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Effects of Gallid Herpesvirus 2 Marek's Disease Challenge Virus and Attenuated Vaccine Virus CVI988/Rispens on Immune Adhesion of Erythrocytes of Chickens

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Abstract: This study was designed to investigate the influence of Gallid herpesvirus 2 Marek's Disease challenge virus and different inoculated routes of attenuated vaccine virus CVI988/Rispens on immune adhesion of erythrocytes in chickens. One day old chicks were randomly divided into a control group (C), an experimental group challenged with Jing-1 challenge strain (V), a second and third experimental group individually injected intra-abdominally (IA) or subcutaneously (SC) with vaccine strain, CVI988/Rispens. The erythrocyte immune adhesion was evaluated with yeast rosette-forming tests of red blood cell complement receptor type 1 (RBC-CR1) and red blood cell immune complex (RBC-IC). It was found that chickens from group V had fewer erythrocytes. During this time, the rate of RBC-IC rosette formation was higher in group V except on days 3 and 20. While the rate of RBC-CR1 rosette formation was lower in group V except on the 26th day when the number of RBC-CR1 rosettes was extremely higher. The rate of RBC-CR1 rosette formation and RBCs count was higher in group IA than that of SC. The results suggest that challenge virus lead to immune suppression, a decline in the immune adhesion of RBCs followed by a rebound during the later stages of infection; effects on RBC immunity were increased after inoculate attenuated vaccines strains; IA vaccination route may enhance the immune adhesion of RBCs comparing with SC route; and an improvement in the RBC immunity adhesion during the later stages of infection may have resulted from a compensatory immune response to immune organ atrophy and the decrease of RBCs.

Key words: Gallid herpesvirus 2 Marek's disease challenge virus, attenuated vaccine virus CVI988/Rispens, erythrocyte, RBC-CR1 rosette, RBC-IC rosette

INTRODUCTION

Marek's disease is a common, malignant disease of chickens. It is caused by the Marek's Disease Virus (MDV), a member of the herpes virus family (Osterrieder *et al.*, 2004). The disease is characterized by lymphocyte hyperplasia, infiltration of mononuclear cells in the splanchnic organs, peripheral nerves, muscles, skin, gonads and iris and coexistent tumors. This highly contagious viral disease which was first reported in 1907, accounts for substantial growth defects and deaths in chickens (Marek, 1907; Biggs, 2001), resulting in serious economic losses to the poultry industry (Morrow *et al.*, 2004).

Siegel *et al.* (1981) put forth the concept of "the red cell immune system". After more than two decades of research, it has become increasingly recognized that the immune functions of Red Blood Cells (RBCs) play a role in the functioning of the whole immune system (Paccand *et al.*, 1990). RBCs are involved in the identification, adhesion, concentration and killing of pathogens; the removal of circulating immune complexes and immune regulation, especially in anti-tumor immune response (Hess *et al.*, 2003).

Some studies also have been carried out on the animals RBC immune system (Jiang *et al.*, 2010).

Erythrocytes in chicken are nucleated throughout the entire life cycle and contain organelles in their cytoplasm which generally characterized as ellipsoid in shape and red color due to contain the most abundant hemoglobins. The major function of these cells is gas exchange, at the meantime, other functions containing interaction with the immune system have been attributed to RBCs (Morera *et al.*, 2011). Given that Marek's disease is the first tumor disease in chicken that can be prevented by vaccination, it can serve as a model for research into the genesis, development and immune response of cancer. In the past, several studies have addressed the adhesion activity of RBCs in response to subcutaneous and *in ovo* inoculation of MD's vaccine in chickens and the immune effect of *in ovo* inoculation of MD's vaccine was good (Bao *et al.*, 2000; Gimeno *et al.*, 2008). Since embryonic inoculation requires complex operations and has a low inoculated efficiency, it is necessary to study the effects of other route of inoculation in chickens. The purpose of our study, therefore, was to explore the effects of virulent MDV as well as attenuated vaccine virus CVI988/Rispens through by intra-abdominal and subcutaneous injection on the RBC immune function of SPF chickens. Our aim was to study of effects on RBC immunity of a MDV

challenge strain and of attenuated vaccines strains, CVI988/Rispens, the role of the vaccine route on RBC immunity and correlation between RBC immunity function and clinical and lesional observations.

