain drastic genetic changes, such as substitutions in amino acid residues 193 and 225 in the HA molecule of clade N A(H3N2) virus, have occurred in the influenza genome that produce little or no fitness cost (i.e., reduction in transmissibility) [3, 9]. In particular, a change at position 193 may have significance because, in resistant strains, the same position was mutated in 3 subtypes, namely, resistant A(H1N1) in the 2006–2007 season, clade N virus with A(H3N2), and clade 1 viruses with A(H5N1) [10]. This specific HA mutation may contribute to the maintenance of adamantane-resistant strains.

Furthermore, after 2 seasons, it was observed that clade N A(H3N2) resistant strains accommodated sensitive strains that had dual mutations in the HA gene but lacked the M2 gene mutation (figure 1). This may indicate that the selection pressure still favors sensitive strains over resistant strains. With respect to recent human influenza, we need to elucidate the mechanism behind the appearance and maintenance of adamantane-resistant strains that acquired little fitness cost.

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Figure. 1. Phylogenetic analysis of the hemagglutinin (HA) gene (HA1 domain) from influenza A (H3N2) and (H1N1) isolates collected in Japan during the 2006–2007 influenza season. Resistant strains are marked with asterisks; sensitive strains are unmarked. Sequences from representative vaccine components and known adamantane-sensitive or adamantane-resistant strains obtained from a genetic database (http://www.flu.lanl.gov/, Influenza Sequence Database at the Los Alamos National Laboratory; http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html, Influenza Virus resource at the National Center for Biotechnology Information) were included. The amino acid substitutions found in each group are indicated by line, and the percentage of bootstrap values over 70% is shown for major branches. *A*, A(H3N2) isolates; *B*, A (H1N1) isolates. Amino acid numbering for A (H1N1) corresponds to the H3 subtype [4, 5].

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Reply to Saito et al.

To the Editor—The rapid emergence and spread of adamantane resistance among human influenza A(H3N2) viruses circulating globally in 2004–2007 and the more recent emergence of adamantane resistance among influenza A(H1N1) viruses in certain geographical regions have been reported by several laboratories in the United States and other countries [1–6]. In their letter, Saito et al. [7] presented data on resistance to adamantanes among seasonal influenza A viruses collected in Japan during the 2006–2007 influenza season. The influenza A(H3N2) subtype was predominant in Japan during this season. Within this subtype, the incidence of adamantane resistance reached ~90%, which was a 25% increase from the previous influenza season. A drastic spike in the incidence of adamantane resistance (an increase from 0% to 64%) was also detected among influenza A(H1N1) viruses.

Our surveillance data support the view of Saito et al. [7] that the incidence of adamantane resistance among A(H3N2) viruses remains high. However, our analysis, which was conducted on a larger number of isolates from different geographic areas, revealed a significant decrease in resistance among A(H3N2) viruses isolated in many regions, compared with data from the 2005–2006 season (table 1 and previously published data [6]).

In addition to observations of Saito et al. [7] regarding influenza A (H1N1) viruses circulating in Japan, our data demonstrate that the incidence of adamantane resistance increased significantly among viruses of this subtype that were collected

Table 1. Incidence of resistance to adamantanes among inflenza A viruses collected worldwide in 2006–2007.

Region	Resistant viruses, no. (%)	
	A(H3N2)	A(H1N1)
Asia	235 (66.4)	118 (73.7)
Europe	71 (35.2)	45 (60.0)
North America	481 (68.0)	519 (2.9)
South America	77 (94.8)	29 (0)
Total	864 (67.2)	711 (18.1)

NOTE. Viruses submitted and tested at the World Health Organization Collaborating Center for Surveillance, Epidemiology, and Control of Influenza at the Centers for Disease Control and Prevention, Atlanta, Georgia.

in 2006–2007 in other countries in Asia, as well as in Europe. In contrast, in the United States, as well as in the other countries of North and South America, the incidence of adamantane resistance among viruses of the H1N1 subtype remained nearly unchanged from the last year. These observations demonstrate a substantial geographical difference in the incidence of resistance, especially among H1N1 viruses.

