FULL PAPER *Immunology*

Prolonged Suppression of Chick Humoral Immune Response by Antigen Specific Maternal Antibody

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ABSTRACT. Although the inhibitory effect of maternal antibodies on active immunization of neonates has been extensively documented, much less attention has been devoted on the exact level of these antibodies which can induce this effect and the extent of such effect. Firstly, laying hens were immunized with dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH).Then, maternal anti-DNP antibodies in chicks derived from these hens were measured by using enzyme-linked immunosorbent assay (ELISA). Chicks with high levels of maternal anti-DNP showed immune suppression, while chicks with low levels of maternal anti-DNP showed normal immune response when they immunized with the same antigen at 1 and 4 weeks of age. Then, different doses of purified maternal anti-DNP were transferred to fertile eggs at 16 days of embryogenesis by in ovo injection and all chicks were immunized with DNP-KLH at 1 and 4 weeks of age. Chicks received 1 mg of anti-DNP showed normal immune response, chicks received 3 mg of anti-DNP showed weak immune response, and chicks received 5 and 8 mg of anti-DNP showed immune suppression. Chicks received 8 mg of anti-DNP were immunized with DNP-KLH at 4 and 7 weeks of age. Their immune response was significantly lower than that of chicks of no-maternal anti-DNP. These results suggested that high levels of maternal antibodies interfere or suppress the immune response of active immunization not only at early period but also at the period in which the maternal antibodies at very low levels.

KEY WORDS: chick, hapten, IgY, immune suppression, maternal antibodies.

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The principal function of the immune system is to protect animals from infectious organisms and from toxic products. Newly hatched birds emerge from the sterile environment of the egg and, are susceptible to many pathogens during the first few weeks of age and like mammals, require temporary immunological assistance because their immune system is not fully developed; hence, passive maternal immunity is thought to be an antigen-specific protection [23]. There are many reports in literature regarding the transfer of pathogen-specific antibodies from hens to their chicks via the egg and their role in the protection of newly hatched chicks from the pathogens. The transfer of maternal immunity from laying hens to newly hatched chicks takes place in a two-step process. The first step is transport of antibodies from maternal serum into the yolk of maturing oocyte in the ovarian follicle. The second step is uptake of antibodies from the yolk into the circulation of newly hatched chicks which occurred predominantly in the last few days before hatching [4, 13, 16, 17]. The amount of antibody transferred to chick can depend on the age of the mother, the time within the laying cycle, and the titers of antibodies within the mother's serum [22]. Maternal antibodies protect the hatchling against environmental pathogens during the period required for the immune system to mature and at the same time they prevent generation of tolerance to the same antigens as they prevent their early interaction with immune cells [2]. It has been reported that the presence of maternal antibodies further limits the immune response of neonates to either natural infection or active immunization [3, 7]. Several studies in animal models have confirmed the biological observation of the inhibitory effect of passive antibodies on vaccine responses. High levels of viral antibodies transferred to calves could interfere with the antibody response to vaccination [20, 21]. Passively-introduced antibodies can protect against infectious diseases and may interfere with antibody response to active immunization in early infancy [5, 6, 10, 12, 14, 15]. Vaccination in poultry farm is considered the novel approach which has been shown to elicit specific immunity against different pathogens. However, maternal antibodies transmitted to the newly hatched chicks are known to provide protection against many serious viral and bacterial diseases during the first period of life, they may interfere with early vaccination programs [22, 24]. The aim of this study is to investigate and confirm the effect of maternal antibodies on chick immune response.

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MATERIALS AND METHODS

Animals: Partially inbred chickens (H-B15 white leghorn; Bu-1[a]) were used in this study. These chickens were bred in our animal facilities and were provided with food and chlorinated water *ad libitum* under the regulation of guideline for the animal experiment in Hiroshima University. The chickens were divided into immunized and non-immunized group to dinitrophenylated keyhole limpet hemocyanin

(DNP-KLH). Eggs derived from the chickens were incubated and hatched in our own facilities. Chicks derived from non-immunized hens were ascertained to be free from maternal anti-DNP antibodies and considered as a control group.

Antigen preparation: DNP-KLH was prepared as described previously [9, 11]. Briefly, in clean, dry and dark container, 200 mg of K_2CO_3 was dissolved in 6 m*l* of distilled water (DW), and then putted on a magnetic stirrer and slowly 200 mg of KLH (Calbiochem Behring Co., Germany) was added and left at room temperature. At the same time, 200 mg of 2, 4-dinitobenzen sulfonic acid sodium salt (DNBS) (Eastman Kodak Co., San Diego, U.S.A.) was dissolved in 4 m*l* of DW. DNBS solution was added into KLH solution. Then, the mixture was stirred in dark place at room temperature for about 18 to 24 hr, and then it was dialyzed against phosphate buffered saline (PBP) at 4°C until obtaining zero value of optical density (OD) at 360 nm against PBS. Finally, the mixture was filtered by using 0.45 μm filter. The protein content of this antigen was determined by OD value measured at 280 nm. Conjugation ratio of hapten with protein was determined as described previously [9, 11]. Final product was DNP_{32}-KLH. Then, the antigen was kept in a refrigerator at 4°C until use. Dinitrophenylated bovine serum albumin (DNP_{28}-BSA) was prepared in the same manner.

