Second, we were unaware of the time of infection of the chickens on each farm. In most cases, the chickens showed no symptoms and paired sera were collected when the H5N2 virus was no longer detected on the farm; therefore, a certain amount of time might have elapsed after exposure to the infected chickens and the infection of several subjects might not be detected through analysis of paired sera. Third, the study on the association between the variables and the neutralizing antibody titer for a single serum sample was based on a cross-sectional observation. Thus, the results did not establish a causal relationship between them. Fourth, the subjects were administered a questionnaire, their previous history of influenza was not checked, and 17% of subjects were excluded from the analysis because of failure to provide paired sera or appropriate data. Fifth, the neutralizing antibody test evaluated antibody titers based on the presence of inhibition of virus multiplication in cells. It was difficult to confirm an infection or the time of infection by using only the neutralizing antibody test because isolation of the virus itself was necessary to verify an infection with influenza viruses.

In conclusion, this study suggested the possibility that the first avian influenza H5N2 infection was transmitted to humans through exposure to infected chickens, and that a history of seasonal influenza vaccination and an age of over 40 years might be associated with positivity for H5N2-neutralizing antibody. Considering the serious impact of an influenza pandemic on public health, further epidemiological and virological examinations are recommended for the benefit of the global community.

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A prime–boost vaccination of mice with heterologous H5N1 strains

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ABSTRACT

We evaluated the priming effect of an H5N1 pandemic vaccine in a mouse model to investigate strategies for influenza pandemic vaccination. For priming, an alum-adjuvanted inactivated whole H5N1 vaccine (NIBRG-14, clade 1) was used. As booster vaccines, several formulations of Indo05/05/2005(H5N1)PR8-IBCDC-RG2 vaccines (clades 2–1) were evaluated, including split, whole, alum-adjuvanted split, and alum-adjuvanted whole vaccines.

Any type of booster vaccination elicited a significant HI antibody response despite the difference in antigenicity between the priming and booster vaccines. The split vaccine elicited a much stronger booster response than the alum-adjuvanted whole vaccine. When the mice were primed with the H1N1 or H3N2 vaccines, this did not affect the booster response to the H5N1 vaccine. These results indicated that an alum-adjuvanted whole vaccine is able to confer immunological memory to haemagglutinin even if the primed and boosted vaccine strains are in different clades and, once vaccinated, a split vaccine is preferred to evoke recall responses.

1. Introduction

Since December 2003, an unprecedented epizootic and highly pathogenic avian influenza A (H5N1) virus has affected poultry and wild birds in more than 60 countries in 3 continents [1]. In addition, there have been 387 confirmed human cases of H5N1 virus infection and its fatality rate is approximately 63% (September 10th, 2008) [2]. Although very few cases of human-to-human transmission have been reported so far [3–5], a research group reported statistical evidence of such transmission in Sumatra [6]. Considering the risk of large scale human-to-human transmission of the H5N1 virus, such as occurred during the 1918 influenza pandemic, it is essential to develop vaccines that could control a possible pandemic. Under these circumstances, the pandemic influenza preparedness action plan of Japan stipulates that, when the Minister of Health, Labour and Welfare declares a phase 4 state, health care workers and public servants may be vaccinated with the stockpile of prototype vaccines as an emergency measure [7]. This means that the stockpiled vaccines will be used as priming vaccines with the hope of achieving cross protection against the pandemic virus and a cross-priming effect when the pandemic vaccine is administered as a booster. Under this action plan, alum-adjuvanted inactivated whole vaccines have been developed in Japan.

Previous investigations have revealed a priming effect before booster injection of homologous strains in clinical trials of the MF59-adjuvanted vaccine [8] and the subunit vaccine [9]. Another clinical trial has also shown a priming effect against a different strain of H5N1, with the priming effect of A/HK/156/97 (H5N1, clade 0) recombinant haemagglutinin vaccine [10]. However, we do not know how an alum-adjuvanted vaccine will be able to act as a primer for booster vaccines with different immunogenicities.

In Japan, the alum-adjuvanted whole vaccine was rationally selected to improve immunogenicity since whole vaccines are generally more immunogenic than split vaccines and aluminum salt, the only licensed adjuvant with a widely accepted safety profile, was expected to reduce antigen dosage, expanding vaccine supply in pandemic. The alum-adjuvanted whole vaccine seems appropriate for a priming vaccine because it enhances cross-reactivity to various H5N1 viruses [11]. In general, split vaccines are less reactogenic than whole vaccines and whole virus vaccines are more immunogenic than split vaccines in naïve vaccines [12–15]. On the other hand, primed vaccinees show no difference of their response to whole and split vaccines [12–15]. Therefore, a split vaccine may be an appropriate booster vaccine if an adequate immune response can be elicited.

In this study, we evaluated several prime–boost regimens using vaccines with different antigenicities because we assumed that administration of a priming pandemic vaccine would be followed by a booster pandemic vaccine. We also evaluated the influence of

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Table 1
Priming effect of alum-adjuvanted whole vaccine to a drifted influenza strain.

Priming	Indo05/PR8-RG2			NIBRG-14		
	Pre	1st	2nd	Pre	1st	2nd
NIBRG-14	25.9 (7.8–85.9)	190.3(46.4–780.1)	264.6 (87.7–798.7)	207.5 (82.2–523.8)	1395.8 (448.5–4344.7)	1076.3(291.9–3969.2)
PBS	5.0 (5.0–5.0)	16.8(6.1–46.4)	174.5(90.9–335.1)	5.0 (5.0–5.0)	5.0 (5.0–5.0)	5.9 (3.9–9.0)

Priming vaccines of NIBRG-14 whole alum-adjuvanted vaccines (NIBRG-14) were injected twice with a 3-week interval. After 4 months, booster vaccines of Indo05/PR8-RG2 whole alum-adjuvanted antigens were injected twice with a 3-week interval. The HI antibody titers (GMT, 95%CL) were measured before the first booster vaccination (pre), 2 weeks after the first booster vaccination (1st), and 2 weeks after the second booster vaccination (2nd). All dosages are 0.2 μg HA/dose.

the formulation and dosage of booster vaccine in order to consider the best strategy for booster pandemic vaccination.

