ELISPOT Assay for Detection of Antibody Secreting Cells to Infectious Bursal Disease Virus in Chickens

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ABSTRACT The ELISPOT assay was used to enumerate antibody secreting cells (ASC) in spleens of chickens infected with infectious bursal disease virus (IBDV). In the first experiment, chickens were orally challenged with IBDV. Spleens were collected 14 d later and used for ELISPOT assay. The assay detected 16 to 28 IgG ASC per 10^6 splenocytes and 3 to 6 IgM ASC per 10^6 splenocytes. In the second experiment, chickens were vaccinated against IBDV and orally challenged with IBDV 14 d after vaccination. Spleens were collected 7 d postchallenge. The ELISPOT assay detected 18 to 128 IgG ASC per 10^6 splenocytes and 4 to 6 IgM ASC per 10^6 splenocytes. The results indicated that the ELISPOT assay can be used to measure isotype-specific antibody responses to IBDV or other avian pathogens.

(Key words: ELISPOT, antibody secreting cells, infectious bursal disease virus)

INTRODUCTION

The ELISPOT assay can be used to enumerate antibody secreting cells (ASC) in various lymphoid organs. It is useful in analyzing the dynamics of a humoral immune response in a particular region such as gut lamina propria or cecal tonsils. It offers many advantages over conventional hemolytic plaque-forming cell assays for enumerating ASC (Sedgwick and Holt, 1983). It can be used to detect antibodies of different isotypes by employing appropriate secondary antibodies. Although ELISPOT assay is widely used in mammalian species, it has not been utilized in studies of avian humoral immune responses.

Infectious bursal disease (IBD) is an economically important disease in the poultry industry around the world. The causative agent, infectious bursal disease virus (IBDV), predominantly affects the bursa of Fabricius, causing lymphocytolysis and subsequent immunosuppression (Fadly et al., 1976; Becht, 1981). As a result, infected birds are susceptible to other infectious agents and also do not respond well to vaccinations against several diseases such as Marek’s disease and infectious bronchitis (Giambrone et al., 1976; 1977). The purpose of the present study was to analyze the humoral immune response to IBDV by enumerating specific ASC using an ELISPOT assay.

MATERIALS AND METHODS

Experimental Design

Specific-pathogen-free (SPF) chickens used in this study were obtained from SPAFAS as fertile eggs and hatched under appropriate conditions. The chickens were reared in Horsfall isolation units with unlimited access to feed and water. The infected and control chickens were housed in separate rooms under similar environmental conditions.

In Experiment 1, three 2-wk-old SPF chickens were orally challenged with 4 × 10^5 embryo lethal dose 50 (ELD50) of IBDV strain STC. Spleens were collected for ELISPOT assay 14 d after challenge. Two uninoculated chickens were used as controls. In Experiment 2, two groups of three 2-wk-old SPF chickens were used. One group of chicken was orally vaccinated twice with IBD vaccine, Clonvac D-78 at a 1-wk interval. The other group was intraocularly vaccinated twice with Clonvac D-78 vaccine. Chickens were orally challenged with 4 × 10^5 ELD50 of IBDV strain STC 14 d after the second vaccination. The other group was intraocularly vaccinated twice with Clonvac D-78 vaccine. Chickens were orally challenged with 4 × 10^5 ELD50 of IBDV strain STC 14 d after the second vaccination. Spleens were collected for ELISPOT assay 7 d after challenge. Spleens were also collected from two control chickens that were neither vaccinated nor challenged.

Abbreviation Key: ASC = antibody secreting cells; ELD50 = embryo lethal dose 50; HBSS = Hank’s balanced salt solution; IBD = infectious bursal disease; IBDV = infectious bursal disease virus; SPF = Specific-pathogen-free.
Purification of Virus

Infectious bursal disease virus strain STC was propagated in 3-wk-old SPF chickens by oral inoculation of chickens with 4 x 10^5 ELD50 of virus and bursae were collected 3 d postinoculation. Virus was purified by the procedures as described previously (Ture and Saif, 1992). Briefly, bursae infected with IBDV were homogenized in TNE buffer (10 mM Tris HCl, 100 mM NaCl, and 1 mM EDTA, pH 7.9) using a mortar and pestle and subjected to three freeze-thaw cycles to obtain a homogenous suspension. Freon (1, 1,2-trichloro trifluoro ethane) was added to the homogenate and the mixture was further homogenized using a homogenizer.4 The mixture was centrifuged at 400 x g for 20 min at 4 C. The aqueous phase was removed and held on ice. Fresh freon was added to the precipitate and the extraction process was repeated three times. The aqueous phases were pooled and centrifuged at 8,500 x g for 3 h at 4 C to pellet the virus. The pellet was suspended in TNE buffer and centrifuged over 30% sucrose at 120,000 x g for 3 h at 4 C. The precipitate was suspended in TNE buffer and protein concentration was estimated using the BIO-RAD protein assay kit.5