On the basis of phylogenetic analysis of the hemagglutinin (HA) gene, we previously reported that the drug-resistant influenza A(H1N1) viruses circulating in several countries in Asia belong to a distinctive genetic group (clade 2a) [6]. In contrast, the HA sequences of the drug-sensitive A(H1N1) viruses that were collected elsewhere (in the United States, in particular) during the same period fell into genetically different groups (clades 1 and 2b). According to the report by Saito et al. [7], all drug-resistant A(H1N1) viruses collected in Japan in 2006–2007 had amino acid changes R192M, A193T, and T197K in the HA molecule. However, given our published [6] and most recent data, not all of the drug-resistant A(H1N1) viruses circulating worldwide during that season shared these 3 amino acid changes. Moreover, our previous and current data indicate that there are no signature amino acid changes in the HA

molecule that are unique to resistant A(H3N2) viruses as well.

In conclusion, we believe that several factors that are not yet well understood may contribute to the emergence and spread of adamantane-resistant viruses regionally and globally. Amantadine use in Asia during the severe acute respiratory syndrome outbreak and the avian influenza scare may have facilitated the emergence and later the spread of adamantane-resistant influenza A(H3N2) viruses in the region. However, it is unlikely to be the only factor that contributed to the alarming global spread of drug-resistant A(H3N2) viruses during 2005–2006. It is unclear whether the changes in the HA molecule alone or in combination with a novel gene constellation ('N-lineage' [8]) played a role in the unprecedented spread of drug-resistant A(H3N2) viruses in other countries where the use of amantadine and/or rimantadine was limited or nonexistent. The dramatic increase in the incidence of resistance to adamantanes among viruses of the other subtype—A(H1N1)—that are currently circulating in some Asian countries raises further concerns. It also challenges our understanding of the mechanisms that underlie viral evolution and the potential effects of drug resistance on viral fitness. Analysis of the mechanisms that drive the emergence and spread of drug resistance, as well as close monitoring of the viruses circulating in different geographic regions, are critical for confronting the global threat of epidemics or even pandemics caused by drug-resistant viruses.

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ORIGINAL ARTICLE

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Protection against systemic fatal pneumococcal infection by maternal intranasal immunization with pneumococcal surface protein A (PspA)

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Abstract *Streptococcus pneumoniae* is a leading causative pathogen responsible for various types of bacterial infectious diseases in children. The aim of this study was to evaluate the protection conferred against fatal pneumococcal infections during infancy by maternal intranasal immunization with pneumococcal surface protein A (PspA). Four-week-old female BALB/c mice were immunized with PspA mixed with, or without, cholera toxin B (CTB) intranasally twice a week for 3 weeks. After the final immunization, they were mated with male mice to obtain offspring. Offspring at 10 days old were intraperitoneally inoculated with a pneumococcus strain, TIGR4, serotype 4. After the infections their survival periods were monitored. Anti-PspA-specific IgG antibody was induced in sera and breast milk at birth and maintained for 14 days during nursing periods in the PspA-immunized mother mice. At birth, offspring delivered from PspA-immunized mother mice had levels of anti-PspA-specific IgG antibody in sera same to those in their mothers on the day of birth. The survival times to death of offspring delivered from PspA-immunized mother mice after systemic fatal pneumococcal infections were significantly extended compared to those of controls. These findings suggest that maternal intranasal immunization with PspA could be an attractive procedure to employ against pneumococcal infections in early childhood.

Key words *Streptococcus pneumoniae* · Maternal immunization · Pneumococcal surface protein A (PspA) · Vaccine

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Introduction

Streptococcus pneumoniae is responsible for a significant proportion of bacterial infectious diseases such as meningitis, otitis media, bacteremia, and pneumonia.[1,2] The current 23-valent pneumococcal capsular polysaccharide vaccine is efficacious in adults.[3] However, the vaccine cannot elicit protective antibodies in infants because of the weak immunogenicity of the T-cell-dependent antigens.[3,4] Protein-conjugated polysaccharide vaccines have been considered as an alternative means to induce protective immunity in infants and children.[5,6] Efforts have been made to develop protein-conjugated polysaccharide vaccines containing up to 11 serotypes. Recent human trials of a 7-valent polysaccharide conjugate vaccine showed the capability to elicit solid protection against invasive pneumococcal infection in children.[7] The protein-conjugated polysaccharide vaccines, however, could not protect against infection with strains of all virulent capsular types, because of the broad diversity of capsular polysaccharides.[8–10]