MATERIALS AND METHODS

One hundred and twenty eight male Hy-Line brown chicks were purchased from the First Breeding Bird House in Shanxi when they were one day old.

Jing-1 challenge strain (isolated in 1974, LD50 3.48, 38 passages) was purchased from the Institute of Animal Husbandry and Veterinary Science, Beijing Academy of Agriculture and Forestry Science and stored in liquid nitrogen (Lot: 200806). The CVI988/Rispens vaccine samples (6000 PFU/feather) were supplied by Merial Canada Inc. (Baie D'Urfe, Quebec, Canada).

Grouping and inoculation: The 128 one-day-old chicks were randomly divided into four groups, 32 chicks to a group. In the virus-challenged group (V), each chick was injected with 0.2 mL of a solution obtained from diluting the Jing-1 challenge strain virus with sterile Hanks solution. In the group C, each chick was given a mock intra-abdominal injection of 0.2 mL of sterile Hanks solution. In the group SC, each chick received 0.2 mL dilution of CVI988/Rispens inoculated subcutaneously into its neck. In the group IA, each chick was inoculated intra-abdominally with 0.2 mL dilution of CVI988/Rispens. All chicks were kept in accordance with normal breeding conditions and the incidence of MD was observed. On day 3, 6, 10, 15, 20, 26, 35 and 45 after inoculation, four chicks from each group were sampled for blood from vein and then were weighed, necropsied when they were sacrificed by cervical dislocation after ether anaesthesia. All procedures were performed according to protocols approved by the Biological Studies Animal Care and Use Committee of Shanxi Province, China.

RBC count: The RBC count in all specimens was determined using an ABX Pentra 60 hematology analyzer (ABX Diagnostics, Irvine, CA).

RBC-CR1 and RBC-IC rosette forming tests: The yeast were washed with 0,1M phosphate-buffered saline (PBS) pH7.4 three times, boiled for 20 mins in a water bath, mixed well and adjusted to a concentration of 2×10^8 /mL. The heparinized blood samples were washed with PBS three times and centrifuged at 2000 r/min for 3 to 5 mins. The cell pellets were resuspended with PBS and prepared to a concentration of 1.25×10^7 /mL. The reagents added and procedures are listed in Table 1. The rates of RBC-CR1 and RBC-IC rosette formation were determined by the previously published method (Guo, 1993).

The binding of erythrocytes to yeast cells were observed under an optical microscope. All smears were stained

Table 1: Mixture of erythrocyte rosette reaction

| | | | | | | | | | | | |
|----------------|--------------------------|-------|-------------------------|-----------------------|-------|-----|-------------|--------|-------------------|-------|---|
| RBC suspension | 1.25x10 ⁷ /ml | 50 µL | Yeast fungus suspension | 2x10 ⁸ /ml | 25 µL | PBS | 0.1M, pH7.4 | 100 µL | 2% Glutaraldehyde | 25 µL | respectively mixed, smeared and stained |
| RBC-CR1 test | 50 µL | 50 µL | | | | | | 100 µL | | 25 µL | |
| RBC-IC test | 50 µL | 50 µL | | | | | | 100 µL | | 25 µL | |

with Wright's stain and erythrocyte rosettes were counted. RBC was characterized as oval in shape and red color in cytoplasm and yeast fungus were blue. That of two or more yeast fungus adhered around the RBC were called erythrocyte rosettes. Two hundreds RBC were counted continuously from each slide and then the rate of erythrocyte rosettes were calculated.

Statistical analysis: All data were presented in mean±standard deviation form and analyzed using SPSS 17.0 for analysis of variance (ANOVA). $P<0.05$ was considered a significant difference and $P<0.01$, a highly significant difference.