Preparation of Splenocyte Suspension

Spleens aseptically removed from challenged or vaccinated and challenged chickens were placed in ice cold Hank’s balanced salt solution (HBSS, containing 140 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1.1 mM MgCl2, 5.6 mM glucose, and 10 mM HEPES, pH 7.4). They were cut into small pieces and squeezed with a 5-mL syringe plunger to extrude cells. The cell suspension was centrifuged at 250 x g for 10 min at 25 C. The cell pellet was resuspended in 5 mL of ACK lysing buffer (0.15 M NH4Cl, 1 M K2CO3, and 0.01 M EDTA, pH 7.2) and incubated at room temperature for 5 min to lyse the red blood cells. The cell suspension was washed twice with HBSS and resuspended in RPMI-1640 containing 5% fetal bovine serum, 10 mM L-glutamine, 2 x 10^-5 M β-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. The cell density was adjusted to 1 x 10^6 cells per milliliter after determination of cell viability using trypan blue dye exclusion method.

ELISPOT Assay

Purified IBDV virus was used to coat 96-well microtiter plates.7 Purified virus was suspended in carbonate-bicarbonate coating buffer (pH 9.6) at a concentration of 100 μg/mL and 100 μL of the virus suspension was applied to each well. The plates were incubated at 4 C overnight. The plates were washed three times with PBS-Tween containing 140 mM NaCl, 5 mM KCl, 10 mM Na2HPO4, 1 mM KH2PO4 and 0.05% (vol/vol) Tween, pH 7.2 and blocked by incubation with 1% BSA in PBS for 1 h at 37 C. The plates were washed twice with PBS-Tween. Splenocyte suspensions in RPMI-1640 medium were added to the wells (1 x 10^5 cells per well) and the plates were incubated at 37 C, 5% CO2 for 4 h. Splenocyte suspensions obtained from each chicken were tested in quintuplicate wells. Negative control wells contained purified virus and received splenocyte suspensions obtained from un inoculated control chickens. At the end of the incubation, the plates were washed five times with PBS-Tween and affinity purified goat anti-chicken IgM6 (1:500) or goat anti-chicken IgG (1:50) was added. After incubation at 4 C overnight, the plates were washed five times with PBS-Tween, and affinity purified alkaline phosphatase conjugated rabbit anti-goat IgG6 was added at a concentration of 1:40,000 (100 μL per well). The plates were further incubated for 2 h at room temperature and unbound antibodies were washed away using PBS-Tween. For detection of ASC, four parts of 2.3 mM 5-BCP8 (alkaline phosphatase substrate) in AMP buffer (1 mM MgCl2·6H2O, 0.01% Triton X-100 (vol/vol) and 0.1% NaN3 (wt/vol), pH 10.5) was mixed with 1 part of 3% agarose6 and 100 μL of this mixture was added to each well. The plates were left at room temperature for 20 min. The plates were then placed in a humid chamber and incubated at 4 C. The plates were examined for formation of spots using an inverted microscope 16 to 18 h after addition of the substrate mixture.

Statistical Analysis

Differences between mean IgG ASC were compared using a one-way ANOVA (SAS Institute, 1990).

RESULTS

Experiment 1

In Experiment 1, splenocytes obtained from chickens challenged with IBDV strain STC were used for detection of ASC. There were 16 to 28 IgG ASC per 10^6 splenocytes and 3 to 6 IgM ASC per 10^6 splenocytes (Table 1). Positive spots were blue in color, circular or oval in shape and had a homogeneously granular appearance with a dark center. The spots were generally 0.1 to 0.5 mm in diameter. Positive spots were not observed in negative control wells for IgM ASC. Occasionally, one or two false positive spots were seen in a few negative control wells for IgG ASC. These spots lacked the characteristic dark center and granular appearance.