In addition to these disadvantages in the current polysaccharide-based pneumococcal vaccines, the matter of immune responses in infants should be discussed more. Children younger than 2 years old usually have lower levels of pathogen-specific IgG antibody in sera, due to the age-related immaturity of their immune responses.[11] Subnormal levels of serum IgG antibody against causative pathogens are also reported in children who are prone to particular infectious diseases such as those who are otitis-prone.[12–16] If these younger children are infected with pneumococci, especially antimicrobial-resistant *S. pneumoniae*, it is difficult to eradicate these pathogens by immunological host defense or by the use of antimicrobials.[17] The demand for protein-based pneumococcal vaccines and their protection against pneumococcal infections during infancy have been further emphasized by studies demonstrating a recent rapid increase in both the prevalence and level of resistance of multiple antimicrobial-resistant pneumococci.[17,18] Pneumococcal surface protein A (PspA) is a surface-exposed protein of *S. pneumoniae* and is a virulence factor. PspA is a highly

immunogenic protein and is expressed in all pneumococcal isolates.[19-23] Thus, PspA is an attractive candidate for future protein-based pneumococcal vaccines.

In this animal study, we evaluated the protection conferred against pneumococcal infections during infancy by maternal intranasal immunization with PspA.

Materials and methods

Maternal intranasal immunization

Four-week-old female BALB/c bjy mice were used in this experiment. Mice ($n = 6$) were intranasally immunized with 1 μg recombinant PspA (rPspA) mixed with 4 μg cholera toxin B subunit (CTB; List Biological Labs, Campbell, CA, USA) on the Mondays and Fridays of 3 consecutive weeks. These immunizations included CTB for the first 2 weeks of immunization. During the last week, for the last two immunizations, mice received antigen alone. Control mice ($n = 6$) received only CTB for the first 2 weeks and only saline for the last week.

After the final immunization, the female mice were mated with male mice for 2 weeks. Approximately 3 weeks after mating, offspring (19 from immunized mothers and 18 from controls) were obtained. Sera and milk were collected from mother mice at birth (day 0) and at 7 and 14 days after birth. Sera were also collected from offspring at birth (day 0), and at 7 and 14 days after birth. Offspring at 10 days old were used for a systemic fatal pneumococcal infection model. All animal studies were conducted in accordance with the guidelines of and after approval of the Wakayama Medical University Animal Care and Use Committee.

Enzyme-linked immunosorbent assay (ELISA)

Anti-PspA-specific IgG antibody in milk and sera of mother mice and offspring were evaluated by a solid-phase enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microplates (MaxSorp; Nunc, Roskilde, Denmark) were coated with 50 μl rPspA (2 μg/ml) in phosphate-buffered saline (PBS) overnight at 4°C. After three washes with PBS containing 0.05% Tween 20 (PBS-T), the wells were blocked for 1 h with casein buffer (0.2% casein, 0.05% Tween 20 in PBS) at room temperature. Then, 50-μl samples diluted with casein buffer were incubated at 4°C overnight. After a washing with PBS-T, the plate was incubated with 50 μl of biotynylated anti-mouse IgG antibody (1: 3000; Southern Biotechnology Associates, Birmingham, AL, USA) diluted in casein buffer for 2 h at room temperature. Then, after a washing with PBS-T, the plate was incubated with alkaline phosphatase-conjugated streptavidin (1: 4000; Southern Biotechnology Associates) for 2 h at room temperature. Color was developed with *p*-nitrophenyl phosphate (PNPP; Sigma Chemical, St. Louis, MO, USA) and the optical density of each well was measured by a spectrophotometer at 405 nm.

Systemic infection model

Offspring ($n = 9$) from immunized mothers and offspring ($n = 8$) from nonimmunized mothers at 10 days old were inoculated intraperitoneally, under anesthesia, with pneumococcal cells at 1×10^4 CFU in 100 μl sterile PBS. The pneumococcal strain used in this study was TIGR4, serotype 4 virulent strain. *S. pneumoniae* were grown at 37°C in Todd-Hewitt broth with 0.5% yeast extract (THY) until mid log-phase and stored in THY broth containing 10% glycerol at −80°C. After the intraperitoneal inoculation, the offspring were monitored for their death every 6 to 8 h. The survival periods were expressed as times from inoculation with pneumococci to death.

Statistics

Comparisons of anti-PspA-specific IgG antibody between two groups were calculated by Student's *t*-test. The comparison of survival between two groups was calculated by the Mann Whitney *U*-test. Statistical values were calculated with Prism 4 (GraphPad Software, La Jolla, CA, USA).