2. Methods

2.1. Vaccines used in animal studies

NIBRG-14, Indo/05/2005(H5N1)/PR8-IBCDC-RG2, A/Solomon Islands/3/2006 (H1N1) IVR-145, and A/Hiroshima/52/2005 (H3N2) IVR-142 were used as the vaccine strains. NIBRG-14 is one of the WHO vaccine reference strains, which is an attenuated form of the A/Vietnam/1194/2004 (H5N1) virus (clade 1) created by reverse genetic engineering at the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK). Indo/05/2005(H5N1)PR8-IBCDC-RG2 (Indo05/PR8-RG2) is an attenuated vaccine strain that was derived from the A/Indonesia/5/2005 (H5N1) virus (clades 2–1) by the Centers for Disease Control and Prevention (CDC, Atlanta, USA). A/Solomon Islands/3/2006 (H1N1) IVR-145 and A/Hiroshima/52/2005 (H3N2) IVR-142 are influenza vaccine strains used in the northern hemisphere during the 2007/2008 season, and these were obtained from the National Institute of Infectious Disease (NIID, Tokyo, Japan). These strains have a PR8 backbone.

The viruses were cultured in embryonated hens eggs, purified from allantoic fluid by zonal centrifugation, and inactivated with formalin to prepare whole vaccine. Split vaccines were prepared by treating the purified viruses with ether and polysorbate 80.

2.2. Immunization

Specific-pathogen-free female BALB/c mice (Japan SLC, Inc.) aged 6–8 weeks were used in all experiments. The number of mice in each group was 7 or 8. As the adjuvanted, 0.3 mg/ml aluminum hydroxide (0.3 mg/ml Al) was used in all experiments. The protocols for these animal experiments were approved by the animal experimentation ethics committee of Kaketsuken (Kumamoto, Japan).

In the first experiment, BALB/c mice received two intramuscular injections into the hind leg quadriceps muscles at a 3-week interval with 0.1 ml of a 0.3 mg/ml Al-adjuvanted NIBRG-14 whole vaccine containing 0.2 μg of haemagglutinin (HA) per dose. As control, 0.3 mg/ml Al in PBS was injected. Four months after the first injection, the mice were re-immunized with 0.3 mg/ml Al-adjuvanted Indo05/PR8-RG2 whole vaccine (0.2 μg HA/dose). In the next experiment, primed BALB/c mice were prepared in a similar fashion. The booster vaccine was Indo05/PR8-RG2 (0.2 μg HA/dose) with four different formulations, which were whole, split, 0.3 mg/ml Al-adjuvanted whole, and 0.3 mg/ml Al-adjuvanted split. In the third experiment, BALB/c mice were primed with 0.3 mg/ml Al-adjuvanted NIBRG-14 whole vaccine (0.2 or 0.01 μg HA/dose). As control, 0.3 mg/ml Al-containing PBS was used. Booster vaccination was conducted with Indo05/PR8-RG2 (0.2 or 0.01 μg HA/dose) of four different formulations as was done in the second experiment. In the fourth experiment, BALB/c mice were primed with two intramuscular doses at a 3-week interval using whole vaccines containing 0.3 mg/ml Al adjuvant (0.2 μg HA/dose) and one of the following viruses: NIBRG-14 (H5N1, clade 1), A/Solomon Island/3/2006 (H1N1) IVR-145, or A/Hiroshima/52/2005 (H3N2) IVR-142. Four months after priming, the mice were boosted twice with 0.3 mg/ml Al-adjuvanted Indo05/PR8-RG2 (H5N1, clades 2–1) whole or split vaccines (0.2 μg HA/dose).

Serum samples were obtained 3 times in each experiment, which were before the 3rd injection (pre), before the 4th injection (1st), and 14 days after the 4th injection (2nd). Serum was obtained from the tail vein (pre and 1st) or the heart (2nd). In all experiments where mice were primed with one dose of vaccine and boosted with several different formulations, the animals were randomized in order to normalize the HI titres before injection of the booster vaccines (Fig. 1).

2.3. Haemagglutination inhibition assay

The immune response elicited after vaccination was evaluated by performing the haemagglutination inhibition (HI) assay. In brief, serum samples were treated with RDE (II) (Denka Seiken, Tokyo, Japan) for at least 16 h at 37 °C, incubated for 1 h at 56 °C, and then incubated with 50% guinea pig erythrocytes (H3N2) or 50% chicken erythrocytes (the other viruses) overnight at 4 °C. The samples were diluted 2-fold from 1:10 at the start in the V-shaped (H3N2) or U-shaped (the other viruses) wells of 96-well plates. The HI titre was defined as the highest dilution that inhibited haemagglutination. Negative samples were assigned titres of 5 for calculation purposes.

2.4. Statistics

The raw haemagglutinin inhibition titres were transformed into $\log_{10}$ values for calculation of the geometric mean titre (GMT). The GMTs of each group of mice were compared with the Bonferroni test. All data handling and statistical computations were done with Microsoft excel (version 2002).

3. Results

3.1. Priming effect of alum-adjuvanted whole vaccine on a drifted influenza strain

To investigate the priming effect of alum-adjuvanted whole vaccine on the recall response evoked by later vaccination with drifted viruses, we immunized mice with NIBRG-14 as the priming vaccine and then boosted these animals with Indo05/PR8-RG2. To simulate the situation in which the time available to prepare a vaccine after the onset of a pandemic is at least 4 months [16,17], we gave the mice an additional booster vaccination at 4 months after priming. Four months after priming with NIBRG-14, the mice retained little cross-reactivity to heterologous Indo05/PR8-RG2, which had an HI titre of 25.9 (Table 1). On the other hand, the HI titre for homologous NIBRG-14 was 207.5 (Table 1). After a single booster vaccination with Indo05/PR8-RG2, HI antibodies for both Indo05/PR8-RG2 and NIBRG-14 showed an increase, although the second dose did not

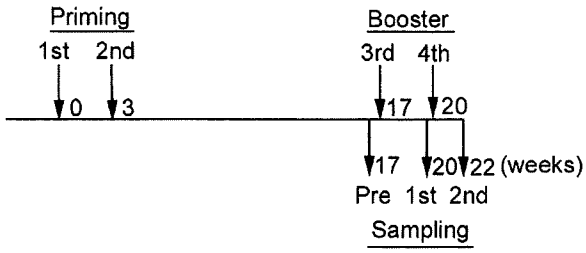

Fig. 1. Vaccination and sampling schedule.

appear to further increase the titres (Table 1). In contrast, naïve control mice given alum PBS required two doses of Indo05/PR8-RG2 to induce a HI antibody response, but the titre was still lower than that in the primed group given a single booster vaccination.