RESULTS

Evaluation of the growth of chickens: On day 26, chicks from the virus-challenged group exhibited substantial pathological changes that were obvious to the naked eye, including testicular swelling and slight kidney swelling. On day 35, testicular swelling, slight liver swelling, bursa of fabricius atrophy and thymus atrophy or degeneration was observed.

Figure 1 depicts the changes in the mean group weight of the four chicks at each time point. It can be seen that the mean group weight from the challenged group increased at a much slower rate than that of the chicks from the group C and vaccination groups, the difference was extremely significant on day 10, 20, 26, 35 and 45 ($P<0.01$) and the difference was significant on day 3 ($P<0.05$). Furthermore, the difference of the mean group

weight of chicks between group IA and group SC was not significant. The mean group weight in four groups was significantly increased between different time point from day 10 onwards and the growth rates of the birds in each group corresponds to growth curve by regression analysis.

Monitoring of RBC count: Figure 2 shows the chicks' RBC count at different time points. As can be seen from Fig. 2, the mean number of RBCs in four chicks from group V remained consistently lower than that of the chicks from group C except on day 15 and the difference was extremely significant on days 20, 26 and 35 ($P<0.01$). The mean number of RBCs in the group IA was higher than that of the group SC and the difference was on days 15 and 20 ($P<0.05$). Moreover, the mean number of RBCs from group V also remained consistently lower than that of the chicks from groups IA and SC except on day 6 and the difference was significant or extremely significant on days 15, 20, 35 and 45 ($P<0.01$, $P<0.05$).

Alternations of RBC-CR1 and RBC-IC rosette forming rates: In order to investigate the dynamics of the RBC immune function, RBC-CR1 and RBC-IC rosette assays were performed (Fig. 5). The results in Fig. 3 and 4 demonstrate that the rate of RBC-CR1 rosette formation in the challenged group was lower than that in the group C and vaccination groups IA and SC on day 15, 20 and 45 ($P<0.01$, $P<0.05$), except on the 26th day when the

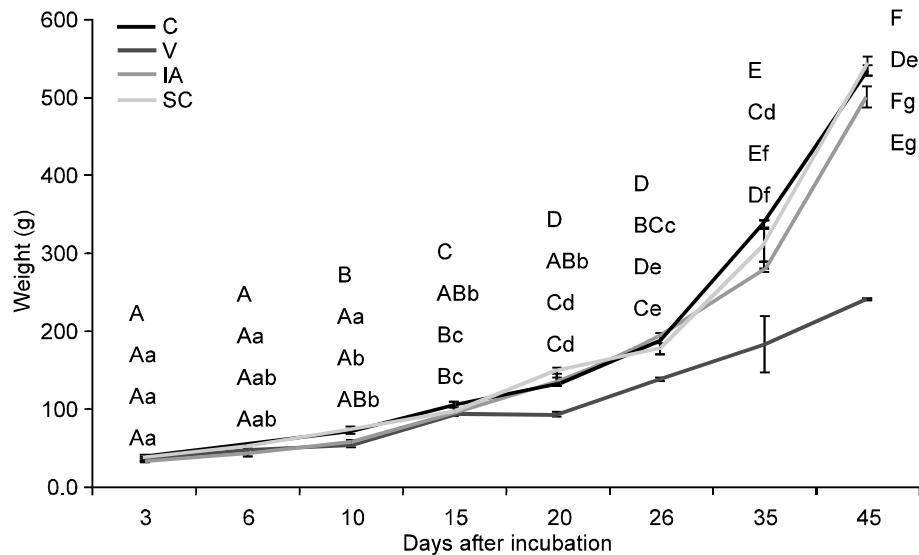


Fig. 1: Monitoring of body weight (g). Diagram with the different capital letters mean extremely significant difference ($P<0.01$) and with the different small letters indicate significant difference ($P<0.05$). The mark order of significant is the same as series. The mean group weight from the challenged group increased at a much slower rate than that of the chicks from the group C and vaccination groups IA and CS, the difference of the mean group weight of chicks between group IA and group SC was not significant and the growth rates of the birds in each group corresponds to growth curve by regression analysis