Results

Anti-PspA-specific IgG antibody in sera of mother mice and offspring

Anti-PspA-specific IgG antibody in sera was highly induced at birth and maintained during the nursing periods among PspA-immunized mother mice, while control mother mice did not have the specific IgG antibody in sera (Fig. 1). Anti-PspA-specific IgG antibody was also induced in the breast milk of PspA-immunized mother mice and maintained for 14 days during the nursing period (Fig. 2).

Anti-PspA-specific IgG antibody in mother mice was transferred to their offspring. In offspring delivered from PspA-immunized mothers, levels of anti-PspA-specific IgG antibody in sera at birth were similar to the levels in the mothers on day 0 (Fig. 3). The specific IgG antibody in the offspring's sera had decreased on day 7 after birth. The levels of Anti-PspA-specific IgG in the sera of offspring on day 7 and day 14 after the birth were lower than those on day 0, although the difference among them was not statistically significant.

Survival after pneumococcal infection

The survival of offspring after infection with pneumococci at 1×10^4 CFU per mouse were evaluated. The median, 75% value, and 25% value of survival times of offspring delivered from PspA-immunized mothers were 32 h, 43 h, and 28 h, respectively. In this model, all mice died after intraperitoneal infection with *S. pneumoniae*. Thus, we compared survival times between the two groups (offspring of immunized and nonimmunized mothers) and statistically

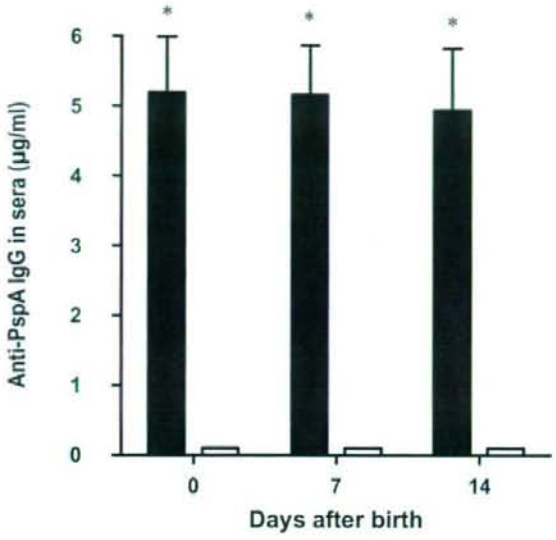

Fig. 1. The levels of anti-pneumococcal surface protein A (*PspA*)-specific IgG antibody in mothers' sera. Values for results are expressed as means ±SE of anti-PspA-specific IgG in mothers' sera. *Closed bars*, immunized mothers (n = 6); *open bars*, nonimmunized mothers (n = 6). *P < 0.01 compared with nonimmunized controls, by Student's t-test

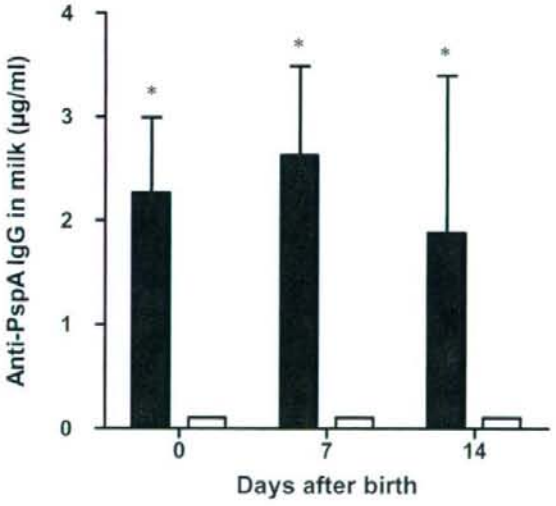

Fig. 2. The levels of anti-PspA-specific IgG antibody in mothers' breast milk. Values for results are expressed as means ± SE of anti-PspA-specific IgG in mothers' breast milk. *Closed bars*, immunized mothers (n = 6); *open bars*, nonimmunized mothers (n = 6). *P < 0.01 compared with nonimmunized controls, by Student's t-test

analyzed the results with the Mann-Whitney U-test. The survival times to death after fatal systemic pneumococcal infections in offspring delivered from PspA-immunized mother mice were significantly extended compared to those of controls (Fig. 4).