3.2. Comparison of the recall response in mice given different booster vaccines

In order to investigate the most appropriate formulation for a booster vaccine, mice primed with alum-adjuvanted whole NIBRG-14 vaccine were boosted with four different Indo05/PR8-RG2 vaccine formulations. As shown in Fig. 2, the mice boosted with split vaccines showed a larger increase in the HI titre than the mice given alum-adjuvanted whole vaccines. Thus, alum-adjuvanted was not effective and rather suppressed the response to booster vaccination. As a consequence, a split vaccine induced the best immune response of the four vaccine formulations. In naïve mice, however, the alum-adjuvanted whole vaccine was the most immunogenic and induced a higher HI antibody titre than the other vaccines.

3.3. Dosage

Next, we tested the dosages for both the priming and booster vaccinations that achieved the optimal antibody response. Mice were primed with the alum-adjuvanted whole NIBRG-14 vaccine and then boosted with alum-adjuvanted whole or split Indo05/PR8-RG2 vaccines at a dosage of 0.2 or 0.01 μg HA (Fig. 3). Primed mice showed a significant response after a single booster injection, whereas naïve mice did not. The highest booster response was achieved in mice primed with alum-adjuvanted whole vaccine (0.2 μg HA) and boosted with split vaccine (0.2 μg HA). When the dosage for either priming or booster vaccination was reduced to 0.01 μg HA, the response was slightly weaker. Even with both

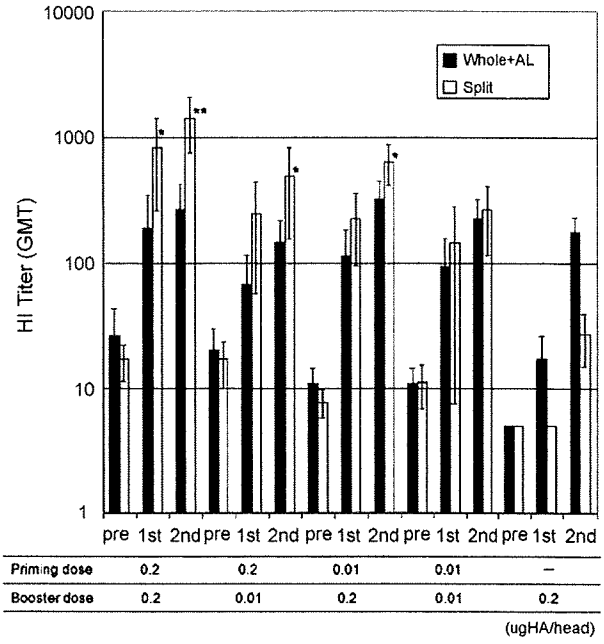

Fig. 3. Dosage strategy of priming and booster vaccination to Indo05/PR8-RG2. The priming vaccination is whole alum-adjuvanted antigens of NIBRG-14. The booster vaccination is whole alum-adjuvanted vaccines (whole + AL) or split vaccines (split) of Indo05/PR8-RG2. Dosages are indicated under the graph and are labeled: priming dose and booster dose, respectively (μg HA/dose). The HI antibody titers were measured before the first booster vaccination (pre), 2 weeks after the first booster vaccination (1st), and 2 weeks after the second booster vaccination (2nd). Error bars represent standard error of the GMT. We compared the statistical difference between the HI titers of the 1st and 2nd primed groups and the 2nd non-primed group injected with alum-adjuvanted whole vaccine by Bonferroni test (*$p < 0.05$, **$p < 0.01$).

priming and booster doses reduced to 0.01 μg HA, the response to a single booster dose of the split vaccine formulation was almost equivalent to that seen in naïve mice immunized twice with the alum-adjuvanted whole vaccine (0.2 μg HA).

3.4. Specificity of the priming effect

Since hetero-subtypic immunity is a well-known phenomenon in mice [18], we examined whether the prime-boost response was induced by hetero-subtypic viruses. Mice primed with an alum-adjuvanted whole vaccine (NIBRG-14, H1N1, or H3N2) were

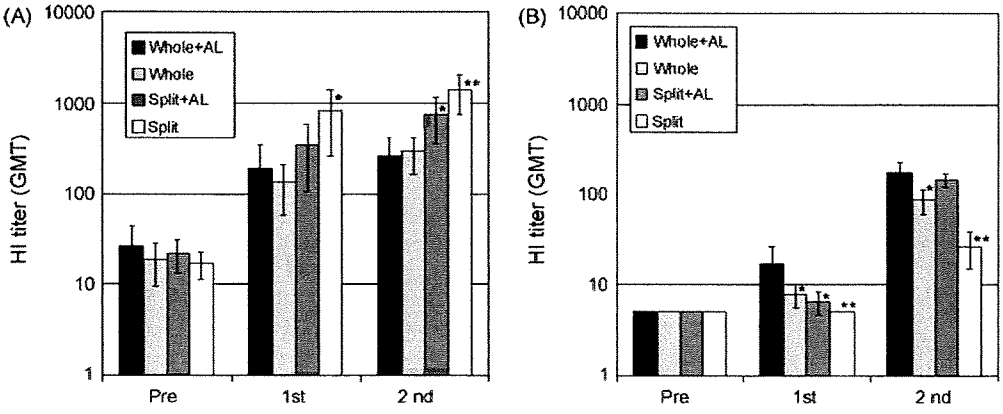

Fig. 2. Comparison of recall response injected with different booster vaccines Priming vaccines of NIBRG-14 whole alum-adjuvanted (A) or alum-containing PBS (B) were injected twice with a 3-week interval. After 4 months, booster vaccines of four formulations of the Indo05/PR8-RG2 vaccine were injected twice with a 3-week interval. The HI antibody titers were measured before the first booster vaccination (pre), 2 weeks after the first booster vaccination (1st), and 2 weeks after the second booster vaccination (2nd). Error bars represent standard error of the GMT. All dosages are 0.2 μg HA/dose. The statistical difference of each group to the alum-adjuvanted whole vaccine group was analyzed by Bonferroni test (*$p < 0.05$, **$p < 0.01$).

