Revaccination with Marek’s Disease Vaccines Induces Productive Infection and Superior Immunity

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Received 2 June 2008/Returned for modification 21 August 2008/Accepted 3 November 2008

The most common lymphoproliferative disease in chickens is Marek’s disease (MD), which is caused by the oncogenic herpesvirus Marek’s disease virus (MDV). The emergence of hypervirulent pathotypes of MDV has led to vaccine failures, which have become common and which have resulted in serious economic losses in some countries, and a revaccination strategy has been introduced in practice. The mechanism by which revaccination invokes superior immunity against MD is unknown. After field trials which showed that revaccination provided protection superior to that provided by a single vaccination were performed, experiments were conducted to explore the interaction between revaccinated chickens and MDV. The results showed that the chickens in the revaccination groups experienced two consecutive productive infections but that the chickens in the single-vaccination groups experienced one productive infection, demonstrating that revaccination of viruses caused the chickens to have productive and then latent infections. Revaccination of the virus induced in the chickens a higher and a longer temporary expansion of the CD8+, CD4+, and CD3+ T-lymphocyte subpopulations, stronger peripheral blood lymphocyte proliferative activity; and higher levels of neutralizing antibody than single vaccination. These findings disagree with the postulate that MDV antigens persist, stimulate the immune system, and maintain a high level immunity after vaccination. The suppression of productive infection by maternal antibodies in chickens receiving the primary vaccination and a lower level of productive infection in the revaccination groups challenged with MDV were observed. The information obtained in this study suggests that the productive infection with revaccinated MDV in chickens plays a crucial role in the induction of superior immunity. This finding may be exploited for the development of a novel MD vaccine that results in the persistence of the antigen supply and that maintains a high level of immunity and may also have implications for other viral oncogenic diseases in humans and animals.

Herpesviruses are important pathogens associated with a wide range of diseases in human and animals. Marek’s disease (MD) is an important, ubiquitous, contagious, and oncogenic disease in chickens caused by Marek’s disease virus (MDV), an alphaherpesvirus (12). Apart from its importance in the poultry industry and for animal welfare (5), MD makes a significant contribution to our understanding of herpesvirus-associated oncogenicity due to the many MD lymphomas with a biological nature similar to that of the lymphoid neoplasia associated with human herpesviruses, such as Epstein-Barr virus (17). Studies have suggested that MD is a natural model for lymphomas that overexpress the Hodgkin’s disease antigen, CD30 (7), and MD in small animals provides a well-defined model of general tumorigenesis and virus-induced lymphomagenesis (5, 7, 12, 17, 22). Unlike human diseases caused by herpesviruses, MD is the first lymphoproliferative disease which is effectively controlled and prevented by a vaccination strategy. The introduction of successful MD vaccines derived from either attenuated serotype 1 MDV (MDV1) (16), avirulent MDV1 (29, 30), or MDV2 or MDV3 (herpesvirus of turkeys [HVT]) (24) has been a singular achievement both for agricultural development and as a model system for studying the prevention of cancer in the natural host. Thus, research on the pathogenesis and immunology of MD has significant importance for comparative medicine in humans and animals. A variety of vaccines and vaccination procedures are practically applied for the effective control and prevention of MD in the field (39). However, since the application of global MD vaccination 30 years ago, oncogenic MDV continuously trends toward increasing virulence, and more virulent MDV strains have emerged. Some of these can break through vaccinal protection, such as very virulent MDV (vvMDV) and very-virulent plus MDV (vv+MDV), which seriously threaten the effectiveness of the existing MD vaccines (16, 22, 24, 29, 30, 39). In some countries or areas, MD vaccine failures caused by vvMDV have become common again, causing huge economic losses, and this is becoming a serious problem in poultry. Given the tendency for MDV to increase in virulence and the economic pressures confronting the poultry industry in some parts of the world (39), it is not realistic to await the arrival of more effective MD vaccines superior to the current “gold standard” vaccine, CVI988. Since the end
of the 1980s, in order to deal with this problem by improving the protective efficacy of the vaccine to reduce the incidence of MD, some countries with high frequencies of MD vaccine failures have introduced a revaccination strategy (39). The common regimens of revaccination are priming of 1-day-old chickens and boosting them at either day 7 or day 14. Statistically, revaccination provides better protection than single vaccination in the field (41).

