after stratification by area. All statistical procedures were performed using SAS software.

Patients of all ages diagnosed with influenza at the clinics were asked to donate pharyngeal swabs and sera. Pharyngeal swabs were refrigerated in buffer and sent to the laboratory of author TK within a few days, and circulating influenza virus was cultured by standards methods. Antigenic similarity of isolated strains to the vaccination strain was determined using ferret sera sensitized to A/Panama/2007/99 (H3N2), the vaccine strain used in the 2003–2004 season in Japan.

Acute sera (at diagnosis) and convalescent sera (4 weeks later) were collected at each clinic, stored at −20 °C, and sent to the laboratory of author AM. Serum hemagglutination inhibition (HI) titers to A/H1N1, A/H3N2, and B influenza viruses were determined using standard methods, with an initial serum dilution ratio of 1:10. Information about vaccination was gathered by interviewing clinic patients, because people in Japan aged <65 years are not subsidized for vaccination by their municipalities. Differences in dilution ratios and HI titers were calculated using the Mann-Whitney *U*-test.

The Ethical Board of the Kyoto Prefectural University of Medicine approved this study.

3. Results

On October 1, 2003, population registries listed 627 men and 828 women aged ≥65 years in area A and 606 men and 849 women aged ≥65 years in area B. Of these, 456 men and 621 women in area A and 503 men and 721 women in area B replied to the questionnaire and were not institutionalized during the entire peak influenza outbreak period. The mean ± S.D. age was 75.1 ± 7.1 years in area A and 75.6 ± 7.1 years in area B. The proportion of vaccinated subjects was 69.3% in area A and 64.2% in area B. According to the questionnaire, 3.8% of those vaccinated in area A and 3.1% of those vaccinated in area B were inoculated twice, with the remainder having single inoculations.

Information regarding vaccination obtained from the municipality offices was compared with self-reports on the questionnaires. Of the 747 subjects in area A who were recorded by their municipality as having been vaccinated, six reported they had not been vaccinated, and 84 did not reply. Conversely, of the 330 subjects who were recorded as having not been vaccinated, 29 reported they were vaccinated, and 21 did not reply. In area B, four of the 786 subjects recorded as having been vaccinated reported they had not been vaccinated, and 106 did not reply. Of the 438 subjects in area B recorded as having not been vaccinated, 31 reported that they had, and 40 did not reply.

The distribution of underlying conditions and behaviors/environments for likelihood of exposure to influenza is shown in Table 1. The prevalence of influenza differed slightly between areas A and B. Table 1 also shows the resulting model of propensity scores as odds ratios, which estimate the tendency of vaccination for subjects having each of the factors listed in the table. For each subject, the propensity score was calculated as a sum of the products of the response (1/0) and coefficient for each parameter.

Overall, 4.1% of the study subjects had a fever of ≥37.0 °C, 1.7% had a fever of ≥38.0 °C, and 1.8% were diagnosed with influenza. These percentages differed between areas (Table 2). The sex- and age-adjusted odds ratio (OR) of vaccination for a fever of ≥37.0 °C during the peak period of influenza outbreak was 0.91 for both areas combined (Table 3). The adjusted OR of vaccination for fever of ≥38.0 °C was 0.81, and that for diagnosis of influenza was 0.88. Those ORs tended to be smaller than 1, but not significantly so, and, after further adjustment of propensity score for underlying conditions, these ORs tended to be lower. Further adjustment of propensity score for likelihood of exposure to influenza did not change the ORs substantially.

From December 1, 2003, to March 31, 2004, 51 patients in area A and 56 in area B were diagnosed with influenza in the clinics. The trends for the number of patients, as well as the trend of ILI per sentinel in Kyoto prefecture [5], are illustrated in Fig. 1. The peak of influenza incidence in area A occurred earlier than that of Kyoto prefecture as a whole, whereas the peak in area B was almost the same as that in Kyoto prefecture. During the peak 4 weeks (i.e., January 19 to February 15) 77% of all influenza patients in area A, and 71% in area B were treated.

Thirteen patients in area A and 23 in area B donated pharyngeal swabs and acute sera. Of these 36 patients, 15 were males and 21 were females, of mean age 46.8 ± 20.7 years (range, 14–95 years). Influenza virus was isolated from nine patients in area A and 16 in area B, and all viruses were of AH3 subtype. Of the 25 isolated strains, 15 (60%) differed at least four-fold in dilution ratio (640 or less) compared with A/Panama/2007/99 (H3N2) virus, the strain used in Japan for vaccination. The dilution ratios did not differ between vaccinated and unvaccinated patients.

Eight of 10 (80%) acute sera of vaccinated patients had HI titers of ≥40 to A/H3N2 influenza virus, whereas 76% (20/26) of unvaccinated patients had HI titers of <40 ($p < 0.001$). HI titer to A/H3N2 virus was significantly higher in convalescent compared with acute sera, both in vaccinated ($p = 0.005$) and unvaccinated ($p < 0.001$) patients. However, HI titers to A/H1N1 and B influenza virus did not increase.

4. Discussion

Under the National Epidemiological Surveillance of Infectious Diseases, clinically diagnosed influenza cases have been reported weekly by sentinel clinics nationwide. The height of the peak in 2003/04 season ranked sixth among the past 10 seasons, and the total number of cases per sentinel was the fourth lowest. During this influenza season in Japan, prefectural and municipal public health institutes reported that 4740 influenza AH3, 290 influenza B, and only five AH1 viruses were isolated. Thus, about 94% of the influenza

Table 1
Distribution of underlying conditions and risks of exposure to influenza, and resulting models of propensity scores

	Area					
	A			B		
		OR	P		OR	P
Number of subjects	1077			1224		
Underlying condition						
Diabetes mellitus						
No	64.9%	1.00		7.2%	1.00	
Yes	8.4	1.02	0.93	65.6	0.83	0.52
Unknown/no response	26.7	1.20	0.55	27.2	0.51	0.03
Asthma						
No	68.7	1.00		68.1	1.00	
Yes	3.9	0.80	0.61	2.1	0.72	0.52
Unknown/no response	27.4	0.96	0.93	29.8	1.39	0.50
Chronic bronchitis						
No	66.2	1.00		67.2	1.00	
Yes	3.0	0.72	0.48	1.7	0.44	0.17
Unknown/no response	30.8	1.30	0.48	31.1	0.54	0.18
Emphysema						
No	65.8	1.00		67.2	1.00	
Yes	4.6	0.93	0.85	1.9	3.73	0.03
Unknown/no response	29.6	0.92	0.83	30.9	2.81	0.02
Easily catch colds						
No	54.4	1.00		66.3	1.00	
Yes	14.5	1.40	0.11	10.9	1.90	0.004
Undetermined	24.1	1.04	0.77	15.6	1.15	0.42
No response	7.0	0.96	0.91	7.2	1.15	0.57
Self-rated health						
Poor	12.5	1.00		8.0	1.00	
Good	21.5	0.94	0.82	22.7	1.19	0.50
Moderate	62.8	1.21	0.36	64.8	1.97	0.004
No response	3.2	1.62	0.30	4.5	1.91	0.08
Likelihood of exposure to influenza						
Subjects often go out into crowds						
No	75.7%	1.00		69.8%	1.00	
Yes	20.9	0.86	0.38	26.5	0.94	0.11
No response	3.4	1.20	0.69	3.7	0.56	0.73
Household members often go out into crowds						
No	43.9	1.00		36.7	1.00	
Yes	49.5	0.89	0.47	54.4	0.74	0.96
No response	6.6	1.04	0.90	8.9	0.52	0.83
Kindergarten to high school student in household						
No	28.3	1.00		19.4	1.00	
Yes	65.6	1.53	0.009	73.8	0.89	0.19
No response	6.1	1.53	0.28	6.8	0.92	0.08

Odds ratios (ORs) for disposing and behavioral factors were calculated separately from vaccinated = 1 and unvaccinated = 0 as a dependent variable by multivariate logistic regression.