Table 2
Strain specificity of second vaccination.

Priming	Booster (Indo05/PR8-RG2)	Indo05/PR8-RG2		
		Pre	1st	2nd
NIBRG-14	Whole + AL	40.0 (20.0–80.0)	320.0(133.2–768.5)	452.5(199.4–1027.0)
	Split	48.8(20.1–118.1)	2152.7(908.8–5098.9)	2560.0(1198.8–5467.0)
H1N1	Whole + AL	5.0 (5.0–5.0)	21.8(11.4–41.9)	160.0(80.0–319.8)
	Split	5.0 (5.0–5.0)	32.8 (20.2–53.3)	262.5(128.6–535.7)
I-RM9	Whole + AL	5.0 (5.0–5.0)	20.0 (9.4–42.7)	246.8(124.0–491.1)
	Split	5.0 (5.0–5.0)	21.8(17.8–26.8)	207.5 (86.7–496.6)
	Whole + AL	5 0(5 0–5 0)	51 9(26 1–103 2)	349 0(215 2–566 0)
PBS	Split	5.0 (5.0–5.0)	5.5 (4.4–6.7)	30.8(13.6–69.7)

The priming vaccination was alum-adjuvanted whole vaccine of NIBRG-14, H1N1 and H3N2. To the control mice, alum-containing PBS was injected. The booster vaccination is whole alum-adjuvanted vaccines (whole + AL) or split vaccines (split) of Indo05/PR8-RG2. The HI antibody (GMT, 95%CL) to Indo05-RG2 was measured before the first booster vaccination (pre), 2 weeks after the first booster vaccination (1st), and 2 weeks after the second booster vaccination (2nd). All dosages are 0.2 μg HA/dose.

boosted with alum-adjuvanted whole or split Indo05/PR8-RG2 vaccines (Table 2).

In order to confirm that priming had elicited an immune response, HI titres for the priming vaccine strain were measured after four months. The results confirmed that the mice had been appropriately primed (data not shown). NIBRG-14-primed mice showed a significant response after a single booster injection of Indo05/PR8-RG2 as both alum-adjuvanted whole and split vaccines. In contrast, when Indo05/PR8-RG2 vaccines were injected into the H1N1- or H3N2-primed mice, there was little response.

4. Discussion

We investigated the effect of a prime–boost vaccination strategy on the immune response to H5N1 influenza viruses in a mouse model. In order to simulate a pandemic situation where influenza viruses from two phases may have differing antigenicity, mice were primed with a Vietnam strain vaccine (NIBRG-14) and then boosted with an Indonesian strain (Indo05/PR8-RG2). The primed animals showed high HI titre responses after only one booster injection, whereas naïve animals revealed a more modest HI response after two injections. Interestingly, split vaccines evoked stronger HI titre responses than whole or alum-adjuvanted vaccines.

The alum-adjuvanted vaccine used in our study has shown the ability to produce effective immunological memory that evokes a significant anti-H5 response after booster vaccination. The memory effect for the HI antibody response is mainly derived from a haemagglutinin and not from other viral components, because vaccination with H1N1 or H3N2 showed little priming effect on the recall response to H5N1 viruses. Although there was weak cross-reactivity of HI antibodies induced by the Vietnam strain for the Indonesian strain, the two viruses have more than 95% identity of their haemagglutinin amino acids and possibly share the same T helper cell and B cell epitopes. With cross-reactive memory, a rapid and vigorous recall response induced by the prime–boost regimen could reduce viral replication in the early stage of infection, limit the spread of infection, and possibly reduce illness and death in a pandemic.

Our results suggested that the split vaccine is the most suitable formation for a booster vaccine, while the alum-adjuvanted whole vaccine elicits the best HI response in naïve animals. It was reported with regard to H1N1 and H3N2 that split vaccines are similar to or more immunogenic than whole vaccines in a primed population, but achieve a worse response in naïve hosts [13–15]. The H5 subtype is even less immunogenic than H1N1 and H3N2 [19,20], but the split vaccines evoked a more vigorous response after booster injection than that induced by the whole vaccines in our mouse model. One possible reason is that the whole virus vaccinations mainly induce an IgG2a antibody response (Th1 response), whereas split vaccines induce a mixed IgG1 and IgG2a response (mixed Th1/Th2 response) [21]. In addition to showing comparable or even better immunogenicity with respect to inducing a humoral recall response, the split vaccine is less reactogenic than the whole vaccine [12–15]. Thus, with respect to both immunogenicity and reactogenicity, a split vaccine is likely to be a better formulation as a booster vaccine for H5 pandemic influenza if the vaccinees have already been primed.

Why does the split vaccine achieve a better response than the alum-adjuvanted vaccine booster injection? The common view is that alum-adjuvanted vaccines enhance the Th2 antibody response and activate DCs. Alum-adjuvanted vaccines also influence Th1 and Th2 responses by promoting the secretion of IL-1β and IL-18 [22], and the presence of alum means that the antigens are slowly released over time [23]. Sustained and slow release could be preferable for stimulating primary immune cells like DCs and other phagocytes, but could be too slow to trigger a rapid recall response.

If the dosage is reduced, the pandemic vaccination strategy could be improved. Our results indicate that priming and booster doses of only 0.01 μg HA still evoked a significant recall response. Although it is difficult to estimate the human response from our murine data, we consider that the vaccine dose can be reduced on the basis of the results of several previous experiments. For example, booster H5N3 vaccination of only 7.5 μg HA also achieved a favorable booster response in human trials [8]. In addition, several other clinical studies on H1N1 and H2N2 influenza have indicated that dose reduction may be feasible for both priming and booster vaccination [17]. When 25 or 90 μg HA of A/HK/156/97 (clade 0) recombinant heamagglutinin vaccine was injected, it was found that a booster dose of rgA/VN/1203/04 (clade 1) alum-adjuvanted split vaccine elicited a reaction irrespective of the priming dose [10].