Even though the MD revaccination strategy has become common practice in some regions of countries with serious problems with MD, the mechanisms by which superior immunity is induced by revaccination have not been explored through the use of laboratory experiments. We generated a hypothesis on the mechanisms by which revaccination against MD provides enhanced immunity. The hypothesis consists of three main points, including factors related to both the vaccine virus and the immunized host: (i) chickens revaccinated with MDV experience a productive infection and then latent infection, which occur following a single MD vaccination or natural MDV infection (5, 7, 12, 17, 22); (ii) the MDV antigens produced during a productive infection caused by revaccinated viruses not only stimulate primary immune responses but also activate the immune memory established by the primary vaccination, resulting in a stronger specific immunity; and (iii) the immune system of chicks 7 days of age or older is more competent than that of 18-day-old embryos or 1-day-old chicks. We postulated that a productive infection after revaccination of MDV occurs and is crucial for improving immunity. With the competent immune system plus the existence of immune memory, the antigens produced during a productive infection resulting from revaccinated MDV are able to stimulate chickens to generate superior specific immunity against the vigorous oncogenicity of vvMDV or vv+MDV. This study was designed to determine (i) whether or not revaccinated viruses infect chicks in a manner similar to that of viruses given by single vaccination or natural MDV infection, (ii) how the host birds respond to revaccinated viruses following the establishment of specific immunity by primary immunization, and (iii) if revaccination induces superior immunity against MD. Elucidation of these points above will explain scientifically the phenomenon observed and will also enrich our knowledge of the immunology of MD and viral oncology.

**MATERIALS AND METHODS**

**Chickens, viruses, vaccines, and antibodies.** One-day-old specific-pathogen-free (SPF) white leghorn chickens free of anti-MDV maternal antibodies (Ab−) and commercial Wolf Mountain chickens with anti-MDV maternal antibody (Ab+) were supplied by Nanjing Biopharmaceutic Ltd.; and a bivalent vaccine, Fc126CVI988/Rispens (MDV1) and cell-free and cell-associated Fc126 (MDV3) were provided by Nan tong Wolf Mountain Breeder Farm, Nantong, China, respectively. MD vaccine stocks not only stimulate primary immune responses but also activate the immune memory established by the primary vaccination, resulting in a stronger specific immunity; and (iii) the immune system of chicks 7 days of age or older is more competent than that of 18-day-old embryos or 1-day-old chicks. We postulated that a productive infection after revaccination of MDV occurs and is crucial for improving immunity. With the competent immune system plus the existence of immune memory, the antigens produced during a productive infection resulting from revaccinated MDV are able to stimulate chickens to generate superior specific immunity against the vigorous oncogenicity of vvMDV or vv+MDV. This study was designed to determine (i) whether or not revaccinated viruses infect chicks in a manner similar to that of viruses given by single vaccination or natural MDV infection, (ii) how the host birds respond to revaccinated viruses following the establishment of specific immunity by primary immunization, and (iii) if revaccination induces superior immunity against MD. Elucidation of these points above will explain scientifically the phenomenon observed and will also enrich our knowledge of the immunology of MD and viral oncology.

**PREPARATION OF VIRUS STOCKS.** Monolayers of primary chicken embryo fibroblasts (CEFs) and monolayers of duck embryo fibroblasts (DEFs) were prepared from 9-day-old SPF chick embryos and 13-day-old duck embryos, respectively. RB1B was propagated in DEFs for 4 to 5 days and then harvested and titrated in DEF monolayers by counting the numbers of PFU. MDV strains Fc126, CVI988, and Z4 were titrated by using CEF monolayers.

**Immunization and challenge.** The regimens used for the grouping, immunization, and challenge of the chickens are shown in Table 1. One-day-old SPF white leghorn chickens or commercial Wolf Mountain chickens were randomly divided into 12 groups. Each group contained 15 chickens and was immunized with different MD vaccines at 1 or 7 days of age, and some groups were boosted at 7 days of age by subcutaneous injection. Each group of SPF chickens was hosted in a separate isolator, and commercial chickens were fed in separate rooms in the experimental animal houses of the Laboratory of Animal Infectious Diseases of the Agricultural Ministry of China, Yangzhou University, Yangzhou, China. Experimental chickens were challenged with vvMDV strain RB1B at 25 days of age by intra-abdominal injection.

**Experimental farm.** One small local breeding farm (Local Chinese Breeder Development Ltd., Yangchen, China) that had had a serious problem with MD for several years was chosen for use in the field trials of MD revaccination. Four flocks were fed in four houses, and each house hosted 2,100 Three Yellows chickens that were highly susceptible to MD. These chickens were vaccinated at 1 day of age or were revaccinated at 7 days of age with either cell-associated Fc126 or CVI988. During the experiments, no attempts were made to change the procedure for cleaning and disinfection. The experimental design is shown in Table 2. Thirty-six unvaccinated chickens (six chickens per cage) were placed with vaccinated chickens in each house as controls, and the rate of mortality from MD was recorded for 6 months.