Table 2
Rate of each outcome by study area and vaccination status

Area	Vaccination	No. of subjects		Mean age ± S.D.	Outcome (%)		
		Men	Women		Fever of ≥37.0 °C	Fever of ≥38.0 °C	Diagnosis of influenza
A	Vaccinated	307	440	75.2 ± 6.9	4.5	1.5	2.8
	Unvaccinated	149	181	74.6 ± 7.7	4.2	2.4	3.0
B	Vaccinated	282	504	76.0 ± 6.8	3.7	1.7	0.8
	Unvaccinated	221	217	74.9 ± 7.3	4.6	1.8	0.9

Table 3
Odds ratios and 95% confidence intervals of vaccination for influenza-related outcomes

Area	Adjusted variables	Outcome		
		Fever of >37.0 °C	Fever of >38.0 °C	Diagnosis of influenza
A	Sex, age	1.11 (0.59, 2.10)	0.70 (0.29, 1.72)	0.90 (0.42, 1.95)
	+Underlying conditions[a]	1.11 (0.58, 2.10)	0.65 (0.26, 1.61)	0.84 (0.38, 1.82)
	+Likelihood of infection[b]	1.10 (0.58, 2.10)	0.64 (0.26, 1.58)	0.81 (0.37, 1.76)
B	Sex, age	0.79 (0.43, 1.43)	0.98 (0.39, 2.42)	0.98 (0.26, 3.58)
	+Underlying conditions[a]	0.69 (0.37, 1.27)	0.97 (0.38, 2.45)	0.84 (0.22, 3.18)
	+Likelihood of infection[b]	0.69 (0.37, 1.26)	0.99 (0.39, 2.50)	0.83 (0.22, 3.16)
A+B	Sex, age	0.91 (0.59, 1.41)	0.81 (0.43, 1.53)	0.88 (0.46, 1.71)
	+Underlying conditions[a]	0.86 (0.55, 1.32)	0.77 (0.40, 1.47)	0.81 (0.41, 1.57)
	+Likelihood of infection[b]	0.86 (0.55, 1.33)	0.77 (0.40, 1.47)	0.78 (0.40, 1.52)

[a] Adjusted for sex, age, and propensity score for underlying conditions.
[b] Adjusted for sex, age, and propensity scores for underlying conditions and likelihood of exposure to influenza.

viruses isolated nationwide during the 2003/04 season were AH3 viruses, with most of the others being B viruses. More than 90% of the AH3 viruses were the A/Fujian/411/2002-like strain, which was less than one-fourth homologous to A/Panama/2007/99 by HI titer [13].

Two major factors affecting the efficacy and effectiveness of influenza vaccines and their measurements are antigenic similarity between vaccinating and circulating strains, and the specificity of outcome measures [1]. In this study, 60% of circulating strains exhibited one-fourth or lower homology to A/Panama/2007/99 by HI titer, similar to that noted throughout Japan. Thus, the efficacy of vaccine during this season may have been attenuated by antigenic drift in circulating strains.

To examine the effectiveness of an influenza vaccine in the general population and to evaluate public health policy, we surveyed all community-living, but not institutionalized, elderly individuals in the study areas. The response rates to the questionnaires in areas A and B were 74% (1077/1455) and 84% (1224/1455), respectively. We hypothesized that subjects who responded to the questionnaire would be more health-conscious and more cooperative with their municipality, and would therefore have a higher tendency to be vaccinated. We found that the rates of vaccination among study subjects in areas A and B were 69.3% and 64.2%, respectively, which are higher than those that observed in the entire population ≥65 years old in these areas (62.1% in area A and 60.8% in area B). Health-conscious people are likely to recall their health conditions, enabling us to more precisely evaluate the effectiveness of the vaccine.

We used a questionnaire of self-reported symptoms to determine clinically defined outcomes relative to several criteria for having influenza [1,3,10,14]. The clinically defined outcomes we used were fever ≥37.0 °C, fever ≥38.0 °C, and diagnosis of influenza at a clinic. A higher fever temperature reduces misclassification of outcome. Although the diagnosis of influenza at a clinic is a more specific outcome, it may increase differential misclassification because it is dependent on a difference between vaccinated and unvaccinated individuals in a behavioral tendency to consult medical facilities, as well as on the tendency of doctors to diagnose influenza. We found that the ORs for fever ≥38 °C were around 0.80, the lowest among the outcomes, whereas the outcomes of fever ≥37 °C and diagnosis of influenza had ORs nearer to 1. Although we did not include respiratory symptoms in the outcome criteria, acute fever in community-living elderly during the study period almost always originated from upper respiratory infection, and individuals with fevers diagnosed as arising from other origins were excluded from the study.

We defined the 4 weeks of peak influenza outbreak, during which about 70% of cases of influenza were diagnosed in clinics, as the period for adopting outcomes. A strict limitation of the peak period and a large outbreak of influenza would lead to a higher specificity. During the 2003/04 season, however, the influenza outbreak was not large both in Kyoto prefecture and throughout Japan [8]. Outbreaks in the study areas were not systemically surveyed, but the trends in clinics were similar. Consequently, the effectiveness of vaccination may have not been evaluated sufficiently.

To minimize misclassifications regarding exposure to vaccine, we obtained information on vaccination from municipality records, which are used for subsidization. People not recorded as vaccinated by their municipality office were regarded as not vaccinated, regardless of their responses on the questionnaire. The few possible exceptions would not affect our results.

Among the potential confounding factors considered were underlying conditions and behaviors/environments related to likelihood of exposure to influenza. The propensity score should be adjusted for these factors, because their prevalence and the outcome criteria were relatively small compared with the prevalence of vaccinated individuals [10,11]. We found that ORs adjusted for propensity scores were modest and stable compared with ORs calculated by the stepwise method (data not shown). ORs of vaccination became lower after adjusting for underlying conditions, probably because elderly individuals having these conditions tended to be vaccinated and also had influenza. In contrast, the adjustment

for likelihood of exposure to influenza did not alter the results. These findings indicate that we would have underestimated the effectiveness of vaccination had we not adequately adjusted for those underlying conditions.

Influenza vaccination has been shown to be effective in the elderly [1] and has been recommended by public health policy [2]. A recent meta-analysis showed that, among elderly community-living individuals, vaccination reduced ILI by 35%, hospitalization for pneumonia and influenza by 33%, mortality following hospitalization for pneumonia and influenza by 47%, and mortality from all causes by 50% [14]. In Japan, however, a prospective study conducted in cooperating clinics failed to show a significant effectiveness of influenza vaccine among outpatients aged ≥65 years, although ILI was reduced 50–80% among people aged <65 years [7]. This may have been due to the small number of ILI patients in this study and the small outbreak during that influenza season [7]. A recent prospective study showed that vaccination significantly reduced the risk of fever ≥38.5 °C during an influenza outbreak in community-dwelling elderly [3]. A large number of people were registered in the study cohort, however, making the design hardly applicable to monitoring public health activities.

A recent ecological study showed that influenza vaccination prevented an unexpectedly small fraction of excess mortality from pneumonia, influenza and all causes [15]. Many of the excess deaths, however, may have occurred in a small subset of vulnerable and unvaccinated elderly, such as those who were hospitalized and/or institutionalized, and these results may not indicate the effectiveness of vaccination among community-living elderly. As in most analytical epidemiologic studies, we limited our study subjects to non-institutionalized elderly, and we controlled for confounding factors such as underlying diseases.

Our finding that vaccination was not significantly effective may be related to the small outbreak of influenza during the 2003/04 season, to the mismatch between vaccinating and circulating strains, and to low specific outcomes. Although prospective surveillance with a symptom diary [3] is considered superior to our method, it requires vastly greater human resources. In addition, the subjects in a prospective study may take more precautions, causing the results to have a stronger self-selection bias than those obtained using our design. The method outlined here can be used by administrative authorities to monitor the effectiveness of influenza vaccination. We utilized about six person-days for preparation of the survey, and about 10 person-days for filing and computer input of the answers. Postage costs totalled about $2700, and we spent several hundred dollars to prepare questionnaires and buy envelopes and address stickers.

In conclusion, we have shown here that influenza vaccination had about 20% effectiveness among elderly community-dwelling Japanese individuals during the 2003/04 influenza season, an effectiveness that was not statistically significant. This relative lack of effectiveness may have been due to misclassification of outcomes due to inaccurate recall by the subjects and/or the low specificity of outcomes. The smallness of the outbreak and a drift in circulating strains may also have affected the results. The framework we developed, however, can be used by public health authorities to monitor the effectiveness of vaccination. To avoid underestimating the effectiveness of vaccination, results should be adequately adjusted for underlying conditions.