In conclusion, we confirmed the advantage of using priming vaccination to create immunological memory for pandemic viruses even if different clade vaccines were used. Our results suggested that an appropriate vaccination strategy was to use a split vaccine instead of an alum-adjuvanted whole vaccine during an influenza pandemic. This strategy will possibly improve the immunogenicity and reactogenicity of vaccines and could also increase the vaccine supply by reducing the dosage necessary for a significant immune response. Clinical trials are needed to confirm our findings in humans and these will provide some answers regarding the appropriate dosage and formulation for influenza vaccines.

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特集I　T細胞への抗原提示をめぐって

細胞質抗原のMHCクラスIIによる提示*

笠井道之**

Key Words：cytosolic antigen, MHC class II-presentation, autophagy, lysosome, protease

はじめに

免疫応答は抗原提示細胞の膜上に提示される非自己抗原に由来するペプチド(抗原ペプチド)または自己抗原に由来するペプチド(自己ペプチド)とMHC分子とが結合した複合体にT細胞が反応することから始まる．抗原ペプチドとMHCクラスI分子との複合体はCD8陽性T細胞に，また，抗原ペプチドとMHCクラスII分子との複合体はCD4陽性T細胞にそれぞれ認識され，獲得免疫応答(細胞障害性免疫応答および液性免疫応答)を誘導する．一方，自己ペプチドとMHC分子との複合体の提示は，T細胞の自己寛容性の獲得とその制御に関与する．

ここで取り上げる細胞質抗原のMHCクラスII分子によるCD4陽性T細胞への提示についても同様である．細胞質に侵入した細菌またはウイルスに由来する抗原ペプチドとMHCクラスII分子との複合体を提示する場合は，CD4陽性T細胞による感染防御(液性免疫と免疫記憶の誘導および制御)に関係する．一方，細胞質に発現した自己蛋白質またはオルガネラに由来する自己ペプチドとMHCクラスII分子との複合体を提示する場合は，自己寛容性を有するCD4陽性T細胞レパートリーの形成(自己寛容性の成立)とその細胞による自己免疫疾患，がん免疫，および移植免疫の制御(自己寛容性の制御)に関係する．

MHC拘束性抗原提示プロセス(抗原の輸送，分解，MHC複合体形成および提示)

抗原提示細胞は主にプロテアソームシステムとリソソームシステムの2つの分解システムで抗原蛋白質をペプチド化する．抗原提示細胞の細胞質(膜蛋白質の細胞質領域と核を含む)に存在する抗原はプロテアソームシステムで分解・ペプチド化された後にMHCクラスI分子と複合体を形成し，CD8陽性T細胞に提示される．一方，その細胞外に存在する抗原(小胞内腔領域に存在する膜蛋白質抗原を含む)はリソソームシステムで分解・ペプチド化された後にMHCクラスII分子と複合体を形成しCD4陽性T細胞に提示される．これら2つのMHC分子拘束性抗原提示プロセスが免疫学における抗原提示プロセスのパラダイムであり，多くの場合はそれに従い免疫反応が誘導される[1]．しかしながら，抗原提示細胞内における蛋白質の輸送とその制御に関する解析が進んだ結果，細胞外に存在する抗原がプロテアソームシステムで分解・ペプチド化された後にMHCクラスI分子と複合体を形成しCD8陽性T細胞に提示されること，また，細胞質に

* Major histocompatibility complex (MHC) class II presentation of cytosolic antigens.

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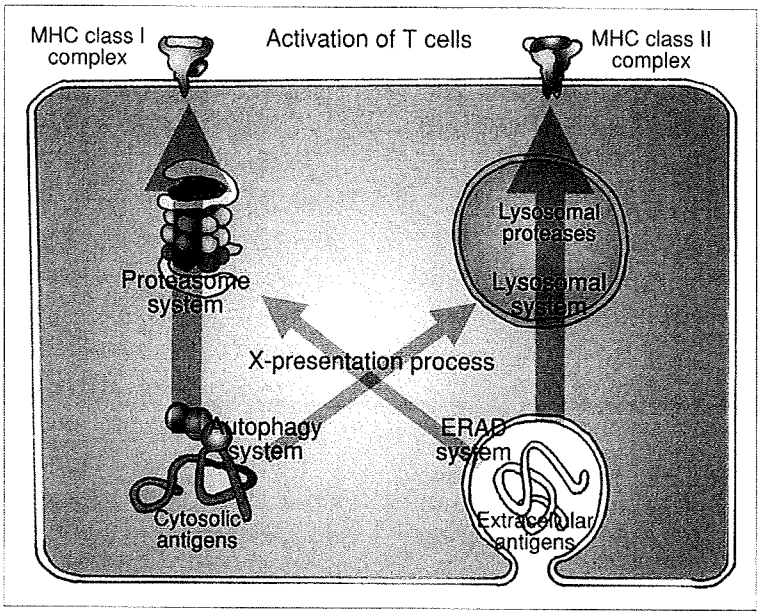

図1　MHC抗原提示プロセス
抗原提示細胞が抗原蛋白質をペプチド化する場合，主にプロテアソームシステム(proteasome system)とリソソームシステム(lysosomal system)の2つの分解システムを用いる．抗原提示細胞の細胞質や核に存在する抗原(cytosolic antigens)はプロテアソームシステムで分解・ペプチド化された後にMHCクラスI分子と複合体を形成し(MHC class I complex)，CD8陽性T細胞に提示される．一方，その細胞外に存在する抗原(小胞内腔領域に存在する膜蛋白質抗原を含む)(extracellular antigens)はリソソームシステムで分解・ペプチド化された後にMHCクラスII分子と複合体を形成し(MHC class II complex)，CD4陽性T細胞に提示される．しかし，細胞外に存在する抗原もER-associated degradation(ERAD)を介してプロテアソームシステムで分解・ペプチド化された後にMHCクラスI分子と複合体を形成し，CD8陽性T細胞に提示される．さらに，細胞質や核に存在する抗原もオートファジー(autophagy)を介してリソソームシステムで分解・ペプチド化された後にMHCクラスII分子と複合体を形成しCD4陽性T細胞に提示される．2つの分解システムを中心にした文字どおりのクロスプレゼンテーション(X-presentation)プロセスがあることが明らかになってきた．