Experiment 2

In Experiment 2, splenocytes obtained from vaccinated chickens challenged with IBDV strain STC were used for
TABLE 1. Number of antibody secreting cells (ASC) per 10⁶ cells in spleens of 2-wk-old chickens orally challenged with 4 × 10⁵ embryo lethal dose 50 (LD₅₀) of infectious bursal disease virus strain STC or vaccinated with Clonvac D-78 and orally challenged with 4 × 10⁵ ELD₅₀ of infectious bursal disease virus strain STC. Each value represents the mean ± SD.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IgG</th>
<th>IgM</th>
</tr>
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<tbody>
<tr>
<td>None (n = 4)</td>
<td>4.75 ± 2.2c</td>
<td>ND¹</td>
</tr>
<tr>
<td>Oral challenge (n = 3)</td>
<td>22.67 ± 6.11b</td>
<td>5.67 ± 2.5</td>
</tr>
<tr>
<td>Oral vaccination + challenge (n = 3)</td>
<td>86.33 ± 39.27a</td>
<td>4.67 ± 1.1</td>
</tr>
<tr>
<td>Intra-ocular vaccination + challenge (n = 3)</td>
<td>45.67 ± 38.77a</td>
<td>3.33 ± 2.3</td>
</tr>
</tbody>
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¹Mean values within a column with no common superscript differ significantly (P < 0.05).

¹Not done.

detection of ASC. There were 81 to 128 IgG ASC and 4 to 6 IgM ASC per 10⁶ splenocytes in chickens orally vaccinated with Clonvac D-78 and challenged with IBDV (Table 1). There were 15 to 88 IgG ASC and 2 to 6 IgM ASC per 10⁶ splenocytes in chickens intraocularly vaccinated with Clonvac D-78 and challenged with IBDV (Table 1).

**DISCUSSION**

An ELISPOT assay was developed and used to detect ASC to IBDV in the present study. The numbers of ASC varied among chickens. This variation may reflect the differences in humoral responses among individual chickens. Differences in the number of infectious virus particles received by each chicken may also account for variation observed between birds. There were more IgG ASC in the spleens of orally vaccinated chickens challenged with IBDV than in those challenged with IBDV alone. This result may suggest an anamnestic immune response. However, the possibility that the age of the birds in the vaccinated group may have contributed to a stronger immune response cannot be ruled out.

The numbers of ASC in the mouse and rat varied from 100 to 400 per 10⁶ splenocytes (Van Loveren et al., 1988, Xu et al., 1991). Such large numbers of ASC were not detected in the experiments described in this report. A possible explanation for this difference could be the parenteral administration of antigens used in the murine species. In addition, IBDV is lymphocytolytic and may cause depletion of lymphocytes resulting in decreased ASC. Chickens used in the present study were orally inoculated and thus might not carry large numbers of ASC in the spleen. Furthermore, particulate antigens such as whole bacterial cells or sheep RBC, may induce a strong humoral immune response that leads to the production of large numbers of ASC in several lymphoid organs.

ELISPOT assays have been used for detection of soluble and particulate antigens in mammalian systems (Franci et al., 1986; Van Loveren et al., 1988; Xu et al., 1991). The ELISPOT assay uses the principle of diffusion in gel ELISA that allows visualization of antigen-antibody reactions (Sedgwick and Holt, 1983). The ELISPOT assay is versatile in that it can be used to detect cells that secrete a particular isotype or allotype. In addition, it can be used to detect lymphokine or cytokine producing cells by employing specific antibodies against these proteins.

To our knowledge such an assay has not been reported for an avian system. ELISPOT assay is invaluable in evaluating the humoral immune responses in terms of ASC. The ELISPOT assay is applicable wherever conventional ELISA would be used. However, it is particularly useful in detecting ASC in the secondary lymphoid organs. For example, it can be used to measure number of ASC in gut lamina propria (Lycke, 1986) and cecal tonsils after oral inoculation of an immunogen. Similarly, it can be used to detect ASC in the Harderian glands of avian species following intraocular inoculation of vaccines or pathogens. Delineation of the source of antibody producing cells may be of value in infections or studies involving comparison of different lymphoid organs in generation of local and systemic antibody responses. The procedure described in this report can be adapted to detect other avian pathogens and also to distinguish the magnitude of antibody responses following local or systemic administration of an immunogen.

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**REFERENCES**


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