Immunization of hens: Six laying hens were immunized with DNP-KLH (1 mg per each hen) emulsified in Freund's complete adjuvant (FCA) (Wako Pure Chemical Industries, Japan) into their peritoneal cavity. Second immunization was performed after 2 weeks, then they repeatedly immunized every 3 weeks using Freund's incomplete adjuvant (FIA) (Wako Pure Chemical Industries, Japan). Immunized laying hens were divided according to the purpose of use into 2 groups: first group was used for production of infertile eggs (collected daily after one week of the second immunization and stored in the refrigerator at 4°C until use for extraction of IgY), second group was used for production of fertile eggs (collected daily after one week of the second immunization and kept in an incubator at 15°C with humidity 60% until use). Chicks derived from immunized hens and non-immunized hens were immunized two times with DNP-KLH (2 mg/kg of body weight) or DNP-BSA (2 mg/kg of body weight) at 1 week and 4 weeks after hatching. The first immunization was given into the peritoneal cavity with the antigen emulsified with FCA. The second immunization was given by the same manner but with FIA instead of FCA.

Purification of chicken IgY from the egg yolk: Chicken IgY was extracted from the egg yolk by the water dilution method as described before [1] with some modifications. Briefly, the tare weight of an empty clean glass beaker was recorded. The egg yolk was separated from the egg white using an egg separator then washed with DW, then rolled onto a clean, dry paper towel for removing the adhering egg white, after position the egg sac near any edge of the paper towel, the egg sac was punctured with Pasteur pipette and finally the egg yolk was collected in the beaker. One volume of the egg yolk was mixed slowly and gently with nine volumes of DW. The pH value of the diluted egg yolk was adjusted to 5.2 by adding 1N HCl drop by drop during stirring it. The beaker was covered and kept in the refrigerator at 4°C for at least 6 hr. Diluted egg yolk was mixed gently before added to the centrifuge tubes (centrifugation for 15 min at 10,000 × g in a refrigerator centrifuge). The supernatant was decanted into a clean beaker, while stirring gently; ammonium sulfate (final percentage was 40%) was added gently and the mixing was continued for at least 30 min. The suspension was centrifuged for 15 min at 10,000 × g in a refrigerated centrifuge. Supernatant was discarded. Equal volume of PBS to the original volume of the egg yolk was added to the pellet and mixed gently until IgY pellet was completely dissolved. The purified IgY solution was dialyzed for 4–5 times against PBS until ammonium sulfate was completely removed. The volume of purified IgY solution was measured after filtration with 0.45 μm filter. IgY purity was measured using sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Finally, purified IgY stored in refrigerator at 4°C until use.

Affinity purification of antigen specific IgY: DNP-BSA was conjugated with swollen CNBr-activated Sepharose 4B beads (GE Healthcare, Sweden) in coupling buffer (bicarbonate buffer) (0.5 M NaCl, 0.1 M sodium bicarbonate, pH 8.3). Free-reacted sites on the beads were blocked with 1 M ethanol amine, pH 8.0, finally the beads were washed with the coupling buffer and then the elution buffer (0.5 M NaCl, 0.2 M glycine-HCl, pH 2.5) four times before washing with PBS and stored in the refrigerator at 4°C until use. DNP-BSA column was washed gently with 5 m*l* of low pH buffer (0.5 M NaCl, 0.2 M glycine-HCl pH 2.5) then gently washed with PBS (20 times of the gel volume). After that IgY solution was added several times to the Sepharose gel in the column. The filtrate was collected. The column was washed with PBS (20 times of the gel volume), then 5 m*l* of the elution buffer (0.5 M DNP-EACA) (Sigma Chemical Co., U.S.A.) was added gently to the Sepharose gel in the column. The filtrate which contains anti-DNP antibodies was collected then dialysed against PBS, concentrated using centrifugal filter device (Amicon ultra-15, ultracel 100k) (Millipore, U.S.A.). Finally the column was washed firstly with 20 m*l* of low pH buffer then with PBS (20 times of the gel volume), then kept in the refrigerator at 4°C with PBS containing sodium azide. The optical density of the concentrated sample was measured at 280 and 360 nm for calculation of the actual amount of anti-DNP antibodies. Finally, the immunoreactivity of anti-DNP antibodies was evaluated by using Enzyme-linked immunosorbent assay (ELISA).