**Sampling and flow cytometry analysis.** Blood samples were randomly collected from five chickens in each group by venipuncture of the jugular vein or wing with heparinized syringes. The isolation of peripheral blood lymphocytes (PBLs) was performed by the use of Ficoll-Paque separation. The PBLs were analyzed by flow cytometry for the expression of MD antigens and for the quantification of the CD3, CD4, and CD8 T-lymphocyte subpopulations (6). Briefly, the PBLs were washed with phosphate-buffered saline with 2% bovine serum albumin and 0.02% sodium azide (PBA). Immunostaining was done on ice in 96-well U-bottom plates (105 cells/well). The washed cell pellets were resuspended in 50 μl primary antibody and were then incubated at room temperature for 30 min. After the cells were washed twice with cold PBA, they were resuspended in 25 μl fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Sigma, Shanghai, China) and incubated on ice for 20 min in the dark. Finally, the cells were washed twice and resuspended in 300 μl of PBA for flow cytometric analysis.

**Lymphocyte proliferation assay.** The PBLs were cultured in triplicate in 100 μl RPMI 1640 supplemented with 10% fetal calf serum, 5 μg/ml concanavalin A
(mitogen concanavalin A; Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin in 96-well flat-bottom plates (8 × 10^5 PBLs/well; 96-well) at 37°C in a 5% CO₂ incubator for 56 h; 10 μl of 5 mg/ml freshly prepared 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in phosphate-buffered saline was added; and the mixture was incubated for 4 h. After the supernatant was discarded, 100 μl of formazan (Sigma) in dimethyl sulfoxide was added, and the plates were incubated at room temperature for 20 min. The supernatant was harvested for measurement of the optical density at 570 nm value on an enzyme-linked immunosorbent assay microreader.

Neutralization test. Blood samples were randomly collected from five chickens from each group by venipuncture at weekly intervals until the termination of the experiment (7 weeks postinfection). Sera isolated from chickens of the same group were pooled for use in the neutralization assays. Cell-free HVT Fc126 was used as the test virus and was diluted to 1,000 PFU/ml in serum-free M199-F10 medium. Serum samples were prepared in triplicate by a twofold serial dilution in serum-free M199-F10 medium. Diluted serum samples (50 μl) and virus dilutions (50 μl) were mixed and incubated at 37°C for 1 h, and then the mixtures were loaded onto the 80% confluent CEF monolayers in 24-well plates and the plates were incubated at 37°C for 1 h. The plates were then incubated at 37°C for 24 h after replacement of the mixtures with maintenance medium (M199-F10 medium with 2% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin). The medium was replaced with 0.5% low-melting-temperature agarose in maintenance medium, and then the plates were incubated for 4 to 5 days. The positive neutralization was set at a serum dilution of at least 1:8, which caused 50% plaque reduction, and the endpoint of the serum dilution was considered the dilution that caused 50% plaque reduction.

RESULTS

Better protection against MD by revaccination in field trials. The rate of mortality from MD on the experimental farm had ranged from 11.7 to 18.5% in different houses in the year before this study, when vaccination with cell-associated Fc126 was applied. Chickens from four flocks with 2,064 chickens each were immunized with the Fc126 or CVI988 MD vaccine at 1 day of age or were boosted at 7 days of age. Thirty-six unvaccinated chickens were housed with each vaccinated flock to indicate the degree of contamination and the virulence of the field MDV strains on the farm. The rates of mortality for each unvaccinated group ranged from 33.3 to 36.1% in the different experimental houses, indicating that the rate of contamination with pathogenic MDV was heavy during the trials. The results of the trials showed that revaccination significantly reduced the rate of mortality from MD by 9.7% to 7.14% (P < 0.005), and revaccination with CVI988 gave the best protection (P < 0.005), with rates of mortality from MD of 1.9% among chickens revaccinated with CVI988 and 6.1% among the chickens in the flock that received a single vaccination (Table 2).