存在する抗原がリソソームシステムで分解・ペプチド化された後にMHCクラスII分子と複合体を形成しCD4陽性T細胞に提示されることが明らかになってきた(図1)．たとえば，細胞外の抗原がファゴサイトーシスまたはエンドサイトーシスにより小胞の内腔側に取り込まれ，小胞膜に存在するSec61を含むER-associated degradation(ERAD)分子群により細胞質側に移動しプロテアソームシステムでペプチド化された後，TAPを介してもう一度小胞の内腔に移動しMHCクラスI分子と複合体を形成する[2]．一方，細胞質抗原はオートファジー(マクロオートファジーあるいはシャペロン分子介在型オートファジー)の経路を介してエンドソーム/リソソーム系の小胞内腔に輸送され，その内腔側で分解・ペプチド化された後にMHCクラスII分子と複合体を形成する[3]．このように，抗原提示細胞の細胞質に存在する抗原もその細胞外に存在する抗原もプロテアソームシステムを介した抗原提示プロセスとリソソームシステムを介した抗原提示プロセスの両方にアクセス可能なクロスプレゼンテーションプロセスが存在することがわかってきた(図1)．

オートファジーは飢餓刺激などの代謝・増殖に関するレセプターからのシグナル伝達[4]，Toll like receptors(TLRs)を含むpattern recognition receptors(PRRs)からの刺激を介した自然免疫系

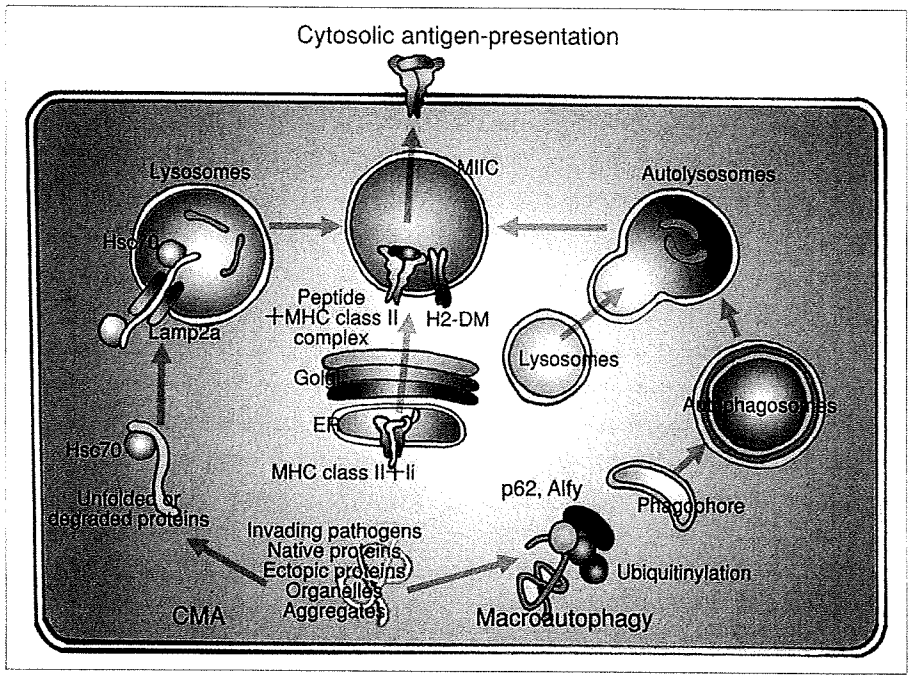

図2　オートファジーを経由する細胞質内抗原のMHCクラスII分子による提示
オートファジーは代謝・増殖に関するレセプターからのシグナル伝達，Toll like receptors(TLRs)を含むpattern recognition receptors(PRRs)からのシグナル伝達，およびIFN-γやTNFファミリーリガンドに対するレセプターからのシグナル伝達などで誘導される．主に，マクロオートファジー(macroautophagy)またはシャペロン介在型オートファジー(CMA)により細胞質内抗原をリソソームシステムに輸送する．細胞質内抗原として，細胞質内に感染・侵入した細菌やウイルスおよびそれらに由来する抗原蛋白質(invading pathogens)，構成的自己蛋白質あるいはオルガネラ(native proteins, organelles)，エピジェネティックに発現した異所性自己蛋白質(ectopic proteins)などに加えてそれらの凝集体(aggregates)がある．CMAのプロセスでは，細胞質内のプロテアーゼで限定的に分解された抗原蛋白質あるいは自己蛋白質がHsc分子にエスコートされてLamp2aのチャンネルを経由してリソソーム内腔側に輸送される．リソソーム内のプロテアーゼでペプチド化された後，MHCクラスII分子やH2-DM分子が存在するリソソーム様小胞(MIIC)と融合し，H2-DM分子の触媒的な作用の下でMHCクラスII分子と複合体(peptide＋MHC class II complex)を形成する．マクロオートファジーのプロセスでは，抗原蛋白質や自己蛋白質はそれらに特異的なE3リガーゼによりユビキチン化された後にp62分子やAlfyにエスコートされLC3分子と相互作用し，三日月型のphagophore形成を経由して脂質二重膜構造のautophagosome内に取り込まれる．さらに，lysosomeと融合しautolysosomeとなり，抗原蛋白質や自己蛋白質はカテプシンをはじめとするリソソーム内の酵素で分解しペプチド化される．AutolysosomeはMIICと融合し，ペプチドはH2-DM分子の触媒的な作用の下でMHCクラスII分子と複合体(peptide＋MHC class II complex)を形成する．