Inoculation of antigen specific IgY: Anti-DNP antibody was injected into eggs either from vein or from yolk. Briefly, the blunt end of the egg was sterilized with tincture of iodine, and then small hole (1 cm × 1 cm) was made by a dental drill. Some of the chicks were injected with purified anti-DNP antibodies (2 mg/0.2 m*l* in PBS) from intravenous route at 18 days of embryogenesis (18E) using 1 m*l* syringe

(30G 1/2 needle; Becton Dickinson, U.S.A.). The other chicks were injected with PBS either containing 1 or 3 or 5 or 8 mg anti-DNP antibodies from this hole into the egg yolk at 16 days of embryogenesis (16E) using 1 m*l* syringe (30G 1/2 needle).

Collection of blood samples: Blood samples were collected every week from each chick from the wing vein using 1 m*l* syringe with 27G needle and stored at 4°C for 1 to 2 hr. Serum was separated from clotted blood by centrifugation at 10,000 × g for 5 min and stored at –30°C until use.

Measurement of anti-DNP antibodies: ELISA was used for measuring anti-DNP antibodies in serum samples. The ELISA method was performed as previously described [26]. Briefly, each well of a 96-well-microplate plate (Nunc, Roskile, Denmark) was coated with 55 μ*l* of DNP-BSA (50 μg/m*l*), incubated overnight at 4°C in a moist chamber. The plate was washed 5 times with washing buffer (0.05% tween 20 in PBS) (350 μ*l*/well). The blocking buffer (PBS containing 25% Block Ace: Dainippon Sumitomo pharmaceutical Co., Japan) was then added to the plate (350 μ*l*/well), then the plate was incubated at 37°C for 2 hr. The blocking buffer was decanted, then the plate was washed 5 time with washing buffer, then 4-fold diluted serum samples (diluted in 10% Block Ace in PBS) were added to the plates (55 μ*l*/well). After incubation at 37°C for 1 hr, the diluted serum samples were decanted and the plate was washed 5 times with washing buffer, then diluted HRP-labeled goat anti-chicken IgY heavy and light chain (Bethyl Inc., Montgomery, TX, U.S.A.) (1/2,000, diluted in 10% Block Ace in PBS) was added to the plate (55 μ*l*/well), then the plate was incubated at 37°C for 1 hr. After decanting diluted anti-chicken IgY solution, the plate was washed 5 times with washing buffer, then the substrate solution (25 m*l* phosphate citric buffer (pH 5.7), 0.01 g of *o*-phenylenediamine (Sigma St Louis, MO, U.S.A.) and 5 μ*l* H_2O_2) was added to the plate (55 μ*l*/well), the plate was left for 10–20 min in the dark place till the appearance of yellow color, the reaction was stopped using 2N H_2SO_4 (55 μ*l*/well). Finally optical density was measured at 490 nm with a micro plate reader (BIO-RAD Model 680, Japan). Each plate contained the dilution buffer only instead of sample was considered as negative control, and the standard purified anti-DNP antibodies (1 mg/m*l*) was considered as positive control. The concentration of serum anti-DNP antibody was measured after conversion of ELISA data into mg/m*l* by using standard anti-DNP antibody sample of known concentration.

Statistical analysis: The mean anti-DNP antibodies titers of the newly hatched chick's sera were compared using Student's *t*-test. All values were expressed as mean ± standard deviation and were considered to be significant at $p<0.05$ and highly significant at $p<0.005$.

RESULTS

Immune response of newly hatched chicks of no-maternal antibodies: Fourteen newly hatched chicks derived from non-immunized hens were divided into 2 groups: non-immunized (5 chicks) and immunized group (9 chicks) with DNP-KLH at 1 and 4 weeks of age. Antigen-specific antibodies (anti-DNP) concentrations (mg/m*l* serum) were measured in serum samples of both groups by using ELISA as mentioned before. The concentration of anti-DNP antibody was zero (mg/m*l*) in the serum of non-immunized chicks till 6 weeks of age. This group of chicks was considered as a negative control group (Fig. 1). On the other hand, the concentrations of anti-DNP antibodies were high in the serum of immunized chicks especially after the second immunization, (0.1661 ± 0.0654 mg/m*l*) at first week after the immunization and gradually decreased (0.09 ± 0.031 mg/m*l*) at the second week. This group of chicks was considered as a positive control group (Fig. 1).