Dynamics of productive infection in chickens caused by revaccinated MDV. Blood samples were collected from immunized chickens at an interval of 3 to 4 days after they were either primed or boosted with one of the MD vaccines. To test for productive infection caused by vaccine viruses in chickens, MDV antigen-positive PBLs were measured by flow cytometry analysis to indicate a productive infection. In experiments with SPF white leghorn chickens (Ab⁻), flow cytometry analysis showed that the vaccines caused a productive infection phase that reached a peak at 7 days postvaccination (dpv), and the productive infection lasted about 1 week. In revaccinated chickens, there were two separate peaks with an interval of 1 week, which equaled the interval between priming and boosting, and the second peak declined to the levels for the control group in a week, indicating that these two productive infections were caused by primed and boosted viruses, respectively, and that the infections then entered latency (Fig. 1A to C). Comparison of the two phases of productive infection in the revaccinated SPF chickens showed that the levels of the two peaks were similar, suggesting that the specific immunity to MD induced by priming with the vaccine was weak at the age (7 days) of revaccination and thus had no significant effect on the productive infection caused by revaccinated viruses.

Analysis of the dynamics of productive infection caused by revaccinated viruses in Wolf Mountain chickens (Ab⁺) was also performed. The experimental Wolf Mountain chickens were from the farm that was heavily contained with pathogenic MDV and that used the cell-associated Fc126 vaccine. In general, similar patterns in the dynamics of productive infection caused by MDV from either priming or boosting vaccines were observed. Revaccination resulted in an independent productive infection phase that reached a peak at 7 days after revaccination and that lasted for 1 week, and then the infection entered a latent stage (Fig. 1D to F). Comparison of the two productive infection phases in revaccinated Wolf Mountain chickens showed that the first peak was lower than the second peak; e.g., in the group revaccinated with CVI988, the proportions of MDV1-positive PBLs were 5.16% ± 1.71% and 7.84% ± 3.5% for the first and second peaks, respectively; in the group revaccinated with the cell-free Fc126 vaccine, the proportions of MDV3-positive PBLs were 5.66% ± 2.31% and 7.14% + 2.9% for the first and second peaks, respectively; and in the group revaccinated with the Z4 + Fc126 vaccine, the difference in the proportions of MDV2-positive PBLs between these two phases of productive infection was smaller than the differences in the proportions of MDV1- and MDV3-positive PBLs (6.08% ± 1.92% for the first peak and 6.84% ± 2.5% for the second peak), indicating that the productive infection caused by priming with MDV was inhibited by anti-MDV maternal antibodies, while the productive infection caused by revaccination with MDV was not significantly affected, that

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**TABLE 2. Results of field trials of MD revaccination**

| House | No. of chickens | Vaccination/dose (PFU) | Revaccination/dose (PFU) | % with MD |
|-------|-----------------|------------------------|--------------------------|----------|
| A     | 2,064           | CVI988/2,000           | None                     | 6.1      |
|       | 36              | None                   | None                     | 33.3 (12/36) |
| B     | 2,064           | CVI988/2,000           | None                     | 1.9      |
|       | 36              | None                   | CVI988/2,000             | 31.0 (11/36) |
| C     | 2,064           | Fc126/2,000            | None                     | 9.7      |
|       | 36              | None                   | Fc126/2,000              | 36.1 (13/36) |
| D     | 2,064           | Fc126/2,000            | None                     | 3.4      |
|       | 36              | None                   | Fc126/2,000              | 33.3 (12/36) |

*One small local breeding farm that had had a serious MD problem for several years was chosen for use in the field trials of MD revaccination. The farm bred Three Yellows chickens that were highly susceptible to MD. On this farm, the rate of mortality from MD ranged from 11.7 to 18.5% in different houses in the year before the cell-associated Fc126 vaccination was applied. Four flocks fed in four houses were vaccinated at 1 day of age or were revaccinated at 7 days of age with either cell-associated Fc126 or CVI988. As controls, groups of unvaccinated chickens were placed with the vaccinated chickens in each house, and the rate of mortality from MD was recorded for 6 months.

* The numbers of chickens at the start of the trial.

* The values in parentheses are the number of chickens with MD/total number of chickens in the group.
homologous maternal antibodies gave higher-level inhibition of MDV infection than heterologous antibodies, and that the maternal antibody caused significant inhibition of infection by the cell-free Fc126 vaccine. These results imply that a large amount of maternal antibodies was consumed by neutralization during the primary productive infection; thus, the secondary productive infection caused by revaccination was not significantly affected.