からのシグナル伝達[5]，あるいはIFN-γやTNFファミリーリガンドに対するレセプターからのシグナル伝達[5,6]で開始される．主としてマクロオートファジーまたはシャペロン介在型オートファジー(CMA)により細胞質内抗原をリソソームシステムに輸送する(図2)．そのシステム内で細胞質内抗原はペプチド化され，MHCクラスII分子と複合体を形成する．MHCクラスII複合体によるCD4陽性T細胞への提示は，病原体感染に対する獲得免疫(液性免疫と免疫記憶)の誘導および胸腺および末梢リンパ組織における自己反応性CD4陽性T細胞の排除(自己寛容性の成立)と抑制性CD4陽性T細胞の形成とその細胞による自己免疫疾患，がん免疫，および移植免疫の制御(自己寛容性の制御)に関係する[7]．このような獲得免疫や自己寛容の成立・制御に関係する

細胞内抗原としては，次の3つがあげられる．①細胞質内に感染・侵入した細菌やウイルスおよびそれらに由来する抗原，②構成的に発現する自己蛋白質あるいはオルガネラ，③エピジェネティックに発現した自己蛋白質である．

言うまでもなく①は獲得免疫に関係し，②と③は自己寛容の成立・制御に関係する．最初に細胞質内に感染・侵入した細菌抗原あるいはウイルス抗原のCD4陽性T細胞への提示について論じるが，これら病原体に由来する抗原のオートファジーを介した輸送・ペプチド化を開始する機構とその制御機構は自己蛋白質のオートファジーを介した輸送・ペプチド化の開始機構や制御機構と共通する部分が少なからずあるからである．

細胞質内の細菌抗原あるいはウイルス抗原のCD4陽性T細胞への提示

細胞内に細菌やウイルスなどの病原体が進入した場合，宿主細胞のPRRsが病原体のpathogen associated molecular patterns(PAMPs)を認識することによりマクロオートファジーを介する病原体殺傷・排除の機構が開始される．その機構に関係するPRRsは4つのカテゴリーに分類される．①TLRs, ②retinoic acid-inducible gene I like helicase receptors(RLRs)，③nucleotide-binding and oligomerization domain-like receptors(NLRs)，④C-type lectin receptors(CLRs)[4,5]である．病原体が宿主細胞の細胞質に侵入後，宿主細胞のPRRsが病原体のPAMPsを認識し，一連のシグナル伝達を経て三日月型のファーゴホア(phagophore)が形成され，病原体を包みはじめる．脂質二重膜構造のオートファゴソーム(autophagosome)へと成長し，完全に病原体を包み込んだ後にオートファゴソームはリソソームと融合し，オートリソソーム(autolysosome)へと成熟する[5]．オートリソソームはファゴサイトーシスに由来するリソソーム(phagolysosome)と同様に病原体を処理する活性化酸素を発生する分子群(NADPH oxidase 2)[7]やカテプシン類による病原体の分解に関与するユビキチン由来のペプチド[8]を備えている上に，病原体に由来する抗原のペプチド化とMHCクラスII複合体形成の場(MIIC)を提供する[3-6]．細胞内に侵入し，増殖するウイルスに由来する抗原蛋白質を提示する場合，たとえばEBAVの場合，宿主細胞のリボソームで合成されたウイルス由来核蛋白質のうち不完全型の蛋白質(ribosomal defective proteins)はユビキチン化された後プロテアソームシステムで分解・ペプチド化されMHCクラスI分子と複合体を形成し，CD8陽性T細胞に提示される．一方，完全な型の核蛋白質はマクロオートファジーによりリソソームに輸送され，autolysosome内のカテプシンなどの分解酵素の作用によりペプチド化された後，MHCクラスII分子と複合体を形成し，CD4陽性T細胞に提示される[9]．EBAV以外に細胞質内に感染・増殖するウイルスとしてHCV，インフルエンザウイルスA型，measles virusなどがあげられる．これらのウイルスに由来する抗原の場合も同様な抗原提示プロセスを受けると考えられる[6]．病原体に由来する蛋白質，脂質成分あるいは核酸成分などのPAMPsがPRRsを刺激することによりマクロオートファジーを誘導するが，同時に抗原蛋白質に特異的なE3リガーゼも誘導し，抗原をユビキチン化すると考えられる．ユビキチン化された抗原蛋白質やその凝集体(aggresome-like induced structures；ALIS)[10]はp62を介してLC3と結合することにより[11]，あるいはautophagy-linked FYVE protein(Alfy)[12]を介してオートファゴソーム内に取り込まれると考えられる．ユビキチン化された抗原蛋白質がプロテアソームシステムで分解されるのかあるいはオートファジーを介したリソソームシステムで分解されるのかの分岐点はユビキチン化抗原蛋白質がユビキチン認識サブユニット(PA700)と相互作用し，プロテアソームのプロテアーゼ活性部分へ運ばれるのか，あるいはp62やAlfyのような蛋白質と相互作用し，オートファゴソーム内部へ運ばれるのか否かにあると考えられる．

細胞質内の自己抗原のCD4陽性T細胞への提示

細胞質内の蛋白質・オルガネラは一定の期間で代謝される．プロテアソームシステムやカルパインのような細胞質内プロテアーゼおよびリソソームシステムがそれに関係する．細胞質内