Effect of maternal antigen specific antibodies on the chick immune response: Thirteen newly hatched chicks derived from immunized hens with DNP-KLH were immunized with the same antigen at 1 and 4 weeks of age. The immune response of these chicks was varied according to the amount of maternal antibodies transferred to them. The immune response of newly hatched chicks with high maternal antibodies was significantly lower than that of the group with no-maternal antibodies, especially after the second immunization ($p<0.005$; 1 and 2 weeks after second immunization) (Fig. 2A). In contrast, newly hatched chicks with low maternal antibodies showed normal immune responses which has no significant difference with that of no-maternal

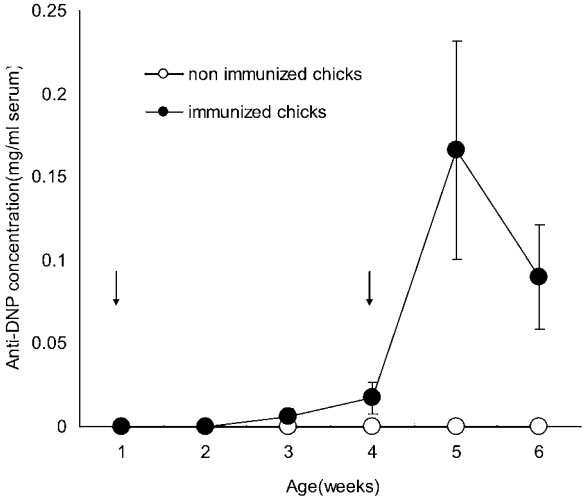

Fig. 1. Immune response of newly hatched chicks derived from non-immunized hens with DNP-KLH and immunized with DNP-KLH at 1 and 4 weeks of age. Anti-DNP antibodies concentrations (mg/m*l* serum) after first and second immunization were measured by using ELISA. Anti-DNP antibodies concentration was zero in the serum samples of non-immunized chicks, but gradually increased in the serum samples of immunized chicks after 2 weeks of the first immunization and reached to the maximum concentration (0.1661 ± 0,0654 mg/m*l* serum) after the second immunization by 1 week, then decreased (0.09 ± 0.031 mg/m*l* serum) after two weeks. The two arrows refer to the time of immunization with DNP-KLH.

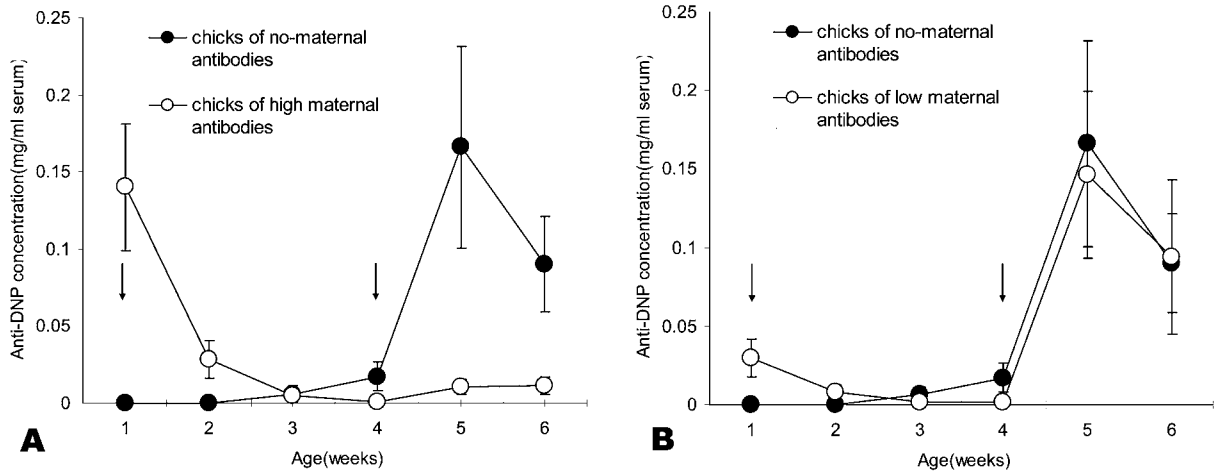

Fig. 2. Immune response of newly hatched chicks derived from immunized laying hens with DNP-KLH and then immunized with the same antigen at 1 and 4 weeks of age. (a) The immune response of the chick with high maternal antibodies was lower than that of the chick with no-maternal antibodies ($p<0.005$ after the first and second week of the second immunization). On the other hand, the chicks with low maternal antibodies showed normal immune response which has no significant difference with that of the chicks with no-maternal antibodies as shown in Fig. 2B ($P>0.05$ after the first and second week of the second immunization). The two arrows refer to the time of immunization with DNP-KLH.

antibodies especially after the second immunization ($p>0.05$ after the first and second week) (Fig. 2B).