Inhibition of productive replication after vvMDV challenge by revaccination. Immunized SPF chickens were challenged with vvMDV strain RB1B at 25 days of age. Four days later, PBLs were isolated at intervals of 3 or 4 days for analysis of the dynamics of the MDV challenge. The results showed that RB1B caused double phases of cytolytic infection, reaching its first peak at 7 days after challenge and its second peak at 17 days after challenge, and the levels of vvMDV infection in the immunized groups were lower than those in the unimmunized chickens (Fig. 2). Comparison of the RB1B cytolytic infection in immunized chickens showed that the levels of the first peaks of infection in groups receiving single or double vaccinations were similar, but the levels of the second peaks of RB1B cytolytic infection in the revaccinated groups were lower than those in the groups receiving a single vaccination; e.g., the proportions of MDV1-positive PBLs were 4.89% ±
1.2% and 4.63% ± 0.85% in groups receiving a single vaccination with CVI988 at 1 day and 7 days of age, respectively, but 3.9% ± 1.1% in the revaccinated group. Among all revaccinated groups, the peak levels of RB1B cytolytic infection in the groups receiving homologous revaccinations with CVI988 (3.9% ± 1.1%) and Fc126 + Z4 (4.2% ± 1.3%) were similar and were lower than the peak level in the group revaccinated with Fc126 (4.9% ± 0.85%); heterologous revaccination with Fc126 and CVI988 (4.1% ± 1.85%) or CVI988 and Fc126 (4.3% ± 1.2%) gave similar results, indicating that revaccination gave moderately higher levels of inhibition of productive replication of oncogenic MDV than the single vaccination (Fig. 2). Similar results were obtained in repeat experiments with Wolf Mountain chickens, but the peak levels of RB1B infection in unvaccinated Wolf Mountain chickens were much higher (14.1% ± 2.9% for the first peak and 12.62% ± 2.6% for the second peak) than those in unvaccinated SPF chickens (8.09% ± 3.2% for the first peak and 9.62% ± 2.5% for the second peak), suggesting that Wolf Mountain chickens are more susceptible to MD than SPF white leghorn chickens.

Induction of longer expansion of T-lymphocyte subpopulations by revaccination. PBLs were isolated from SPF chickens at 7 dpv, and the dynamics of the T-cell subpopulations were analyzed by flow cytometry. The results showed that in the chickens receiving a single vaccination, the CD3 subpopulation started to increase at 7 dpv, reached a peak at 14 dpv, and then declined to the level in unimmunized chickens afterwards (Fig. 3A and B). Compared with the results obtained with a single vaccination, the revaccination significantly extended the period of expansion of the CD3 subpopulation (>2 weeks in the groups receiving revaccinations but 1 week in groups receiving a single vaccination). The dynamics of the patterns of the CD4 (Fig. 3C and D) and the CD8 (Fig. 3E and F) subpopulations were similar to those for the CD3 subpopulation in the groups receiving either a single vaccination or revaccination. In terms of the expansion levels, revaccination moderately increased the levels of CD3 and CD8 T-cell expansion in PBLs but significantly increased the level of CD4 T-cell expansion, e.g., 85% ± 3.5% CD3 T cells in the group receiving a double vaccination with CVI988 and 82% ± 3% in the group receiving a single vaccination with CVI988 at 1 day of age, 28% ± 1.5% CD8 T cells in the group revaccinated with CVI988 and 26% ± 1.5% in the group receiving a single vaccination with CVI988 at 1 day of age, and 61% ± 3.3% CD4 T cells in the group revaccinated with CVI988 but 49% ± 2.5% in the group receiving a single vaccination with CVI988 at 1 day of age. Overall, revaccination resulted in a longer expansion of the T-cell subpopulations than a single vaccination, with CVI988 having a stronger ability to stimulate T-cell expansion than Fc126 (58% ± 2.5% CD4 T cells in the group receiving a double Fc126 vaccination). Similar dynamics of the patterns of these T-cell subpopulations were observed in experiments with Wolf Mountain chickens (data not shown).

Increase in proliferative activity of PBLs by revaccination. After immunization, PBLs were isolated from the chickens and used to test the PBL proliferative activity by the MTT method. The results showed that the PBL proliferative activity was detectable at 7 dpv and the activity reached a peak level at 14 dpv. The period of proliferation of PBLs isolated from the groups receiving a revaccination was longer than that of PBLs...
isolated from chickens receiving a single vaccination, and the levels of PBL proliferative activity in the groups receiving a revaccination were higher than those in the groups receiving a single vaccination (Fig. 4). Among the groups receiving a single vaccination, the levels of PBL proliferation induced by CVI988 and Fc126 + Z4 were similar and were higher than the level induced by Fc126. Among the groups receiving revaccinations with homologous vaccines, Fc126 + Z4 (Fig. 4B) and CVI988 (Fig. 4C) induced higher levels of PBL proliferative activity than Fc126 (Fig. 4A), and revaccination with heterologous vaccines Fc126 and CVI988 also induced higher levels of PBL proliferative activity than a single vaccination with the Fc126 vaccine (Fig. 4D).