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Laboratory and Epidemiology Communications

Isolation of Influenza Virus Type AH3 from a Traveler Returning from Vietnam in July 2005 in Osaka, Japan

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On 28 June 2005, the World Health Organization announced that an additional case of human infection with AH5N1 avian influenza was confirmed in Vietnam and that the new case brought the total, in Vietnam, since mid-December 2004, to 60 cases, of which 18 had been fatal. A man who visited Vietnam from 4 July 2005 returned to Osaka, Japan, on 8 July. As he felt a fever beginning on 7 July, he saw a doctor soon after his flight to Japan. He was diagnosed with influenza type A infection using a rapid influenza test. Although he declared that he was traveling around a coast area and that he had not been near chickens in Vietnam, the doctor reported this case to the Izumi Health Center of Osaka Prefecture, because they could not completely deny the possibility that his influenza was caused by highly pathogenic avian influenza (HPAI). On the afternoon of 8 July, the gargle from the patient was transported to the Osaka Prefectural Institute of Public Health. The specimen was inoculated onto Madin-Darby canine kidney (MDCK) cells. Simultaneously, viral RNA was extracted with QIAamp Viral RNA Mini Kit (QIAGEN K.K., Tokyo, Japan) for analysis by RT-PCR. To obtain an amplicon on a part of the HA1 region of the HA gene in influenza A virus, we used primer pairs as follows: 5′-CAGATGCAGACACAATATGT-3′ and 5′-AAACCGGC AATGGCTCCAAA-3′ for H1, 5′-CAGATTGAAGTGACTA ATGC-3′ and 5′-GTTTCTCTGGTACATTCCGC-3′ for H3 and 5′-CATACCCAACAATAAAGAGG-3′ and 5′-GTGTTC ATTTTGTTAATGAT-3′ for H5 (1,2). We carried out RT-PCR with Ready-to-Go RT-PCR Beads (Amersham, Biosciences UK Ltd., Buckinghamshire, UK) and by using a DNA thermal cycler (Perkin-Elmer Corp., Norwalk, Conn., USA)

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Table 1. The HI test results of isolated virus against refrence antisera

	Antisera					
	A/Moscow/ 13/98	A/New Caledonia/ 20/99	A/Wyoming/ 03/2003	A/Panama/ 2007/99	B/Brisbane/ 32/2002	B/Johannesburg/ 5/99
Antigen						
A/Moscow/13/98(H1N1)	1,280	nt	nt	nt	nt	nt
A/New Caledonia/20/99(H1N1)	nt	320	nt	nt	nt	nt
A/Wyoming/03/2003(H3N2)	nt	nt	2,560	nt	nt	nt
A/Panama /2007/99(H3N2)	nt	nt	nt	1,280	nt	nt
B/Brisbane/32/2002	nt	nt	nt	nt	10,240	nt
B/Johannesburg/5/99	nt	nt	nt	nt	nt	>20,480
Isolated virus						
A/Osaka/27/2005	<10	<10	1,280	<10	<10	<10

nt: not tested.

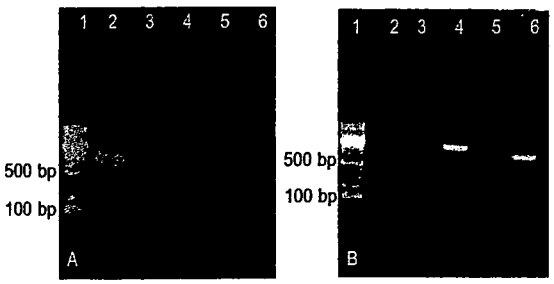

Fig. 1. RT-PCR for a part of the HA1 region of the HA gene in influenza A virus. (A) The RT-PCR products from the extract of the gargle were electrophoresed in 1% agarose gel. Lane 1, marker; lane 2, positive control and primer pairs for H5; lane 3, primer pairs for H1; lane 4, primer pairs for H3; lane 5, primer pairs for H5; lane 6, negative control and primer pairs for H5. (B) The RT-PCR products from the extract of the culture fluid showing the CPE were electrophoresed in 1% agarose gel. Lane 1, marker; lane 2, negative control and primer pairs for H5; lane 3, primer pairs for H1; lane 4, primer pairs for H3; lane 5, primer pairs for H5; lane 6, positive control and primer pairs for H5.

consisting of 1 cycle at 42°C for 30 min, 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec, 45°C for 30 sec, and 72°C for 1 min. However, no PCR products were detected from the extract of the gargle (Figure 1A).

On 11 July, as we observed the cytopathic effects (CPE) in the MDCK cells inoculated with the specimen, we performed a hemagglutination (HA) test with the supernatant of viral culture using erythrocytes of chickens and humans (blood group O). The fluid agglutinated human erythrocytes but not chicken ones. We peformed a hemagglutination inhibition (HI) test with human erythrocytes against the reference antisera provided by the National Institute of Infectious Diseases (NIID). As shown in Table 1, the isolate was reacted with anti-A/Wyoming/03/2003 (H3N2) serum only. We also carried out RT-PCR with the culture fluid showing CPE by the method described above. The RT-PCR products were specific for H3 (Figure 1B). The RT-PCR product was purified in an agarose gel and sequenced directly with BigDye Terminator Cycle Sequencing Ready Reaction Kits with AmpliTaq DNA Polymerase, FS (PE Applied Biosystems, Foster City, Calif., USA) by using an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). A BLAST search showed that the sequence of the isolate possessed 98% homology with A/New York/10/2004 (H3N2). These results indicated that the isolated virus was a human influenza virus type AH3 [named A/Osaka/27/2005(H3)].

According to the Infectious Disease Surveillance Center, NIID, influenza epidemic seasons in Vietnam are from June to August and from December to March in usual years. In July 2005, an influenza epidemic occurred in Vietnam. As there was no influenza virus activity in Osaka in this month, it is concluded that the patient was infected with influenza virus type AH3 in Vietnam. This is one of the cases in Japan. Some papers have already reported that influenza virus type AH3 was isolated from travelers when they came back to Japan from abroad (3-5). Since HPAI is spreading in Vietnam and its neighbor countries now, it is important that we examine whether influenza of returnees from these countries is conventional human influenza or HPAI, even if patients have no history of contact with humans or chickens infected with HPAI. Furthermore, the examination should include detection of viral antigens, amplification of viral RNA and viral isolation for definite diagnosis.

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解　説

ウイルスとは－その生態と検出－

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THE SOCIETY FOR ANTIBACTERIAL AND ANTIFUNGAL AGENTS, JAPAN.

NOTE *Immunology*

Anti-Influenza A Virus Activities of Mannan-Binding Lectins and Bovine Conglutinin

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ABSTRACT. Mannan-binding lectin (MBL) and bovine conglutinin (BKg) belong to the collectin family, which is involved in first-line host defense against various infectious agents. We have previously reported that human MBL inhibited type A influenza viral hemagglutination, infection and spreading to adjacent cells without complement activation. In this study, we investigated the direct antiviral activities of bovine MBL, rabbit MBL and BKg. All collectins used in this study inhibited viral infectivity and hemagglutination at concentrations of 0.02–0.3 μg/ml. They also demonstrated inhibitory activity against viral spreading. Like human MBL, bovine MBL and BKg showed antiviral activities at their physiological concentrations. These results suggest that mammalian MBLs and BKg may inhibit the spread of influenza A virus through the bloodstream.

KEY WORDS: conglutinin, influenza A virus, mannan-binding lectin.

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Collectins are a family of C-type lectins that are structurally characterized by a collagen-like region and a carbohydrate recognition domain [6]. Serum collectins, mannan-binding lectin (MBL) and bovine conglutinin (BKg), and pulmonary collectins, surfactant protein A and D (SP-A and SP-D), play important roles in first-line host defense against a variety of microorganisms, including bacteria, fungi, and viruses by binding to sugars on their surfaces [10]. Although these collectins have different structures and molecular weights [17], they can bind to mannose and inhibit type A influenza viral hemagglutination and infectivity [2, 4, 14, 16]. Guinea pig MBL can neutralize viral infectivity and lyse influenza virus-infected cells by activating complement [3, 27]. In addition, human MBL, human SP-D and BKg act as opsonins, enhancing the activation of neutrophils by influenza A virus and protecting them from deactivation by the virus [14–16]. Human SP-D and BKg, but not human MBL, can induce the formation of viral aggregates and increase the internalization of viruses by neutrophils [12].