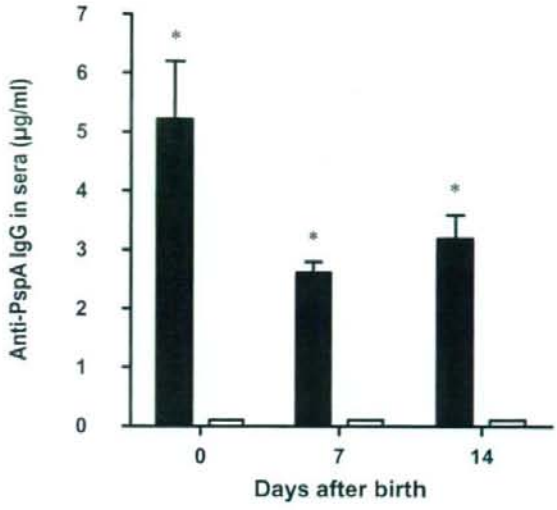

Fig. 3. The levels of Anti-PspA-specific IgG antibody in offspring sera. Values for results are expressed as means ± SE of anti-PspA-specific IgG in offspring sera. *Closed bars*, immunized mothers (n = 19); *open bars*, nonimmunized mothers (n = 18). *P < 0.01 compared with non-immunized controls, by Student's t-test

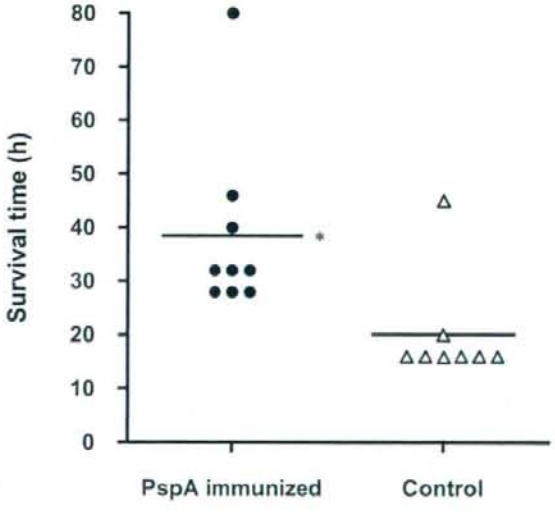

Fig. 4. Survival times of mice after systemic fatal pneumococcal infections. Survival times of offspring after systemic fatal pneumococcal infections are shown. *Closed circles*, offspring delivered from PspA-immunized mother mice (n = 9); *triangles*, offspring delivered from nonimmunized mother mice (n = 8). *P < 0.01 compared with offspring delivered from nonimmunized controls, by Mann Whitney U-test

Discussion

Recurrent bacterial infections are considered to correlate with subnormal levels of serum IgG antibody against causative pathogens, due to age-related immaturity.[11] Virolainen

et al.[23] showed that children who were infected most frequently with pneumococci had the lowest titer of antibody to PspA among children with invasive pneumococcal infections. In addition, young children whether or not they are healthy, show the lowest serum IgG level in their lives at age less than 2 years. Thus, it is important to induce effective protective immune responses against pneumococci during early childhood. In the present study, we report the induction of specific immune responses in the sera and colostrum of mother mice and the transfer of specific antibody from mothers to neonate mice by maternal intranasal immunization with PspA; this resulted in the prolongation of survival time after lethal pneumococcal infections.

Recent studies on the development of an effective mucosal vaccine have focused on the common mucosal immune system (CMSI).[24,25] Yamamoto et al.[26] reported that oral immunization with PspA and cholera toxin (CT) could induce PspA-specific immune responses in both saliva and sera. However, the oral route usually required relatively high amount of antigens for immunization. Kurono et al.[27] reported the benefit of intranasal application to evoke specific mucosal immune responses, as well as systemic immune responses, by the use of the outer membrane proteins (OMP) of *Haemophilus influenzae*. Our previous study[28] showed that intranasal immunization with the OMP P6 of *H. influenzae* and CT every 2 days for 2 weeks elicited anti-P6-specific IgG antibodies as early as day 7, and these peaked on day 21. An application of this immunization schedule for maternal immunization also induced anti-P6-specific IgG in mother's sera at birth and maintained the responses for 14 days during the nursing period.[29] The anti-P6-specific IgG in sera of offspring delivered from P6-immunized mothers was increased at birth and until day 14. In the present study, we evaluated anti-PspA-specific IgG in mother's sera on days 0, 7, and 14 after birth, in which periods the specific immune response would be stable and most efficient for the protection of offspring against pneumococcal infections. In the present study, intranasal immunization with PspA and CTB induced anti-PspA-specific IgG antibody in both breast milk and sera. The intranasal application is easy to repeat and requires only a small amount of PspA, such as 1 μg, for each immunization. In this study, we used CTB as a typical mucosal adjuvant to evoke specific immune responses. Recent studies have demonstrated several mucosal adjuvants, such as attenuated mutant CT, flt3 ligand, and the CpG motif.[30-32] It would also be of further interest to apply these mucosal adjuvants for maternal intranasal immunization.