で構成的に発現している蛋白質・オルガネラがリソソームシステムで分解・ペプチド化され，MHCクラスII分子と複合体を形成しCD4陽性T細胞へ提示される場合，2つのタイプのオートファジーがこれに関与する(図2)．一つは細胞質内のプロテアーゼによる限定分解を受けた後，CMAによりリソソームシステムに運ばれさらにペプチド化された後にMHCクラスII分子と複合体を形成する[3][13]．もう一つは細胞質内の自己蛋白質やオルガネラがユビキチン化の修飾を受けた後にマクロオートファジーによりリソソームシステムに運ばれペプチド化され，MHCクラスII分子と複合体を形成する[3][14]．抗原提示細胞からアフィニティーカラムで精製したMHCクラスII複合体から酸溶出されるペプチドの解析では，細胞外の抗原や細胞膜蛋白質に由来するペプチドに加えて細胞質や核の蛋白質(自己蛋白質)に由来する自己ペプチドがMHCクラスII複合体から酸溶出される全ペプチドのおよそ20%を占める[15]．さらに，抗原提示細胞を飢餓状態にしてマクロオートファジー出現頻度を高めた場合，MHCクラスII複合体から酸溶出される自己ペプチドの量とその種類が増加する[15]．マクロオートファジーは代謝・増殖シグナルや病原体に由来するPAMPsのPRRs刺激で誘導されるばかりでなく，IFN-γやTNF-αなどのサイトカインやCD40Lなどのリガンドの刺激により特異的に誘導される[5][6]．さらに，異所性自己蛋白質やそれに対するE3リガーゼが発現誘導される環境もマクロオートファジーを誘導すると考えられる．したがって，構成的な自己蛋白質も異所性自己蛋白質もそれらに選択的なE3リガーゼによりユビキチン化され，p62やAlfyなどのエスコート蛋白質によりオートファゴソームに取り込まれた後，MHCクラスII分子に拘束して提示されると考えられる[16]．たとえば，胸腺髄質上皮細胞やがん細胞では，構成的に発現する自己蛋白質に加えてエピジェネティックに発現誘導される異所性自己蛋白質もマクロオートファジーを介してリソソームシステム内に輸送され，その内部でペプチド化された後にMHCクラスII分子に拘束して提示されると考えられる[4][6][16]．

胸腺上皮細胞内のマクロオートファジーを介したMHCクラスII抗原提示プロセス

胸腺微小環境を構成する皮質部上皮細胞および髄質上皮細胞は飢餓状態でなくてもマクロオートファジーを起こす[17]．マクロオートファジーを介した皮質部上皮細胞上の自己ペプチドとMHCクラスII複合体の提示は未熟T細胞のMHC拘束性の獲得に関与し，一方，および髄質部上皮細胞上の自己ペプチドとMHCクラスII複合体の提示は自己反応性未熟T細胞の排除に関与することが示唆されている[18]．しかしながら，これまで胸腺上皮細胞内においてオートファゴソームがMHCクラスII複合体形成の場であるH2-DM陽性コンパートメント(MIIC)にアクセスする直接的な証拠はいままで示されていなかった．筆者は，飢餓状態下にあり通常よりも多くマクロオートファジーが誘導されている新生児胸腺の凍結切片を抗LC3モノクローナル抗体(4E12)とMIICのマーカーとなるH2-DM分子に対する抗体(抗H2-DM抗体)で蛍光二重染色を行い，LC3分子とH2-DMとの共存性を調べた．図3のA-mに示すように皮質部上皮細胞も髄質部上皮細胞もLC3分子とH2-DM分子の共存が確認された．さらに，B-mに示すように，胸腺上皮細胞内のLC3陽性コンパートメントとH2-DM陽性コンパートメントの共存が認められ，実際にオートファゴソームがMIICにアクセスすることを示唆する結果が得られた．

おわりに

細胞質抗原のMHCクラスIIによる提示は感染防御や自己寛容の成立・制御に重要な役割を果たしている．細胞質抗原は2つのオートファジープロセスを介してMHCクラスII提示プロセスの主体となるリソソームシステムに取り込まれるが，その制御機構については十分な解明がなされていない．とくに，自己蛋白質が恒常的なマクロオートファジーを介してMHCクラスII分子に拘束して提示される場合と，免疫学的環境下で誘導されたマクロオートファジーを介してMHCクラスII分子に拘束して提示される場合におけ

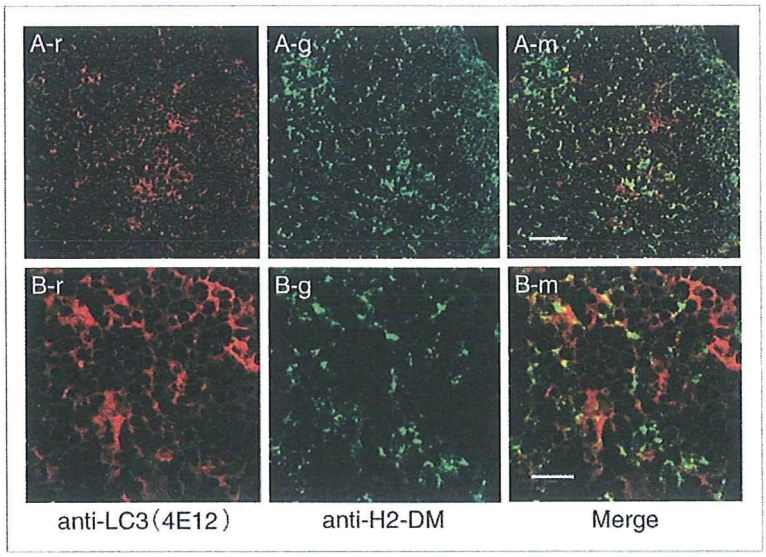

図3　胸腺上皮細胞内のマクロオートファジーを介したMHCクラスII抗原提示プロセス

マクロオートファジー不全マウスは出生後，飢餓状態に陥り死亡する．この現象にもとづいて生後1日目のマウス胸腺から凍結切片を作製し，抗LC3抗体(4E12)とMHCクラスII複合体形成の場であるコンパートメント(MIIC)のマーカー分子であるH2-DM分子に対する抗体(抗H2-DM抗体)との二重蛍光免疫染色を行った．予想どおりにLC3分子(赤色)は胸腺皮質部上皮と髄質部上皮に存在し(A-r)，一部はH2-DM陽性上皮細胞(A-g)と共存していた(黄色：A-m)．LC3陽性コンパートメント(赤色：B-r)は胸腺上皮細胞内でH2-DM分子陽性コンパートメント状(緑色：B-g)と共存している(黄色：B-m)．A-mにおける白線は50μmを示し，B-mにおける白線は20μmを示す．

るマクロオートファジー誘導と抗原提示プロセスの分子機構の相違を明らかにする必要がある．同時に，両者の環境下における自己蛋白質の発現様式の相違とMHCクラスII分子上に提示される自己ペプチドレパートリーの相違も解析する必要がある．以上の解析からLC3を中心とするオートファゴソーム形成の分子制御が細胞質内蛋白質のMHCクラスII分子に拘束した提示とそれに対する免疫応答に関係することが明らかになるであろう．

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