Recently, we have focused on the direct anti-influenza virus activities of human MBL independent of complement activation and opsonization, and showed that human MBL had an ability to inhibit viral spreading from primary infected cells to noninfected cells, which was designated as viral growth inhibition (GI) activity, as well as inhibit viral hemagglutination and infectivity directly [19]. However, whether other animal serum collectins exert direct inhibitory effects against influenza A virus has not been definitely determined. We have previously determined the primary structure and the complement activating ability of bovine MBL and rabbit MBL [20, 21], and characterized the biochemical and biological activities of BKg [29, 30]. In this study, we investigated the direct antiviral activities of bovine MBL, rabbit MBL and BKg against influenza A virus.

Bovine MBL, rabbit MBL and human MBL were purified from each serum sample using mannan-agarose (Sigma-Aldrich Corp., St. Louis, MO, U.S.A.) as previously described [19–21]. BKg was purified from heat-inactivated (56°C, 30 min) bovine serum [29]. The identities of purified collectins were confirmed by N-terminal amino acid sequence analyses and their protein concentrations were quantified by SDS-PAGE, followed by Coomassie brilliant blue staining (data not shown).

The hemagglutination inhibition (HI) test was done by a standard microtiter method [19]. Influenza A virus A/Ibaraki/1/90 (H3N2) used was sensitive to human MBL [19]. Viral solution (4 HA units) was incubated with serial dilutions of collectins for 30 min at 37°C, and then mixed with 0.5% chicken erythrocytes. The HI activity of collectins was determined after incubation for 1 hr at room temperature.

To examine the neutralizing activity of collectins, the rapid focus reduction neutralization test was performed as described before [19]. Briefly, Madin-Darby canine kidney (MDCK) cells were cultured as a monolayer in a 96-well microtiter plate (Asahi Techno Glass Corp. Tokyo, Japan). Influenza A virus A/Ibaraki/1/90 (H3N2) (about 100 focus forming units; FFU) was mixed with each dilution of collectins at 37°C for 1 hr, inoculated onto the MDCK cell monolayer washed with phosphate-buffered saline (PBS), and incubated at 37°C in 5% CO_2 for 1 hr. After washing with PBS, cells were cultured with viral growth medium containing 0.5% tragacanth gum (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The growth medium was composed of modified Eagle's medium (MEM) (Invitrogen Corp., Carls-

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bad, CA, U.S.A.) supplemented with 0.2% bovine serum albumin (Fraction V) (Sigma-Aldrich Corp.), 0.1% glucose, 3% tissue culture vitamins (MP Biomedicals, LLC., Irvine, CA, U.S.A.), 2 μg/ml acetyltrypsin (Sigma-Aldrich Corp.) and 5 μg/ml amphotericin B (Bristol-Myers K. K., New York, U.S.A.). After 24 hr, the cells were washed with PBS and fixed with absolute ethanol. The infected cells were immunostained using the monoclonal antibody against influenza A virus, subsequently rabbit anti-mouse IgG, goat anti-rabbit IgG and peroxidase-rabbit anti-peroxidase complex. All the antibodies used for staining were purchased from MP Biomedicals, LLC. The color reaction was conducted by incubating with 0.3 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Ltd.) and 0.01% H_2O_2 in PBS. The numbers of stained foci were counted under a light microscope. The neutralizing activity was presented as the percent reduction of FFU compared with the FFU in samples not incubated with collectins.

The GI test was performed by the immunohistochemistry assay as described previously [19]. Influenza A virus A/Ibaraki/1/90 (H3N2) (about 50 FFU) was inoculated onto monolayers of MDCK cells in 24-well microplates and incubated at 37°C in 5% CO_2 for 1 hr. After washing with PBS, cells were incubated with viral growth medium containing 0.5% tragacanth gum and several dilutions of collectins for 3 days. The virus-infected areas were immunostained as described above, scanned using HP DeskScan II software and a Scanjet II cx (Hewlett-Packard Co., Palo Alto, CA, U.S.A.), traced and estimated quantitatively using Color-it (MicroFrontier Inc., Winterset, Iowa, U.S.A.) and NIH Image 1.60 software. The GI activity was determined as the percentage of viral infected areas compared to those without collectins.

The HI activity of bovine MBL, rabbit MBL, human MBL and BKg against influenza A virus A/Ibaraki/1/90 (H3N2) was examined. ALL MBLs and BKg inhibited viral hemagglutination (Table 1). The minimum concentration of collectins causing HI was within the range of 0.02–0.15 μg/ml. BKg showed more potent HI activity than MBLs. The HI activity was inhibited in the presence of 10 mM EDTA, 100 mM D-mannose or 50 mM *N*-acetyl-D-glucosamine (GlcNAc) (data not shown).

To measure the neutralizing activity of collectins, the rapid focus reduction assay was done. Bovine MBL and rabbit MBL had the ability to neutralize the infectivity of the influenza A virus in a dose-dependent manner, as human MBL and BKg (Fig. 1 and Table 1). The range of collectin concentrations required for 50% FFU reduction was 0.08–0.3 μg/ml. These neutralization tests were designed to prevent contamination of any serum complement components. Therefore, these results show that the neutralizing activity of both bovine MBL and rabbit MBL is independent of complement activation.

To investigate whether bovine MBL, rabbit MBL and BKg could inhibit viral spreading to adjacent cells, the GI test was performed. The areas infected with influenza A virus were reduced when medium containing collectins was overlaid on MDCK cells after viral infection (Fig. 2). Among the investigated collectins, bovine MBL and human MBL at a concentration of 1.0 μg/ml showed the most potent GI activity, reducing the infected areas to less than 5% of the controls. Rabbit MBL inhibited viral growth moderately (reduction to 16% of control growth) at 1.0 μg/ml and BKg showed the lowest GI activity (32% of control growth) at 2.0 μg/ml. When 100 mM mannose or 50 mM GlcNAc was added to the overlay medium containing 1.0 μg/ml of MBL or 2.0 μg/ml of BKg, respectively, viral infected areas recovered to 40–90% of the control area. These results indicate that the GI activity of MBLs and BKg is also dependent on their lectin activity.

Binding of human MBL, human SP-D and BKg to viral hemagglutinin (HA), which is one of major envelope glycoproteins of influenza A virus, is important for viral neutralization and inhibition of viral hemagglutination activity [2, 13]. On the other hand, antibody against viral neuraminidase (NA), which is another envelope glycoprotein, is known to inhibit viral release from infected cells [22]. Human MBL and rat SP-D inhibit viral NA activity by bind-

Table 1. The HI and neutralizing activities of collectins against influenza A virus A/Ibaraki/1/90 (H3N2)

	HI activity[a)]	Neutralizing activity[b)]
Human MBL	0.15	0.3
Rabbit MBL	0.08	0.2
Bovine MBL	0.08	0.08
BKg	0.02	0.2

a) The minimum concentration showing HI activity (μg/ml).
b) The minimum concentration causing 50% FFU reduction (μg/ml).

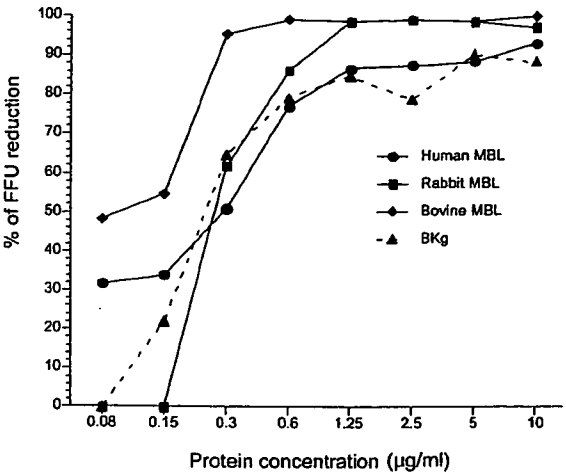

Fig. 1. The neutralizing activity of human MBL, bovine MBL and rabbit MBL and BKg against influenza A virus A/Ibaraki/1/90 (H3N2). The activity was estimated by the rapid focus reduction neutralization test and expressed as the percentage reduction of FFU compared with FFU from cells infected in the absence of collectins. FFU is the mean of triplicate results in a single experiment.