Immune response of newly hatched chicks received different doses of anti-DNP antibodies via in ovo injection at 16E: The immune response of the newly hatched chicks was different according to the amount of maternal antibodies transferred to them (as obtained in the previous experiment). Therefore, we injected the fertile eggs of non-immunized hens with different doses of anti-DNP antibodies at 16E (Due to the transfer of maternal antibodies mainly begin before hatching by three days). We transferred the purified anti-DNP antibodies by two routes. The first route was through the superficial veins of embryos at 18 days of embryogenesis. Injection of 2 mg anti-DNP antibodies/0.2 m*l* in PBS was enough to suppress the immune responses of newly hatched chicks especially after the second immunization when they immunized with DNP-KLH at 1 and 4 weeks of age (data not shown). This method was difficult, time consumed and led to high mortalities among embryos. Therefore, we used the transferring route via egg yolk (easy method, quick and give high percentage of hatchability). Results obtained from two routes were completely same. Therefore, we used in ovo injection in this study. The newly hatched chicks were divided into four groups according to the amount of anti-DNP antibodies injected per each egg. First group consists of 14 chicks derived from injected eggs with 1 mg of anti-DNP antibodies, 9 chicks were immunized with DNP-KLH at 1 and 4 weeks of age and the remaining 5 chicks were not immunized and considered as a control for this group. All chicks, immunized with DNP-KLH, showed a normal immune response especially after the second immunization. Anti-DNP response in immunized chicks was not significantly different from that of chicks derived from non immunized hens or that in chicks with low maternal antibodies ($p>0.05$ after the first and second week of the second immunization) (Fig. 3A). Second group consists of 7 chicks derived from injected eggs with 3 mg of anti-DNP antibodies. Four chicks were immunized with DNP-KLH at 1 and 4 weeks of age and the remaining 3 chicks were not immunized and considered as a negative control. The immune response of the immunized chicks were significantly lower than that in the positive control group ($p<0.05$ after the first and second week of the second immunization) (Fig. 3B). Third group consists of 10 chicks derived from injected eggs with 5 mg of anti-DNP antibodies, 7 chicks were immunized with DNP-KLH at 1 and 4 weeks of age and the remaining 3 chicks were not immunized as a negative control. Unfortunately one chick of the control group was died after 2 weeks of hatching and 3 chicks from the 7 immunized chicks were died before the second immunization. The immune response of the remaining immunized chicks was significantly different from that in the positive control group ($p<0.005$ after the first and second week of the second immunization) and also not significantly different from the immune response of chicks with high maternal antibodies ($p>0.05$ after the first and second week of the second immunization) (Fig. 3C). Fourth group consists of 8 chicks derived from injected eggs with 8 mg of anti-DNP antibodies, 5 chicks were immunized with DNP-KLH at 1 and 4 weeks of age and the remaining 3 chicks were not immunized as a negative control. The immune responses of all immunized chicks were significantly lower than that in positive control group as same as that in high maternal antibodies group ($p<0.005$ after first week and second week of the second immunization) (Fig. 3D).