Anti-PspA-specific IgG antibody in maternal sera was shown to be transferred to the offspring via the placenta.[33] Yamauchi et al.[29] reported the importance of IgG antibody, rather than SIgA, in breast milk to maintain specific IgG in the sera of offspring mice. Differing from that in humans, mouse colostrum and breast milk contain high amounts of IgG antibody compared to amounts of IgA and IgM antibodies. In mice, IgG antibody in the mother's sera is transferred from mother to fetus through the placenta by the neonatal Fc receptor, FcRn, which is expressed in the yolk sacs of mice and rats.[34,35] Moreover, IgG antibody in breast milk is also transferred from the intestine lumen to the systemic circulation in neonate mice.[36] This transport of IgG antibody is mediated by FcRn expressed in the intestine of mice and rats.[37,38] Hotomi et al.[28] reported that anti-P6 specific IgA were not detected in sera of offspring delivered from mothers intranasally immunized with P6 of *H. influenzae*, while anti-P6 specific IgA were induced in mothers' sera and breast milk. Thus, we focused on the anti-PspA-specific IgG in the offsprings' sera transferred from the mothers' sera, and we examined the protection of offspring against fatal pneumococcal infections. Although the immune systems are different in mice and humans, in both mice and humans maternal intranasal immunization can induce specific immune responses in the mother, and these can be effectively transferred to their infants. The findings of the present study strongly suggest that maternal intranasal immunization would be an attractive procedure to protect against *S. pneumoniae* infections in early childhood, because transplacental immunoglobulin (Ig) is transferred during pregnancy and after birth the neonate can obtain Ig via breast milk.[39-41]

In the present study, we further evaluated the protection conferred against fetal pneumococcal infections by maternal intranasal immunization. We inoculated the TIGR4 strain (possessing serotype 4 capsular polysaccharide) intraperitoneally because intravenous application would have been too difficult in these small offspring. As for the virulence of pneumococci to mice, the serotype 3 strains cause rapid sepsis and death, whereas death resulting from infections with type 6A and 6B strains usually does not occur for a few more days. Serotype 4 strain shows relatively high virulence and causes death.[42]

Offspring delivered from mother mice intranasally immunized with PspA were protected from systemic pneumococcal infections. Ogunniyi et al.[43] reported the enhanced survival times of mice after intraperitoneal infections by systemic immunization with PspA. Roche et al.[44] also reported that the survival times of mice after challenge with pneumococci intravenously at 2-log higher than the 50% lethal dose were enhanced by subcutaneous immunization with PspA. The original evidence that PspA could elicit protective immune responses came from passive protection experiments with monoclonal antibodies to PspA.[45,46] The protective capacity of the sera is reported to be much more strongly correlated with serum levels of antibody to PspA than with the serum levels of antibody to the relevant capsular antigen.[20] PspA can interfere with the fixation of complement C3 and potentially block downstream events leading to opsonization and phagocyte chemotaxis.[47] PspA-specific IgG antibody will inhibit the pathogenic role of PspA and enhance opsonophagocytosis. Based on the sequences of the alpha helical region of a major cross-protective epitope, PspA is divided into six clades that comprise three PspA families. Families 1 and 2 represent about 99% of pneumococci, while family 3 comprises about 1% of pneumococci (our unpublished data). PspAs are all cross-reactive immunologically, although they have been found to be structurally and antigenically variable.[21] Although the cross-reactivity of PspA-specific IgG in off-

spring was not addressed in the present study, some studies have reported that immunization with PspA could provide cross-protection against heterologous serotypes of *S. pneumoniae*.[45]

PspA has recently undergone phase I clinical trials in humans and has been found to be safe and highly immunogenic.[33] A human phase I trial with recombinant truncated PspA (family 1, clade 2) provided sera which showed that post-immune sera could protect mice from fetal pneumococcal infection. The mouse model reported in the present study indicates that, for protection against sepsis, a vaccine containing PspA may be ideal for eliciting protection against invasive disease. A previous study revealed that antibody to PspA could overcome the anticomplementary effect of PspA, allowing for increased complement activation and C3 deposition on pneumococci.[46] Also, antibody to PspA enhanced the killing of pneumococci by apolactoferrin via the blocking of the bactericidal active site of lactoferrin responsible for pneumococcal killing.[48] These findings strongly suggested that the anti-PspA antibody can act as a bactericidal antibody and/or could be involved in opsonophagocytic killing. Additional testing will be required to arrive at the optimal minimal composition of a vaccine to prevent pneumococcal infections.