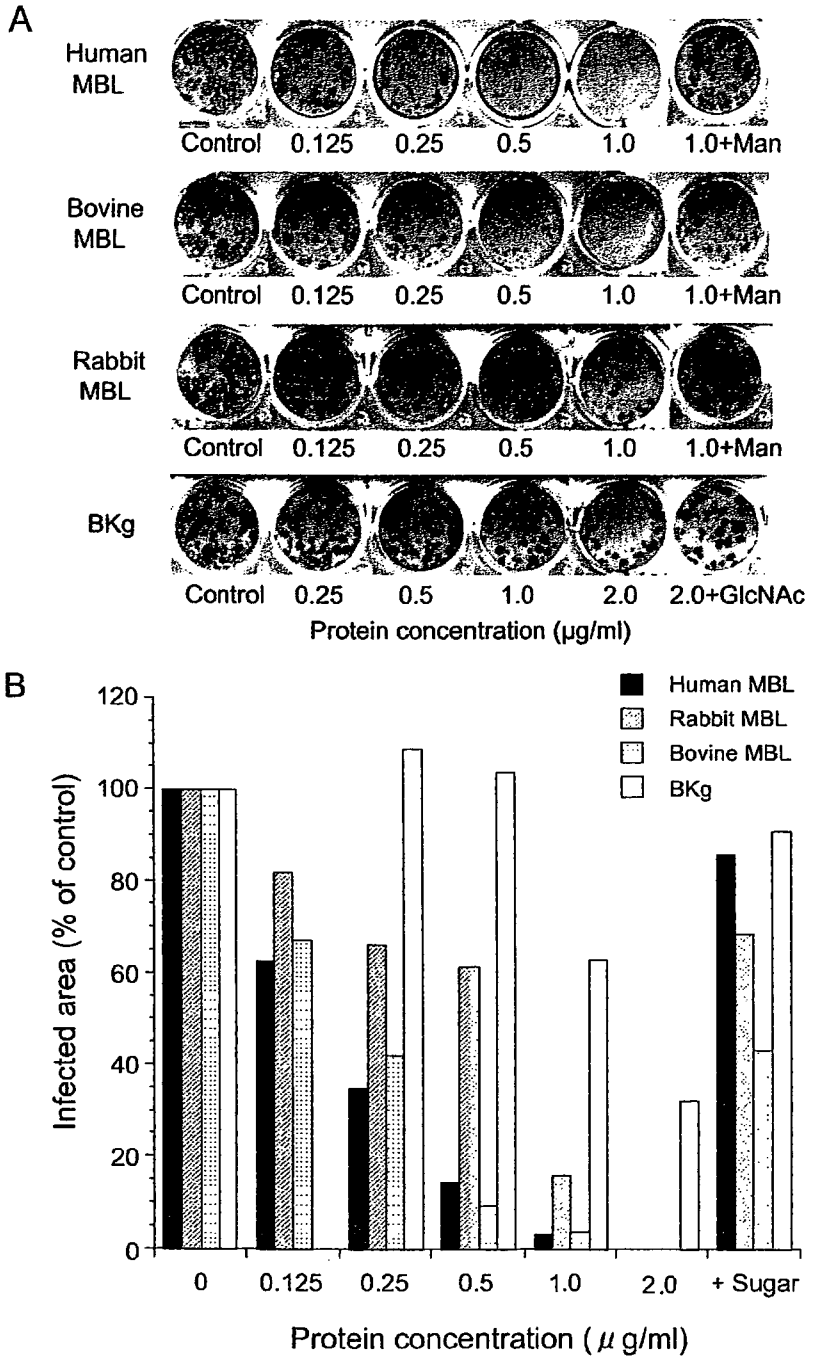

Fig. 2. The viral growth inhibition (GI) activity of human, bovine and rabbit MBL and BKg. MDCK cells were incubated with influenza A virus A/Ibaraki/1/90 (H3N2) (50 FFU/well) for 60 min, washed with PBS, and then incubated with viral growth medium containing 0.5% tragacanth gum and diluted collectins (0–2 μg/ml) with or without 100 mM mannose (Man) or 50 mM GlcNAc for 3 days. The infected foci were immunostained using the monoclonal antibody against influenza A virus (A). The infected area is depicted as a histogram of percentages of the control (B). Values are expressed as the mean of duplicate results in a single experiment.

ing to NA [26, 28]. Our recent lectin blot studies using native and recombinant truncated human MBL and human SP-D lacking the collagen-like region indicated that they bound to both viral HA and NA of A/Ibaraki/1/90 (H3N2), whereas BKg bound only to HA [7–9, 19]. Although human SP-D has a size and oligomeric structure similar to those of BKg [17], it strongly reduced the infected areas to less than 5% of the control areas at 2.0 $\mu g/ml$ (data not shown). Therefore, it is considered that binding of collectins to both HA and NA might be important for their GI activity, where binding to NA might be involved in the enhancement. NA has many N-glycosylation sites, like HA [31]. The N-glycosylation site at residue 165 of HA is conserved in all H3 subtype influenza viruses and its high-mannose sugar chain at this site is essential for viral neutralization by collectins [2, 28]. Therefore, further studies will be required to identify the N-glycosylation site playing a crucial role in the GI activity of collectins.

BKg showed potent neutralizing activity, like MBLs, but required a higher concentration for inhibition of viral spreading to adjacent cells. The concentrations of MBL and BKg in the serum of healthy cows were reported to be 2.37 ± 0.87 and 56.5 ± 14.4 $\mu g/ml$, respectively [1]. Therefore, bovine MBL and BKg were thought to exert both neutralizing and GI activities under physiological conditions. It has been reported that cattle can be infected with both swine and human influenza A viruses in experimental studies [5, 25]. However, influenza A virus has rarely been isolated from cattle infected naturally [11, 24]. In addition, low levels of BKg were reported to be associated with increased susceptibility to respiratory infection [18]. In the case of MBLs, MBL-insufficient children have an increased risk for acute respiratory tract infection, including viral infections [23]. MBL is also detected in the bronchoalveolar lavage from mice infected with influenza A virus that is highly virulent for mice [28]. Therefore, these serum collectins may act as one of the first line host defense agents against influenza virus infection in cattle.

In this study, we investigated the direct anti-influenza virus activities of bovine MBL, rabbit MBL and BKg. Bovine MBL and BKg, like human MBL, could neutralize viral infectivity and inhibit viral spreading at their physiological concentrations in serum. These results suggest that mammalian MBLs and BKg may inhibit the spread of influenza A virus via the bloodstream by direct viral neutralization and inhibition of viral spreading to adjacent cells.

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トリインフルエンザと
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4歳未満児における不活化インフルエンザワクチンに対する免疫応答

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Key words: influenza vaccine, young child, immunogenicity, HI antibody

要　旨

小児科診療所（6施設）を受診した4歳未満の小児259例（0歳64，1歳65，2歳64，3歳66）を対象に，不活化インフルエンザワクチン（2005/06シーズン）に対する免疫応答を検討した．接種は規定どおりに行い，接種前（S0），1回目接種4週後（S1），2回目接種4週後（S2）に血清を採取し，赤血球凝集抑制抗体価（HI価）を測定した．HI価の幾何平均，応答率（response proportion：HI価4倍以上上昇），達成率（achievement proportion：接種後　HI価1:40以上）を算出した．また，HI価上昇倍数を従属変数，接種前HI価と年齢を独立変数とした分散分析を行った．対象者のうち，過去3年以内にワクチン接種を受けたと報告した者は，0歳児0%（0/64），1歳児26%（17/65），2歳児72%（46/64），3歳児77%（51/66）であった．HI価の幾何平均は，ワクチン株や接種回数（S1，S2）に拘らず，0歳児および1歳児では，2歳児・3歳児に比べて常に低値を示した．2回接種後の達成率は，0歳児23～52%，1歳児49～58%，2歳児67～89%，3歳児71～85%であった．年齢と接種前HI価を同時に考慮した分散分析の結果，HI価上昇倍数に対して接種前HI価は常に強い関連を示した．2回接種後のHI価上昇倍数（S2/S0）に対する年齢の効果は，A（H1）およびBについて有意であった（p=0.000，0.002）．インフルエンザワクチンに対する免疫応答は，接種前抗体価と年齢の影響を強く受ける．2回接種しても，0歳児の50～80%，1歳児の40～50%が防御レベルの抗体価を獲得できなかった．