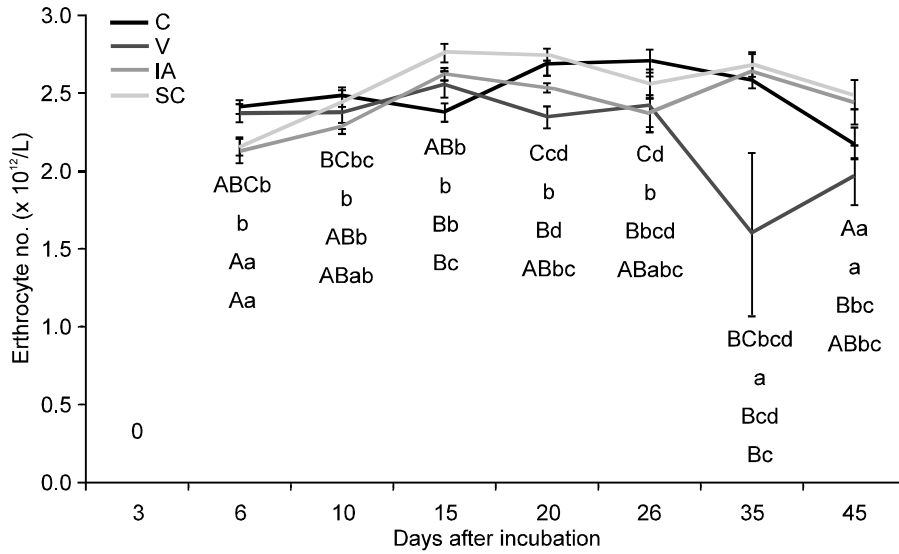


Fig. 2: Monitoring of erythrocyte numbers ($\times 10^{12}/L$). Diagram with the different capital letters mean extremely significant difference ($P < 0.01$) and with the different small letters indicate significant difference ($P < 0.05$). The mark order of significant is the same as series. The mean number of RBCs in four chicks from group V remained consistently lower than that of the chicks from group C except on day 15 and from vaccination groups IA and SC except on day 6 and the mean number of RBCs in the group IA was higher than that of the group SC

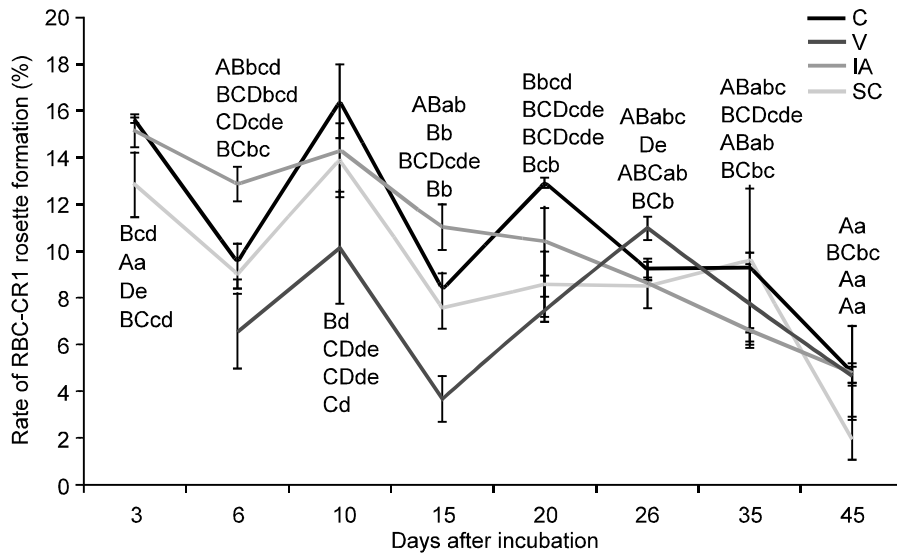


Fig. 3: Monitoring of rate of RBC-CR1 rosette formation in chickens (%). Diagram with the different capital letters mean extremely significant difference ($P < 0.01$) and with the different small letters indicate significant difference ($P < 0.05$). The mark order of significant is the same as series. The rate of RBC-CR1 rosette formation in the challenged group was lower than that in the group C and the vaccination groups on day 15, 20 and 45 ($P < 0.01$, $P < 0.05$), except on the 26th day when the number of RBC-CR1 rosettes was extremely higher in group V than in the group C and the vaccination groups ($P < 0.01$). In addition, the rate of RBC-CR1 rosette formation in group IA remained higher than that in group SC except on day 35