In conclusion, the findings of the present study suggest that maternal intranasal immunization would be an attractive procedure against pneumococcal infections in early childhood, because transition of the specific antibody can be expected through the placenta and the mother's milk.

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REVIEW ARTICLE

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Clinical bacteriology and immunology in acute otitis media in children

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Abstract Acute otitis media (AOM) is the most common disease seen in childhood. *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* (NTHi), and *Moraxella catarrhalis* are the most frequent pathogens of all AOM episodes. The high prevalence of drug-resistant pathogens such as penicillin-resistant *S. pneumoniae* (PRSP) and beta-lactamase producing or nonproducing ampicillin-resistant *H. influenzae* (BLPAR or BLNAR) is causing serious clinical problems worldwide. PRSP and BLNAR have become important risk factors for intractable clinical outcome of AOM. PRSP causes a three times higher incidence of intractable AOM than susceptible strains. BLNAR strains show penicillin-binding protein gene mutation and are not only resistant to ampicillin, but also have reduced susceptibility to cephalosporin. The resistant *H. influenzae* pathogen has shown clonal dissemination in Japan in ways different from those of penicillin-resistant *S. pneumoniae*. Protection against AOM due to these pathogens may depend on pathogen-specific antibodies. Pneumococcal capsular polysaccharides (PCPs) are type specific and poorly immunogenic in children younger than 2 years old. Approximately 50% of otitis-prone children showed subnormal levels of anti-PCP IgG2 antibody. In our immunological study in children with otitis media, however, otitis-prone children were not unusually vulnerable to infections except those resulting in otitis media. This fact seems to refute the presence of a broad immunological deficit in these children. Some pathogen-specific antibodies may be directed against protein immunogens such as pneumococcal surface protein A (PspA) of *S. pneumoniae*, P6 of NTHi, and UspA of *M. catarrhalis*. The levels of antibody to P6 of NTHi in healthy children were significantly higher than those in the otitis-prone children after the age of 18 months. In general, individual antibody levels in otitis-prone individuals did not have an age-dependent rise. The failure to develop a good antibody response to common antigens such as PspA and P6 may enable the pathogen to cause persistent or recurrent disease.

Key words Acute otitis media · penicillin-resistant *Streptococcus pneumoniae* (PRSP) · β-lactamase nonproducing ampicillin resistant (BLNAR) · Otitis prone · P6 · PspA

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Clinical bacteriology in acute otitis media

Bacteria are found in 50%–90% of cases of acute otitis media (AOM) with or without otorrhea.[1] *Streptococcus pneumoniae* (*S. pneumoniae*) and *Haemophilus influenzae (H. influenzae)* are the leading causative pathogens responsible for AOM, and they frequently colonize in the nasopharynx.[1,2] These two notorious pathogens have long been susceptible to β-lactams, and AOM caused by them had easily been improved by oral antimicrobial therapy. However, antimicrobial-resistant pathogens, especially penicillin-resistant *Streptococcus pneumoniae* (PRSP), has become the major cause of intractable otitis media.[2] Antimicrobial resistance in *H. influenzae* has also evolved significantly during the last 20 years, while ampicillin (AMP) had long been considered the drug of first choice for the treatment of infection due to *H. influenzae*.[3]

The mechanism of the resistance of *S. pneumoniae* to β-lactams is the stepwise alterations in the high molecular weight penicillin binding proteins (PBPs) and the reduction of the binding affinity of β-lactams to the PBPs. Among several PBPs, 1A, 2X, and 2B have transpeptidase activity and contain the conserved amino acid motif of SXXK, SXN, and KT(S) G in an active serine residue. *S. pneumoniae* acquires exogenous low affinity genes and causes genetic mutations that alter PBP affinity for β-lactams.[4–7]

Two well-known mechanisms of resistances to β-lactams in *H. influenzae* have been reported. One is the production