〔感染症誌　81：284～290，2007〕

序　文

近年，乳幼児においてはインフルエンザ関連の入院頻度が高いことが相次いで報告されている[1)~3)]．中でも1歳未満児の入院頻度は特に高く，成人ハイリスク者にも匹敵する[2)]．また，2003/04シーズンに米国においてインフルエンザ関連で死亡した小児の約半数は，インフルエンザ罹患前は，特別な基礎疾患を有しない健常児であったことが報告された[4)]．基礎疾患のない健常児においてもインフルエンザの予防が重要であることから，米国予防接種諮問委員会（US-ACIP）は2004/05シーズンより，6～23カ月の乳幼児をワクチン接種勧告（recommendation）の対象とした[5)]．さらに，同委員会は2006/07シーズンより，勧告接種の対象を5歳未満児にまで引き上げることを決定した[6)]．しかし，高齢者におけるインフルエンザワクチンの免疫原性および有効性についてはすでに多数の報告があるものの[6)]，乳幼児は免疫系の成熟度，インフルエンザウイルスへの感染歴，ワクチン接種歴など種々の点で成人とは異なるため，この年齢層におけるワクチン

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の免疫原性および有効性は必ずしも確立しているとは言い難い[7)~9)]．その上，我が国で規定されているワクチンの接種量は諸外国に比べて少なく[6)]，現行接種量で発病予防に十分な免疫の誘導が得られるかどうかについては未だ議論中である．しかしながら研究実施は容易ではないため，我が国では乳幼児を対象とした免疫原性の研究はほとんど実施されていない．

対象と方法

1．対象

対象は，2005年10月から11月に，ワクチン接種を希望して小児科診療所（6施設）を受診した4歳未満の小児である．接種時に明らかな発熱を呈している者，重篤な急性疾患にかかっていることが明らかな者，ワクチンの成分によるアナフィラキシーの既往者，その他，担当医師が接種を不適当と判断した者は対象から除外した．年齢階級毎に50例を確保することとし，急性疾患罹患などにより予期せぬ接種漏れや採血漏れの可能性を考慮して30%増の計259例をエントリーした（1歳未満64例，1.0～1.9歳65例，2.0～2.9歳64例，3.0～3.9歳66例）．結果として，全員プロトコール通り採血を終了した．エントリー時に，対象者の生年月日，過去3年以内の接種歴，前シーズンのインフルエンザ様疾患の罹患歴を調査した．

2．ワクチンおよび接種

2005/06シーズン用の市販インフルエンザHAワクチン（ビケンHE01A）を使用した．ワクチン株は，A/New Caledonia/20/99（H1N1），A/New York/55/2004（H3N2），B/Shanghai/361/2002であり，各株のHA含量は1株あたり30μg/mLであった．1回あたりの接種量は，1歳未満0.1mL，1歳以上0.2mLとし，4週間の間隔をあけて2回（2 doses）皮下接種した．2回目の接種は11月末までに完了した．

3．血清採取，抗体価測定

接種前（S0），1回目接種（1st dose）の4週後（S1），および2回目接種（2nd dose）の4週後（S2），計3回採血した．各施設で採取した血清は直ちに回収し，試験センター（医療法人相生会　臨床薬理センター）において−70～−80℃で凍結保存した．赤血球凝集抑制抗体価（HI価）は，ヒトO型赤血球を用いて定法により測定した．測定は，全採血終了後，一括して財団法人阪大微生物病研究会観音寺研究所で行った[10)]．

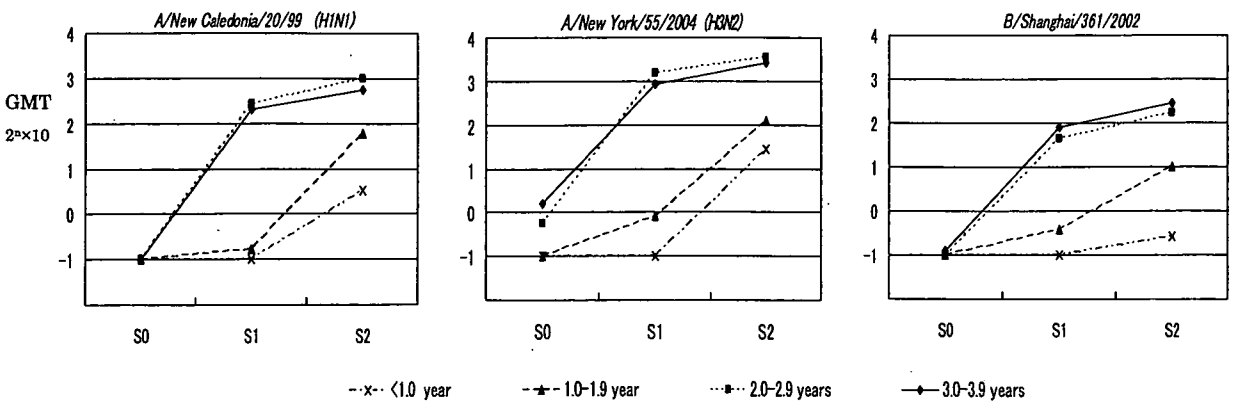
4．解析

HI価の幾何平均，応答率（response proportion：HI価4倍以上上昇の割合）[11)]，達成率（achievement proportion：HI価1：40以上の割合）[11)12)]を算出した．接種前にほとんど抗体を有しない者（1回目接種前の抗体価1：10未満）と，測定可能なレベルの抗体を有する者（1：10以上）に層化し，各年齢層毎に1doseによる応答率（S1/S0）と達成率（S1）を計算した．傾向性の検定にはMantel-extension法を用いた．さらに，免疫応答に対する接種前HI価および年齢の効果を検討するため，HI価の上昇倍数を従属変数，年齢（4レベル）と接種前HI価（3レベル）を独立変数とした分散分析を行った．なお，計算上HI価<1：10は，1：5として取り扱った．計算においてHI価は対数変換して処理した．検定はすべて両側検定とし有意水準は5%とした．解析にはSAS Ver. 9.1.3(SAS Institute Inc., Cary, NC, USA.）を用いた．

5．倫理的配慮

研究内容について説明した後，対象者の保護者から文書による同意を得た．なお本研究のプロトコールについては医療法人相生会臨床試験審査委員会の承認を得た．

Fig. 1 Geometric mean of HI antibody titer against inactivated influenza vaccine by age group



Blood samples were collected before the first vaccination (S0), 4 weeks after the first vaccination (S1), and 4 weeks after the second vaccination (S2).

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Table 1 Response proportion: frequency of subjects with HI titer rise ≧ 4-fold

Age (year)	N	A/New Caledonia/20/99 (H1N1)			A/New York/55/2004 (H3N2)			B/Shanghai/361/2002		
		S1/S0	S2/S1	S2/S0	S1/S0	S2/S1	S2/S0	S1/S0	S2/S1	S2/S0
< 1.0	64	6 (9)	35 (55)	37 (58)	7 (11)	35 (55)	44 (69)	3 (5)	18 (28)	20 (31)
1.0 — 1.9	65	26 (40)	31 (48)	50 (77)	25 (39)	28 (43)	50 (77)	23 (35)	18 (28)	39 (60)
2.0 — 2.9	64	53 (83)	12 (19)	59 (92)	45 (70)	5 (8)	48 (75)	46 (72)	7 (11)	52 (81)
3.0 — 3.9	66	48 (73)	5 (8)	54 (82)	33 (50)	8 (12)	41 (62)	39 (59)	5 (8)	42 (64)
Total	259	133 (51)	83 (32)	200 (77)	110 (43)	76 (29)	183 (71)	111 (43)	48 (19)	153 (59)
Trend P *		0.000	0.000	0.000	0.000	0.000	0.377	0.000	0.000	0.000

Note : The distribution of subjects is expressed as the number and percentage in parentheses.
* Mantel-extension method

Table 2 Achievement proportion: frequency of subjects with postvaccination HI titers ≧ 1 : 40

Age (year)	N	A/New Caledonia/20/99 (H1N1)			A/New York/55/2004 (H3N2)			B/Shanghai/361/2002		
		S0	S1	S2	S0	S1	S2	S0	S1	S2
< 1.0	64	1 (2)	2 (3)	24 (38)	2 (3)	2 (3)	33 (52)	0 (0)	3 (5)	15 (23)
1.0 — 1.9	65	0 (0)	18 (28)	38 (58)	9 (14)	18 (28)	35 (54)	8 (12)	22 (34)	32 (49)
2.0 — 2.9	64	3 (5)	50 (78)	57 (89)	23 (36)	47 (73)	52 (81)	8 (13)	39 (61)	43 (67)
3.0 — 3.9	66	11 (17)	51 (77)	56 (85)	29 (44)	45 (68)	48 (73)	15 (23)	43 (65)	47 (71)
Total	259	15 (6)	121 (47)	175 (68)	63 (24)	112 (43)	168 (65)	31 (12)	107 (41)	137 (53)
Trend P *		0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000