**Higher levels of neutralizing antibody in revaccinated chickens.** Virus-neutralizing (VN) antibodies were detectable at 14 dpv, and the levels of neutralization activity continued to increase in all vaccinated groups until the termination of the experiments, when the chickens were 7 weeks old. Compared with the levels of VN antibodies obtained after the chickens received a single vaccine, the levels of VN antibodies increased faster after the chickens were boosted with MD vaccines, and at the end of the experiment, the levels of VN antibodies in the revaccinated groups were 8 log2 units higher than those in the chickens that received a singly vaccination (7 log2 units) (Fig. 5A). The levels of maternal VN antibodies in Wolf Mountain chickens were 5 log2 units at 1 day of age and decreased to 4 log2 units at 14 days of age and undetectable levels (3 log2 units or less) at 21 days of age in the control group (Fig. 5B). The levels of VN antibodies in immunized chickens (Ab) first decreased and then gradually increased from 3 weeks of age.

**FIG. 3.** Flow cytometry analysis of T-lymphocyte subpopulations in PBLs isolated from experimental SPF white leghorn chickens after immunization. The vaccines used and the ages at which the chickens were immunized are shown. 1d, vaccination at 1 day of age; 7d, vaccination at 7 days of age; 1 + 7d, vaccination at 1 day of age and revaccination at 7 days of age; control, no vaccination.
until 5 weeks of age. Comparison of the revaccinated groups either with or without maternal antibodies found that the levels of VN antibodies were similar at the termination of the experiments but that the levels of VN antibodies in Ab/H11001 chickens receiving a single vaccination were lower than those of Ab/H11002 chickens at the termination of the experiments, suggesting that maternal antibodies neutralize some primed vaccine viruses and that revaccination compensates for the virus loss caused by neutralization by providing an extra amount of viral antigens to stimulate or restimulate the host immune system so that the chickens develop stronger humoral immune responses.

**DISCUSSION**

Natural phenomenon may not be reproducible under laboratory conditions. The results of the field trials performed in the study described here correspond to those gained from empirical experience and statistical evidence from the field that revaccination provides greater protection than a single vaccination (41), which has not been consistently proved in individual laboratory trials (39). Unlike the well-controlled conditions in the laboratory, complicated factors in the field...
affect the efficacy of revaccination, such as the variable virulence and early exposure to pathogenic MDV, infections with other pathogens that cause immunosuppression, and interference from other vaccines (39). In the quantitative experiments, the small number of chickens (e.g., 30 to 50) used in the laboratory may be not enough to obtain statistically significant results that allow investigators to reach a valid conclusion. Therefore, not all natural phenomena are reproducible under laboratory conditions. MDV causes a persistent infection in chickens, and virulent MDV sheds from the birds for a long period after infection, which leads people to postulate that MDV antigens persist and maintain a high level immunity after vaccination and, thus, that revaccination is unnecessary. Nevertheless, revaccination is still being applied in some areas or regions with serious problems with MD, and the postulate that vaccinal MDV antigens persist in chickens after vaccination and the mechanism by which revaccination enhances immunity against MD warrant investigation. After evaluation of MD revaccination in field trials on a farm with a serious problem with MD, this study mainly focused on the interaction between vaccinated hosts and MDV to test if revaccinated MDV causes a productive infection and vaccine viral antigens persist in PBLs after immunization and to test whether revaccination induces stronger immune responses than a single vaccination. Clarification of these questions may provide some information of significance to both providing an understanding of the mechanism by which revaccination enhances immunity to MD and guiding the practice of revaccination.

Productive infection caused by revaccination is crucial to superior immunity against MD. In this study we observed that revaccination caused chickens a phase of productive infection like that which occurs after chickens receive a single vaccination. The proportions of MDV antigen-positive PBLs ranged from 6.1% ± 1.5% to 8.0% ± 3.1% in immunized chicks, demonstrating that vaccine viruses efficiently replicate in PBLs during the productive infection phase but replicate less efficiently than pathogenic MDV (9.6% ± 2.5%). It has been reported that in chicks infected with virulent MDV (strain HPRS-16) or vvMDV (strain C12/30), the proportions of pp38-positive splenocytes reach 6.3% ± 2.8% and 10.7% ± 0.7%, respectively, at 6 days postinfection (dpi), suggesting that infection with MDV strains with higher levels of virulence cause higher levels of productive infection (3). Empirically, the rate of isolation of vaccinal or pathogenic viruses from PBLs is quite low, and much less than 1% of the PBLs produce MDV plaques if one plaque is formed from a single MDV-infected PBL. This might be because virus isolation is not as sensitive as flow cytometry analysis or not all viral antigen-positive PBLs can release infectious virions to generate MDV plaques when these PBLs are cultured on CEFs or DEFs in vitro.