number of RBC-CR1 rosettes was extremely higher in group V than in the group C and vaccination groups ($P < 0.01$). In addition, the rate of RBC-CR1 rosette formation in group IA remained higher than that in group

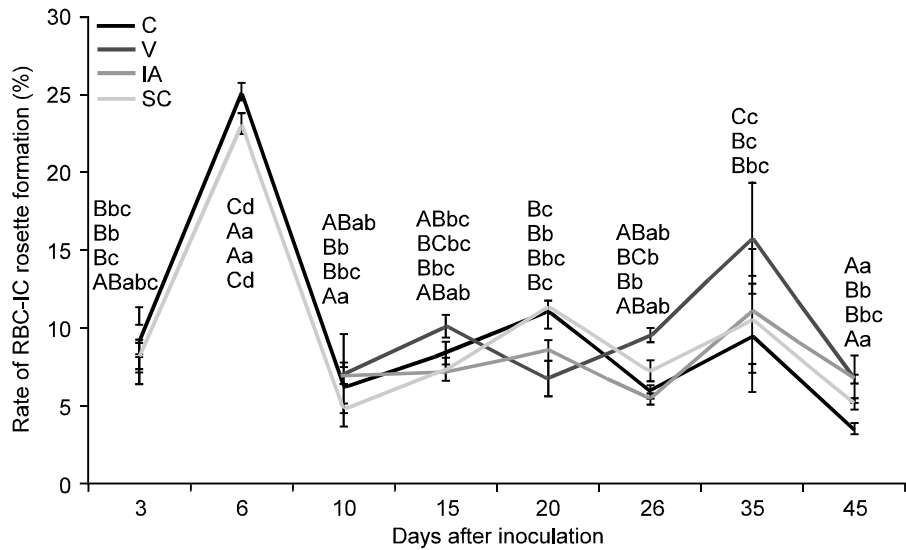


Fig. 4: Monitoring of rate of RBC-IC rosette rosette formation in chickens (%). Diagram with the different capital letters mean extremely significant difference ($P < 0.01$) and with the different small letters indicate significant difference ($P < 0.05$). The mark order of significant is the same as series. The rate of RBC-IC rosette formation in the group V was higher than that in groups C and vaccination groups except on days 3, 6 and 20. There was not statistics significant in the rate of RBC-IC rosette formation between group IA and SC except on days 10 and 45

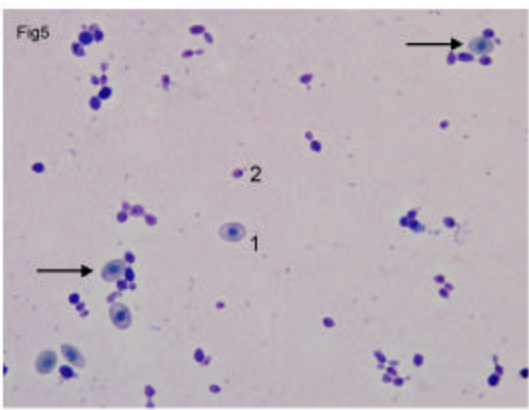


Fig. 5: Erythrocytes binding to yeast cells. Chick erythrocyte: The chick erythrocytes appeared to be ellipsoid - shaped with hyperchromatic nuclei and red distinct cytoplasm. Yeast cells: round, oval - shape or ellipse, blue in color. Erythrocyte rosettes are indicated with arrows (1000x)

SC except on day 35 and on day 3, the difference was highly significant ($P < 0.01$), the difference was significant on days 15 and 20 ($P < 0.05$).