Note : The distribution of subjects is expressed as the number and percentage in parentheses.
* Mantel-extension method

Table 3 Achievement proportion (postvaccination HI titers ≧ 1 : 40) and response proportion (HI titer rise ≧ 4-fold) against A/New Caledonia/20/99 (H1N1) after the first dose by age group and prevaccination titer

Age (year)	S0 < 1:10			S0 ≧ 1:10		
	N	Response proportion	Achievement proportion	N	Response proportion	Achievement proportion
< 1.0	63	6 (10)	2 (3)	1	0 (0)	0 (0)
1.0 — 1.9	61	22 (36)	14 (23)	4	4 (100)	4 (100)
2.0 — 2.9	43	36 (84)	29 (67)	21	17 (81)	21 (100)
3.0 — 3.9	33	26 (79)	20 (61)	33	22 (67)	31 (94)
Total	200	90 (45)	65 (33)	59	43 (73)	56 (95)
Trend P *		0.000	0.000		0.484	0.247

Note : The distribution of subjects is expressed as the number and percentage in parentheses.
* Mantel-extension method

成 績

1. 基礎情報

対象者の平均月齢は，0歳児で9.0カ月，1歳児で17.8カ月，2歳児29.3カ月，3歳児41.4カ月であった．昨シーズンインフルエンザ様疾患に罹患したと報告した者は，0歳児11% (7/64)，1歳児63% (41/65)，2歳児53% (34/64)，3歳児61% (40/66)，過去3年以内にワクチン接種を受けたと報告した者は，0歳児0% (0/64)，1歳児26% (17/65)，2歳児72% (46/64)，3歳児77% (51/66) であった．

2. HI価の幾何平均 (Fig. 1)

ワクチン株や接種回数に拘らず，0歳児および1歳児の幾何平均は，2歳児および3歳児に比べて常に低値を示した．2歳児と3歳児では，1st doseにより幾何平均は大きく上昇し良好なHI価誘導を認めたが，2nd doseによる上昇幅は僅かであった．一方，0歳児と1歳児では1st doseによる上昇幅は小さく，2st doseで大きな上昇を認めたものの，最終的に2st dose接種による獲得抗体価は2歳児と3歳児に比べて低かった．

なお，1歳児のHI価誘導は0歳児より高いが，接種量が等しい2歳児・3歳児より低値であった．

3. 応答率 (Table 1)

1st doseによる応答率 (S1/S0) をA(H1) についてみると，0歳児9%，1歳児40%，2歳児83%，3歳児73% であり，年長児ほど良好な応答率を示した (Trend p=0.000)．この傾向は，いずれのワクチン株でも同様であり，応答率は，0歳児5〜11%，1歳児35〜

Table 4 Effects of prevaccination titer and age on HI antibody response to inactivated influenza vaccine

Test antigen: variables	Sum of squares	Degree of freedom	Mean square	F	P	R^2
		S1/S0				
A/New Caledonia/20/99 (H1N1)						
Age *	117.11	3	39.04	41.62	0.000	0.353
Prevac titer **	8.34	2	4.17	4.45	0.013	
Residual	237.28	253	0.94			
Total	366.75	258				
A/New York/55/2004 (H3N2)						
Age	40.33	3	13.44	16.37	0.000	0.335
Prevac titer	39.90	2	19.95	24.29	0.000	
Residual	207.83	253	0.82			
Total	312.46	258				
B/Shanghai/361/2002						
Age	48.23	3	16.08	15.73	0.000	0.340
Prevac titer	48.58	2	24.29	23.77	0.000	
Residual	258.52	253	1.02			
Total	391.53	258				
		S2/S1				
A/New Caledonia/20/99 (H1N1)						
Age	4.48	3	1.49	2.03	0.110	0.276
Prevac titer	27.09	2	13.55	18.47	0.000	
Residual	185.59	253	0.73			
Total	256.28	258				
A/New York/55/2004 (H3N2)						
Age	20.32	3	6.77	7.89	0.000	0.315
Prevac titer	28.16	2	14.08	16.40	0.000	
Residual	217.15	253	0.86			
Total	316.95	258				
B/Shanghai/361/2002						
Age	0.17	3	0.06	0.08	0.970	0.178
Prevac titer	23.60	2	11.80	16.58	0.000	
Residual	180.06	253	0.71			
Total	219.09	258				
		S2/S0				
A/New Caledonia/20/99 (H1N1)						
Age	40.30	3	13.43	12.61	0.000	0.158
Prevac titer	20.56	2	10.28	9.65	0.000	
Residual	269.44	253	1.06			
Total	319.97	258				
A/New York/55/2004 (H3N2)						
Age	1.34	3	0.45	0.38	0.765	0.115
Prevac titer	35.13	2	17.56	15.05	0.000	
Residual	295.32	253	1.17			
Total	333.70	258				
B/Shanghai/361/2002						
Age	20.40	3	6.80	5.26	0.002	0.126
Prevac titer	17.98	2	8.99	6.96	0.001	
Residual	326.96	253	1.29			
Total	374.04	258				

Two-way analysis of variance for unbalanced data, using the sum of squares associated with Type II estimable functions for each effect.

* Categorized into four levels : < 1.0, 1.0-1.9, 2.0-2.9, and 3.0-3.9 years old.

** Categorized into three levels : H1N1 : < 1 : 10, 1 : 10, and ≧ 1 : 20 for S0 ; < 1 : 10, 1 : 10-1 : 40, and ≧ 1 : 80 for S1 ; H3N2 : < 1 : 10, 1 : 10-1 : 160 and ≧ 1 : 320 for S0 ; < 1 : 10, 1 : 10-1 : 80 and ≧ 1 : 160 for S1 ; B : < 1 : 10, 1 : 10-1 : 20 and ≧ 1 : 40 for S0 ; < 1 : 10, 1 : 10-1 : 40 and ≧ 1 : 80 for S1.

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40%，2歳児70～83%，3歳児50～73%であった．

一方，2nd doseによる応答率（S2/S1）をA(H1)についてみると，0歳児55%，1歳児48%，2歳児19%，3歳児8%であり，年長児ほど低い（p＝0.000）．この傾向もまた，いずれのワクチン株においても同様であり，0歳児28～55%，1歳児28～48%，2歳児8～19%，3歳児8～12%であった．

2 doses接種による応答率（S2/S0）は，A(H1)およびBで年長児ほど高い傾向を示したが（Trend p＝0.000），A(H3)については有意差を認めなかった．

4. 達成率（Table 2）

接種前（S0）の達成率を全対象者においてみると（表中下から2行目），A(H1)6%，A(H3)24%，B12%であり，A(H3)に対する達成率が高値を示した．接種前（S0）の達成率を年齢別にみると，A(H1)では，0歳児2%，1歳児0%，2歳児5%，3歳児17%であり，年長児ほど高くなる．この傾向はいずれのワクチン株についても同様であり，特にA(H3)では3歳児の達成率は44%と高値であった．

接種回数ごとの達成率をA(H1)についてみると，0歳児において接種前（S0）2%，1st dose後（S1）3%，2st dose後（S2）38%であり，達成率の上昇幅はS1で小さく，S2で大きい．一方，3歳児における同様の達成率は，接種前(S0)17%，1st dose後(S1)77%，2st dose後（S2）85%であり，上昇幅はS1で大きく，S2で小さい．このように達成率の上昇幅は，若年ではS1＜S2，年長になるほどS1＞S2となり，この傾向はいずれのワクチン株についても同様であった．

2 doses後の達成率は，0歳児23～52%，1歳児49～58%，2歳児67～89%，3歳児71～85%であり，0歳児と1歳児の達成率は2歳児・3歳児に比べて低かった．