Cellular immune response plays crucial roles in protection from herpesvirus infections in general (28, 34). This type of response is especially important in providing protection from infection with MDV due to the strict cell-associated nature of MDV infection (32). In MDV-infected chickens, CD8 cytotoxic T lymphocytes are expanding when the virus enters latency and then significantly decrease to levels similar to those in uninfected chickens (19, 32). CD4 T cells expand to a peak level of 58 to 65% at 16 dpi and then rapidly decrease (50%) in a week; in contrast, the CD4 T-cell subpopulation was found to range from 45 to 50% during the period of the experiments (23). It has been observed that VN antibodies modulate the early stage of MDV infection and that maternal antibodies decrease the rate of mortality, delay the onset and increase the latent period to death, lower the frequency of clinical signs, and reduce the numbers of virus-infected cells (8, 10, 20, 27), indicating that humoral immune responses are also essential for protection from MD. In this study, we observed that revaccination induced a longer period of T-cell expansion and PBL proliferation and moderately increased the levels of T-cell expansion, PBL proliferative activity, and neutralizing antibodies compared with the results obtained after administration of a single vaccination. These could contribute to the improvement of protection against MD, as a greater reduction of the level of productive infection in revaccinated groups challenged with vvMDV was observed. The suppression of primary infection by maternal antibodies was also observed, and this negative effect on vaccination for MD could be reduced by the productive infection caused by revaccinated viruses. Overall, two productive infection phases in the revaccination groups induced higher levels of immune responses than one productive infection phase in the groups receiving a single vaccination, suggesting that the productive infection caused by revaccination plays a crucial role in the induction of superior immunity.

Temporary high-level cellular immunity to MD by transient MDV antigen supply. The life cycle of oncogenic MDV can be divided into four phases (12). Early productive replication occurs between 3 and 7 dpi; this is followed by the onset of latency starting at 6 to 8 dpi. The third and fourth phases are secondary productive replication and transformation, respectively, in susceptible chickens infected with virulent MDV. In chickens infected with avirulent MDV or HVT, only productive replication and latency occur. During the productive infection, vaccine viruses productively replicate as nonenveloped virions and antigens are synthesized, but during latency, MDV stays nonproductive and no viral or tumor-associated antigens are produced (13, 36). Unlike virulent MDV, which can effectively spread horizontally between weeks 3 and 5 after infection, resulting in infection of other chickens or reinfection (4, 14), the horizontal transmission of avirulent strains is very poor or absent (24). Serotype 2 MDV and CVI988 spread horizontally 2 weeks after immunization (30, 31), but neither attenuated MDV nor HVT does so (4, 14), even though viral antigens can be detected for a limited period in the feather follicle epithelium of chickens infected with HVT (15, 18, 31, 40). The CVI988 strain initially spread well by contact, but partial flock vaccination does not work in practice, as the spread of the vaccine strain was very poor and not rapid enough to precede infection with virulent strains from the field (1, 29). By 2 weeks after vaccination, specific immunity to MD has been developed (31, 33), which inhibits reinfection caused by limited cell-free vaccine viruses inhaled from dust or dander (8, 26, 40), resulting in the failure of the reinfection. This study demonstrated only one phase of productive infection in the chickens receiving a single vaccination and two phases of productive infection in revaccinated chickens within 7 weeks after immunization, suggesting that the productive infection caused by vaccine viruses shed from vaccinated chickens is not successful or that reactivation of latent viruses in PBLs does not occur; thus,
there is no persistent antigen supply after vaccine viruses enter latency.