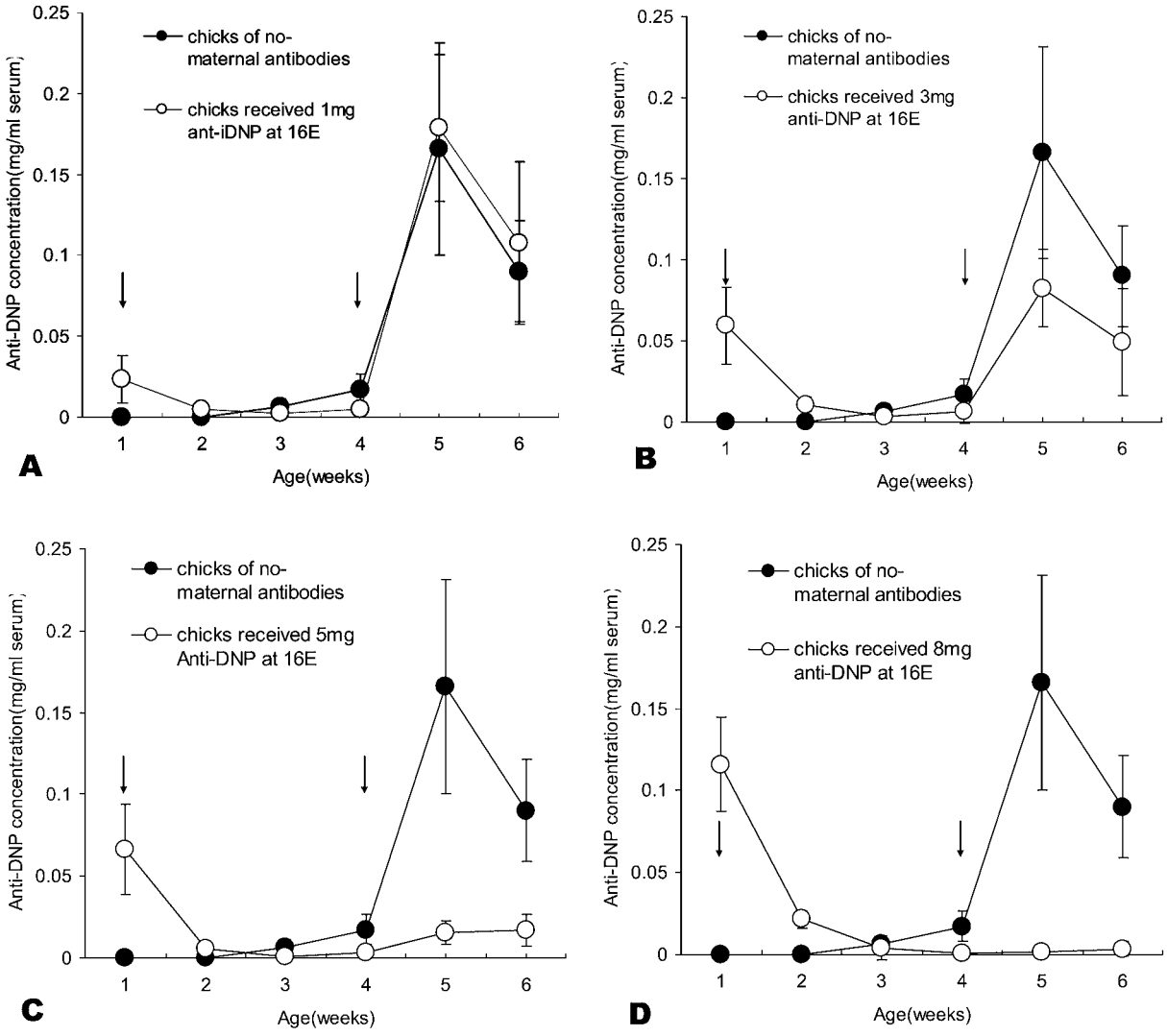

Fig. 3. Immune response of newly hatched chicks derived from injected eggs with different doses of anti-DNP antibodies at 16E and immunized with DNP-KLH at 1 and 4 weeks of age. (A) Chicks received 1 mg of anti-DNP, showed a normal immune response especially after the second immunization, and the immune responses were not significantly different from that of the positive control group ($P>0.05$). (B) The immune response of chicks received 3 mg of anti-DNP antibody was significantly different from that of the positive control group ($P<0.05$ after first and second week of the second immunization). (C) The immune response of chicks received 5 mg of anti-DNP antibody was significantly lower than that in the positive control group ($P<0.005$ after first and second week of the second immunization). (D) The immune response in chicks received 8 mg of anti-DNP antibody was significantly lower than that of positive control group ($p<0.005$ after first week and second week of the second immunization). The two arrows refer to the time of immunization with DNP-KLH.

Immune response of newly hatched chicks received anti-DNP antibody via in ovo injection at the day of hatching: After induction of immune suppression in newly hatched chicks by injection of 8 mg anti-DNP antibodies via in ovo injection at 16E, we performed an additional experiment through which we made sure that the transfer of maternal antibodies not only occur before hatching by three days but also may be occur after hatching. In this experiment, 8 newly hatched chicks derived from non immunized hens were injected with 8 mg anti-DNP antibodies by in ovo injection at the day of hatching then divided into two groups: first group consisted of 3 chicks, were not immunized with DNP-KLH and considered as a negative control, second group consisted of 5 chicks, and were immunized with DNP-KLH at 1 and 4 weeks of age. Unfortunately 1 chick from the first group was died after 1 week of hatching and 2 chicks from the second group were died after 2 weeks from the first immunization. The remaining chicks in the second group showed immune suppression especially after the second immunization and significantly lower than that