5. 接種前抗体価別，応答率と達成率（Table 3）

いずれのワクチン株においても同様の傾向を認めたので，A(H1)に対するHI価の測定結果のみTable 3に示す．接種前HI価（S0）1:10未満では，応答率，達成率ともに年長児ほど高いという傾向を認めた（Trend p＝0.000）．これは，Table 1およびTable 2と同様の結果である．接種前HI価（S0）1:10以上については，0歳児で僅か1例，1.0～1.9歳では4例と極めて少数であった．このように，接種前HI価で層化して年齢と抗体応答の関連をみる場合，唯一用いることができるカットオフ値は1:10であり，それでも年齢分布に極端な偏りが生じるため安定した比較は困難であった．従って，接種前HI価と年齢の影響を同時に考慮するには数学モデルによる解析が必要と思料され，分散分析を行った．

6. 分散分析（Table 4）

HI価上昇に対する接種前HI価の効果は，接種回数やワクチン株に拘らず，常に有意であった（p＝0.000～0.013）．1st doseによるHI価上昇（S1/S0）に対して，年齢および接種前HI価は，それぞれ独立して有意な効果を示した．しかし，2nd doseによるHI価上昇（S2/S1）に対し，年齢の効果は，A(H3)についてのみ有意であり，A(H1)およびBに対する年齢の効果は明らかではなかった．2 doses後のHI価上昇（S2/S0）に対する年齢の効果は，A(H1)，Bでは有意差を示したが（p＝0.000，0.002），A(H3)については有意に到らなかった．

考　察

先行研究と同様[13][14]，年少児ほど防御レベルのHI価獲得は困難であった．2 dosesによる応答率（S2/S0）および達成率（S2）から，4倍以上のHI価上昇を示しても，防御レベルに達しない者を認めた．2回接種によっても，防御レベルのHI価を獲得できなかった者は，およそ0歳児の50～80%，1歳児の40～50%であった．

若年小児（6歳未満2,913例）を対象とした前向き観察研究では，インフルエンザ様疾患に対するワクチン有効率は，2歳以上で33%（95%信頼区間：21～44%），2歳未満では－7%（－44～20%）であり，2歳以上については有意なワクチンの有効性を認めたが，2歳未満における有効性は検出されていない[7]．また，乳幼児（6～23カ月786例）を対象とした2シーズンにわたる無作為化比較試験では，培養陽性インフルエンザに対する有効率は，1年目66%(34～82%)，2年目－7%（－247～67%）であり，1年目については有効性を認めたものの，2年目については有効性を認めていない[8]．さらに，6カ月から8歳の小児29,726例を対象とした後ろ向き研究では，2歳未満のsub-group（5,139人）におけるワクチン有効率は，インフルエンザ様疾患に対して25%（0～44%），肺炎またはインフルエンザに対して49%(9～71%)である[9]．乳幼児におけるワクチンの有効性は不安定となりやすく，先行研究の結果は必ずしも一致しない．これは，若年小児においては罹患の測定方法，流行規模，ワクチン株と流行株の合致度など，研究環境の影響を受けやすいためと考えられる．さらに，本研究では年少児ほど応答率および達成率が低く，接種しても防御レベルのHI価獲得には到らない例が多くみられた．これも，若年小児のワクチン有効性を不安定にする要因と考えられる．なお，0歳児と1歳児だけの免疫応答を比較して接種量の影響を指摘した報告がある[15]．しかし，本研究では，0歳児で特に低い免疫応答を認めたことに加え，1歳児でも接種量が等しい2歳児・3歳児に比べて免疫応答が低いという特徴的所見を認め

た．若年小児における免疫応答の差は接種量のみで説明できず，年齢あるいは年齢と関連する何らかの因子も関与していると考えられる．

HI価の幾何平均および応答率の結果から，1st doseによるHI価の上昇程度は年長児の方が年少児に比べて良好であった．これは年長児ほど接種前に抗体を保有している者が多いためと考えられる．一方，2nd doseによるHI価の上昇程度は，年長児の方が年少児に比べて低い．これは，"law of initial value"または"negative feed back"と呼ばれる現象（抗体応答の頭打ち現象）を明瞭に示している[11]．つまり，年長児においては，1st doseにより高いレベルのHI価が得られたため，2nd dose後のHI価上昇は僅かであったと考えられる．また，この現象が2歳・3歳という低年齢で生じることは特記すべきであろう．

A(H3)に対しては，対象者の24%が接種前に防御レベルのHI価を有していた．接種前のHI価保有割合が，A(H1)やBに比べてA(H3)で高値であったのは，過去3シーズン中2シーズンの主流行株がA(H3)であったためと考えられる[16]．対象を抗体陰性者に限定すれば，年齢の影響のみを観察することが可能であるが，例数の確保が困難となる．そのため，解析の段階では年齢と接種前HI価の影響を同時に考慮し，2要因の独立した効果を検討した．

分散分析の結果，HI価上昇に対する接種前HI価の効果は，接種回数やワクチン株に拘らず常に有意であり，年齢とは独立した強い影響を認めた．最終的に，2 doses後のHI価上昇（S2/S0）に対する年齢の効果は，A(H1)とBについては有意であったが，A(H3)については有意差を認めなかった．但し，A(H3)についても，1st dose（S1/S0）および2nd dose（S2/S1）でのHI価上昇に対する年齢の効果は有意であり，これは以下のように解釈できる．1st doseによるHI価上昇（S1/S0）は，年少児が僅少，年長児が良好，一方，2nd doseによるHI価上昇（S2/S1）は，年少児が良好，年長児が僅少であった．このため，1st doseと2nd doseの影響を別々に観察すると年齢の効果は有意となるが，2 doses接種によるHI価上昇（S2/S0）をみると1st doesと2nd doseにおける年齢の効果は互いに打ち消され消失する．こうした現象がA(H3)で強く現れたのは，接種前のHI価レベルが高値であったこと，特に2歳児・3歳児においては接種前のHI価保有割合が高かったことが影響したためと考えられる．

以上のことから，(1)0歳児では抗体応答が低い，(2)1歳児の抗体応答は0歳児より高いが，接種量が等しい2歳児・3歳児より低い，(3)若年小児における抗体応答の差は接種量のみでは説明できず，年齢あるいは年齢と関連する何らかの因子が影響している，(4)ワクチンに対する免疫応答においては，既存抗体と年齢の両者が強く関与している，と考えられる．

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Immunogenicity of Trivalent-Inactivated Influenza vaccine Among Children Less Than 4 Years old

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We studied the immunogenicity of trivalent-inactivated influenza vaccine. Subjects were 259 children under 4 years old who visited six pediatric clinics to undergo influenza vaccination. Age distribution was 64 aged <1.0, 65 aged 1.0–1.9, 64 aged 2.0–2.9, and 66 aged 3.0–3.9 years, including subjects who had been previously vaccinated within the last three years, 0% (0/64) aged <1.0, 26% (17/65) aged 1.0–1.9, 72% (46/64) aged 2.0–2.9, and 77% (51/66) aged 3.0–3.9 years old. Two doses of vaccine were given subcutaneously four weeks apart. Dosage was 0.1mL for children under 1 year old, while for children aged one year or older, dosage was 0.2mL, based on standard Japanese recommendations. To measure hemagglutination inhibition (HI) antibody titer, triplet sera were obtained before vaccination (S0), 4 weeks after the first vaccination (S1), and 4 weeks after the second vaccination (S2). The geometric mean of HI antibody titer, the response proportion (titer rise≧4-fold), and the achievement proportion (postvaccination titer ≧1 : 40) were calculated by age group. Analysis of variance was used to estimate the independent effect of age and prevaccination titer on antibody increase. The geometric means of HI antibody titer were lower among the two younger age groups than among the two older age groups, regardless of vaccine strain or when blood samples were collected. The achievement proportion after 2 doses of vaccine in the <1.0, 1.0–1.9, 2.0–2.9, 3.0–3.9 year age groups were 38%, 58%, 89%, and 85% against A (H1); 52%, 54%, 81%, and 73% against A (H3); and 23%, 49%, 67%, and 71% against B. Regarding the analysis of variance, prevaccination titer consistently indicated strong effects on antibody increase, regardless of vaccine strain or combination of paired sera. After two doses of vaccine (S2/S0), significant effects of age on antibody induction were shown against A (H1) and B ($p = 0.000$ and 0.002). Thus, the immunogenicity of trivalent-inactivated influenza vaccine was strongly influenced by prevaccination titer and age. Even two doses of vaccine did not induce a protective antibody level in about 50 to 80% of subjects among infants aged <1.0 year, and 40 to 50% among children 1.0–1.9 year old.