The mechanism by which high-level immunity to MD is maintained for a long period is not known. In the field, after vaccine viruses enter latency, it is likely that the major antigen supply for the maintenance of high-level immunity is not vaccination but is infection with wild MDV strains because the wild-type strains have a greater ability than vaccine viruses to induce productive infection and spread horizontally. Chickens infected with wild-type strains shed viruses as early as 14 dpi and effectively spread horizontally between weeks 3 and 5 after infection, resulting in consecutive infection or reinfec-
tion, which presents antigens that prime and boost the immune system (39). Interestingly, it has been reported that challenge with virulent MDV strains markedly increases the level of shedding of vaccine viruses, which may be helpful for the regular MDV antigen supply (21). Therefore, infection with a field MDV strain is beneficial to the maintenance of high-level immunity, but it may cause a serious MD problem if the in-
fec tion of hypervirulent MDV occurs at an age at which chick-
ens are susceptible to MD and prior to the establishment of full vaccinal immunity. It has been suggested that long-term im-
mediate immunity is likely to require periodic stimulation of the immune system by cells that express MDV antigens (2). Re-
cently, it has been reported that the maintenance of high levels of CD8 T cells specific for persistent viruses requires regular contact with viral antigens (37, 38). The experiments per-
formed for this study showed that vaccination induced the temporary expansion of either CD4 or CD8 subpopulations and PBL proliferative activity, and revaccination made the expansion and proliferation longer but not persistent. These findings are consistent with the finding that the maintenance of high levels of CD8 T cells specific for persistent infections requires regular contact with antigens (37, 38). A single vacci-
nation induces a single phase of productive infection in chick-
ens, and revaccination induces an extra phase of productive infection, indicating that there is an interruption of or a gap in the antigen supply after primed vaccine viruses enter latency and prior to infection with field MDV strains (ages 1 to 3 weeks). This antigen supply interruption or gap makes the hosts stay at a quiet stage of immunity when they are still very suscepti-
able to MD. Revaccination fills the antigen supply gap between priming vaccination and wild-type MDV infection and so induces superior immunity against MD. Therefore, the opti-
um age for revaccination is 1 to 2 weeks. The current views that MDV antigens persist, regularly stimulate the immune system, and maintain a high level of immunity against MD after vaccination need further experimental studies and a re-
evaluation of the revaccination strategy on farms with a serious problem with MD.

Generation of stronger immunity by a competent immune system with revaccination. Immunocompetence is important in the pathogenesis of MD, as chickens with immunologic incompetence fail to enter a period of latent infection with MDV; thus, the continuation of MDV cytoplastic infection induces even more destruction to the immune system and further immunosuppression (9). Resistance to MD is ac-
quired gradually and parallels the increase in the level of immune competence (12). Therefore, chickens immunized with an MD vaccine develop stronger immunity at 7 days of age than 18-day-old embryos or 1-day-old chickens. This has been demonstrated by Spencer et al. (35), who showed that vaccination for MD at 23 days of age provides better protec-
tion than vaccination at 2 days of age. Because of the age-
dependent resistance to MD (11), the earlier that the induction of specific immunity is, the more protection against MD that there is. Early exposure to pathogenic MDV is undoubtedly one of the most important causes of excessive MD in vacci-
nated chickens, since up to 7 days is required to develop de-
tectable immunity (40). Virus exposure in the field usually occurs very soon after the hatching or placement of chickens, and the shorter that the interval between vaccination and ex-
posure to pathogenic MDV is, the poorer that the level of protection is (25). Given these points, revaccination should be performed after the establishment of immune memory after primary vaccination and prior to virulent MDV infection, e.g., at 1 to 2 weeks of age.

In conclusion, the most important findings to emerge from the present study are that chickens revaccinated with an MD vaccine experience productive infection, vaccine viral antigens are not persistent in chickens after vaccine viruses enter latency, and the antigen supply produced by revaccination can invoke superior immunity against MD at the age when chick-
ens are still very susceptible to MD. The information produced in this study enriches the current theory on the immunology of MD, provides a scientific basis for revaccination for MD, is useful for guiding revaccination practices, and will be valuable in the development of a new generation of vaccines capable of providing a consistent supply of MDV antigens. The information may also have significance in both preventive viral oncol-
ogy and comparative medicine.

ACKNOWLEDGMENTS

We thank the staff working in Venugopal Nair’s Laboratory of Viral Oncogenesis at the Institute for Animal Health, Compton, United Kingdom, for their technical support and discussions. We also thank Hugh Field of the Department of Veterinary Medicine, University of Cambridge, Cambridge, United Kingdom, and Wayne Kimpton of the Faculty of Veterinary Science, University of Melbourne, Melbourne, Australia, for their valuable discussions. We appreciate Shengxi Li of the Department of Public Health, University of Cambridge, for statist-
ic analyses.

This work was supported by a grant from the Wellcome Trust (United Kingdom) (grant GR061948-1RDA) and a grant from the Natural Science Foundation of China (grant 30270983) to Changxin Wu.

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