On the other hand, the rate of RBC-IC rosette formation in the group V was higher than that in groups C, IA and SC except on days 3, 6 and 20, the difference was highly significant on days 10 and 45 ($P < 0.01$). There was not

statistics significant in the rate of RBC-IC rosette formation between group IA and SC except on days 10 and 45.

DISCUSSION

In this experiment, the body weight of one-day-old chicks infected with MDV increased much more slowly than that of chicks in the control group and vaccination groups, indicating that Marek's disease affected the growth and development of the chicks. The autopsy of the chicks on the 26th day after infection revealed that pathological changes had occurred mainly in the testes and kidneys. On day 35, the clinical signs became more obvious. Besides the testis, liver and kidneys, pathological changes were also observed in the immune organs, such as bursa of Fabricius atrophy and thymus atrophy or degeneration. In the meantime, compared with group C, IA and SC, the number of RBCs had declined sharply to the lowest level which in accordance with the report, that Gilka and Spencer (1995) have described immune mediated anaemia in chickens infected with highly pathogenic MDV that was attributed to erythrophagocytosis by Kupffer cells in the liver and macrophage-like cells in the spleen. These phenomena suggest that by this time, the disease had resulted in severe immune suppression.

Compared with groups C, IA and SC, the group V showed a higher rate of RBC-IC rosette formation. This occurrence was due to the infection caused by the vMDV which activated the immune system and led to an

increase in the number of complement fragment C3b receptors (C3bRs) on RBCs binding to immune complexes. In the later stages of infection and with the development of Marek's disease, the number of circulating immune complexes *in vivo* continued to increase and to occupy more C3bRs, leading to a significantly higher rate of RBC-IC rosette formation as compared to the others groups.

In the early stages of infection, the rate of RBC-CR1 rosette formation in the challenged group was lower than that in groups C, IA and SC and the immune functions of the RBCs were crippled, findings that were consistent with previous studies (Gao *et al.*, 1999; Bao *et al.*, 2000). But comparing with the previous studies, the test term of this study was long, time points was compact and virus-challenged age was one day-old. Moreover, it is worth noting that in the later phases of infection, the rate of RBC-CR1 rosette formation in the virus-challenged group was close to and, on day 26, even higher than that of groups C, IA and SC ($P < 0.01$), indicating an improvement in the immune function of the RBCs. This may have resulted from a compensatory immune response to immune organ atrophy and the decrease of RBCs, but the actual mechanism has yet to be confirmed and requires further studies to do so. Although the binding of erythrocytes to yeast cells was observed under the microscope during rosette assay, it is still unclear whether the binding was mediated by C3bRs or simply due to direct adhesion.

We also compared the effects of different vaccination route (intra-abdominal and subcutaneous inoculation) on the growth of chicks and the immune function of RBCs. At all the experiment stage, although the mean body weight of the chicks in group IA were lower than that of group SC, the difference was not significant. As far as the rate of rosette formation was concerned, the rate of RBC-CR1 rosette formation in group IA was higher than that in group SC and, on days 3, 15 and 20, the difference was highly significant or significant. Moreover, the number of RBCs in group IA was higher than that in group SC and the difference was significant on days 15 and 20. Taken together, these observations indicated that intra-abdominal vaccination is preferable to subcutaneous inoculation since it showed superior results in reinforcing the immune function of the chicks' RBCs. But intra-abdominal injections need more accuracy to be performed at a large scale. People need to be trained before using this method. This will remain in the domain of research.

In conclusion, our study showed that challenge virus directly affected the growth rate of chicks, leading to immune suppression, a decrease in RBC count and a decline in the immune function of RBCs followed by a rebound during the later stages of infection; effects on

RBC immunity was increased after vaccinating attenuated vaccines strains; intra-abdominal vaccination route may enhance the immune function of RBCs and an improvement in the RBC immunity function during the later stages of infection may have resulted from a compensatory immune response to immune organ atrophy and the decrease of RBCs.

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