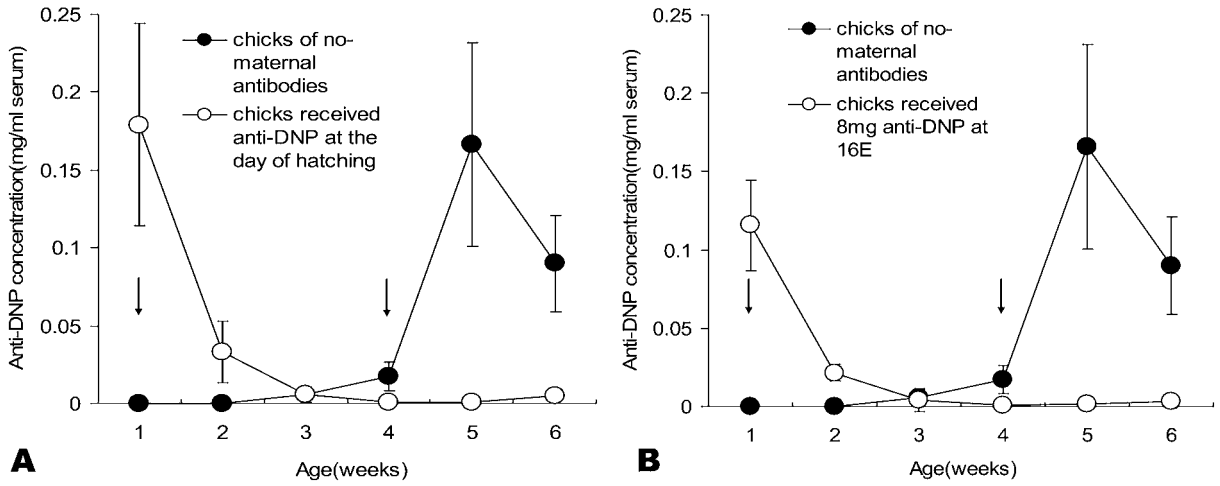

Fig. 4. Immune response of newly hatched chicks injected with anti-DNP antibodies via in ovo injection at the day of hatching and immunized with DNP-KLH at 1 and 4 weeks of ages. (A) The immune response of all immunized chicks was significantly lower than that of the positive control group ($p<0.005$ after the first and second week of the second immunization) and as same as the immune response of chicks derived from injected eggs with 8 mg anti-DNP antibodies via in ovo injection at 16E (B) ($P>0.05$ after the first and second week of the second immunization). The two arrows refer to the time of immunization with DNP-KLH.

of the positive control group ($p<0.005$ after the first and second week) and as same as that of chicks derived from injected eggs with 8 mg anti-DNP antibodies via in ovo injection at 16E ($p>0.05$ after the first and second week) (Fig. 4 and 3D).

Chicks received 8 mg of anti- DNP (purified from the egg yolk of hyper immunized laying hens to DNP-KLH) were also immunized with DNP-BSA. As shown in Table 1, suppression of Anti-DNP antibodies response was observed in the immune response to DNP-BSA especially after second immunization.

Immune response of chicks received anti-DNP antibody at the periods in which the almost completely decreased levels of maternal antibodies: Anti-DNP antibodies concentration reached to the minimum in the serum samples of chicks with high levels of maternal antibodies by the end of the forth or the fifth week age (data not shown). Eight newly hatched chicks were divided into two groups; first group consisted of 5 chicks and received 8 mg of anti-DNP antibodies by in ovo injection at the day of hatching, the second group consisted of 3 chicks and did not receive maternal anti-DNP. The two groups were immunized with DNP-KLH at 4 and 7 weeks of age. Then, anti-DNP concentrations (mg/m*l* serum) were measured in serum samples of both groups by using ELISA. We found that the immune response of the first group which received maternal anti-DNP was significantly lower than that of the second group (of no-maternal anti-DNP) ($p<0.05$ after 2 and 3 weeks of the first immunization and after 1 week of the second immunization (Fig. 5). This result indicates that the chick would not respond against DNP as in case of chicks with no-maternal antibodies, at the periods in which the almost completely decreased levels of maternal anti-DNP antibodies even if the chick received high dose of anti-DNP antibodies at hatch-

Table 1. Percent suppression of maternal anti-DNP antibodies on the production of anti-DNP antibodies in chicks

Chicks were immunized with	Percent suppression of anti-DNP
DNP-KLH	98.9%
DNP-BSA	98.2%

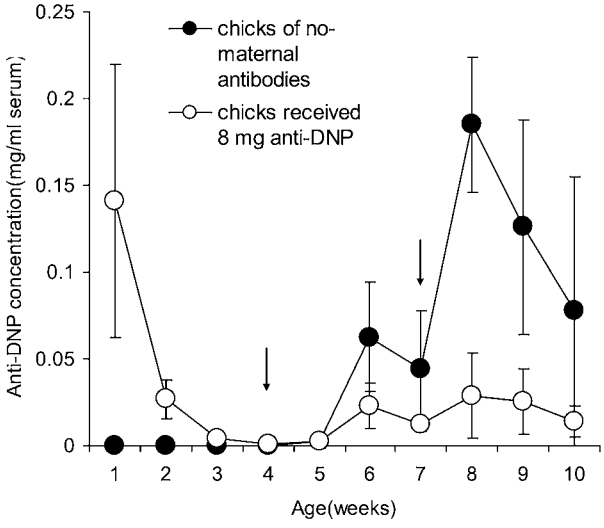

Fig. 5. Immune response of chicks which received 8 mg of maternal anti-DNP antibodies and immunized with DNP-KLH at 4 and 7 weeks of age. Anti-DNP concentrations were measured by using ELISA. The immune response of the chick which received maternal anti-DNP was significantly lower than that of the control group (no-maternal anti-DNP) ($p<0.05$ after two and three weeks of the first immunization and after one week of the second immunization).

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Anti-DNP antibody concentration in the serum samples of 18 non-immunized chicks (derived from immunized laying hens with DNP-KLH) was performed by ELISA every week until 6 weeks after hatching. Different concentrations of anti-DNP antibodies were observed in all chicks, 7 chicks of high maternal anti-DNP antibodies, 5 chicks of moderate maternal anti-DNP antibodies and 6 chicks of low maternal anti-DNP antibodies. The difference was clearly appeared at 1 and 2 weeks of age, but all concentrations almost reached to the minimum by the end of the third week's age. Therefore, we measured the immune response of chicks to DNP-KLH at 4 and 7 weeks of age (in the presence of very low level of their maternal anti-DNP or after withdrawal of these antibodies).

DISCUSSION

The immune system of newly hatched chicks is not yet fully developed, which makes the chicks relatively susceptible to infections. Maternal antibodies provided by the dam compensate this immaturity and provide early age protection against pathogens. Although maternal antibodies protect the newly hatchlings against infectious diseases, they may interfere with earlier vaccine-mediated protection [8, 18, 19, 25]. Vaccination of chicks hatched with high levels of maternal antibodies was ineffective in inducing adequate primary response, and for yet unknown reasons, weakened the secondary immune response of the chicks [18]. To avoid the controversial effect of the maternal antibodies, it was better for chicks left unvaccinated for the first two or three weeks after hatching [8]. The impact of maternal antibodies on the developing immune system of newly hatched chicks remains an important area of investigation. Therefore, the purpose of this study was to investigate and confirm the effect of maternal antibodies on chick immune response. The first experiment revealed that newly hatched chicks that received high amount of maternal anti-DNP antibodies and anti-KLH showed immune suppression when immunized with DNP-KLH at 1 and 4 weeks of age. In contrast newly hatched chicks of low levels of maternal anti-DNP antibodies could show normal immune response especially after the second immunization. Moreover, anti-DNP antibodies production in the case of immune response against DNP-BSA was also suppressed in the presence of maternal anti-DNP and anti-KLH antibodies. This indicates that the antibodies against carrier protein is not involved in suppression on the production of anti-hapten antibodies.

In the second experiment, we confirmed these observations through in ovo injection of fertile eggs derived from non immunized hens with different doses of purified maternal purified anti-DNP antibodies at 16E due to the transfer of maternal antibodies mainly occur during the last three days of incubation as stated before [17]. We found that in ovo injection of 5 or 8 mg anti-DNP antibodies could interfere or suppress the immune response of the newly hatched chicks when immunized with DNP-KLH at 1 and 4 weeks of age, and chicks derived from injected eggs with 1 mg anti-DNP antibodies could show normal immune response but chicks derived from injected eggs with 3 mg anti-DNP antibodies showed weak immune response especially after the second immunization.

After induction of complete immune suppression in newly hatched chicks by injection of 8 mg anti-DNP antibodies via in ovo injection at 16E, we injected small group of chicks with 8 mg anti-DNP antibodies through in ovo injection at the day of hatching, and then immunized them with DNP-KLH at 1 and 4 weeks of age. We found that all chicks showed immune suppression. This finding indicates that transfer of maternal antibodies may occur after hatching. Our result coincided with the report by Good *et al.* [11] who stated that the newly hatched chick does not absorb all its yolk sac antibodies until about 24 hr after hatching.

Although the maternal antibodies present at very low level at 4 weeks age, we surprised that the immune response of chicks received maternal antibodies was significantly lower than that of no-maternal antibodies when they immunized with DNP-KLH at 4 and 7 weeks of age.

In conclusion, high levels of maternal antibodies (5 mg or more/egg yolk) suppress the immune response of newly hatched chicks not only at early period after hatching but also may extend to the period of presence of low level of such antibodies. Maternal antibodies transfer to the newly hatched chicks not only at the last three days before hatching but also after hatching.

It is reported that chicks would not produced anti-TNP antibodies well when chicks were given the maternal antibodies derived from the hen which was highly immunized with TNP hapten, [16]. It might be caused by anti-idiotypic antibodies. However, we indicated here that the suppression was observed by purified anti-hapten antibodies only. This means that this suppression is not caused by anti-idiotypic antibodies. The immunological mechanisms responsible for the suppression of active immune response by passively acquired antibodies remain unclear. It might be occurred by the neutralization of antigen by maternal antibodies, by the stimulation of antigen specific tolerance, or by suppressor cells or regulatory cells. It should mention that this suppression by maternal antibodies was observed in the chick at 7 weeks-old. This age of chick do not have detectable maternal antibodies. The suppression in this period may not take place by the maternal antibodies themselves. This study tested and confirmed the effect of maternal antibodies on chick immune response. The mechanisms of immune suppression will be explored in our next study.

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