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Identification of target cells for Goose parvovirus infection in the immune system organs

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Summary. – Target cells for Goose parvovirus (GPV) in natural infection are still unknown. In this study, immune system organs namely the spleen, bone marrow, thymus, bursa of Fabricius, and blood of experimentally GPV-infected goslings were examined by an immunoassay and flow cytometry for the presence of viral antigen and by a PCR for viral genome. The results indicated that the virus replicated in some cells of the spleen and bone marrow, but not in peripheral blood lymphocytes (PBLs). These data suggested that some cell populations in the spleen and bone marrow were targets for GPV infection. In addition, the immunoassay used for the detection of GPV was found comparable with a PCR in reliability and sensitivity.

Keywords: Derzsy's disease; Goose parvovirus; immunoassay; PCR

Introduction

GPV infection known as Derzsy's disease, goose hepatitis, or goslings plague, characterized by anorexia, prostration, and death within 2 to 5 days, is caused by an important viral pathogen GPV (Derzsy, 1967; Kisary and Derzsy, 1974). The first description of the virus came from China as early as in 1956 and since then, it has been reported from most of the goose and Muscovy farming countries in the world (Gough, 1997).

GPV (the family *Parvoviridae*, the genus *Dependovirus*) can replicate without the aid of a helper virus (Kisary, 1979) and at the nucleotide level is most closely related to the Adeno-associated virus type 2 (Fauquet *et al*., 2004).

Natural target cells for the most of parvoviruses except GPV have been properly defined. The target cells for Human parvovirus B19 (B19) are macrophages, follicular dendritic cells, T and B cells (Brown and Young, 1997; Takahashi *et al*., 1998). Minute virus of mice (immunosuppressive strain) replicates in mouse T lymphocytes and hematopoietic precursors (Gardiner and Tattersall, 1988) and macrophages have been identified as the host cells for Aleutian mink disease virus (Alexandersen *et al*., 1988; Mori *et al*., 1991). Feline panleukopenia virus and Canine adeno-associated parvovirus infect certain cells of the bone marrow, thymus, and PBLs (Truyen and Parrish, 1992). Generally, all of the described parvoviruses showed tropism of immuno-associated cells. However, whether GPV replicates in immunocytes and has a tropism for immune system organ(s) *in vivo* is not clear.

In this study, immune system organs of goslings were examined to detect the target cells for GPV infection by an immunoassay and flow cytometry (FACS) for the presence of viral antigen, and by a PCR for viral genome. The immunoassay used for detection of GPV was evaluated in relation to a PCR.

Materials and Methods

Embryonated eggs and virus. 12-day-old goose embryonated eggs without maternal antibody against GPV were obtained from an experimental animal farm affiliated to Yangzhou University. Virulent GPV strain GPVSYG97-7 was used in this study. It was

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Abbreviations: FACS = flow cytometry; GPV = Goose parvovirus; MAb = monoclonal antibody; PBLs = peripheral blood lymphocytes; p.i. = post infection

a field isolate from a goose farm in the region of Jiangsu province, China, obtained by our laboratory in 1997 (unpublished data). After 30 passages in goose embryonated eggs with the virus inoculation in the allantoic cavity, it was propagated in large amount and stored at -70°C until use. The collected allantoic fluid was titrated and the titer was expressed as 50% embryo infectious dose (EID_{50}).

Infection of animals. 12 goslings four-day-old without any maternal antibody against GPV were kept in the separate isolated cages. The birds were randomly divided into two groups. One group was infected with 10^4 EID_{50} of GPV subcutaneously. The other group was used as a control and the animals were injected with saline. Two non-infected birds were kept together with the infected ones (Table 1).

Samples collection. After 3 days post infection (p.i.) some goslings showed classical symptoms of the GPV infection and died up to 5 days p.i. The survived goslings were sacrificed and the spleen, bone marrow, thymus, bursa of Fabricius were harvested. Heparinized peripheral blood samples were also collected and PBLs were isolated by Ficoll-Paque density centrifugation. The tissues of thymus, bursa of Fabricius, and spleen were grinded into small clumps or single cells and filtered through the filter cloth (100 μm pore size). The resulting cell suspension was centrifuged and the red blood cells were sedimented at the bottom, so it was easy to obtain the cells of upper layer. The bone marrow was clamped out of the bone with tweezers and directly smeared onto the slides for the immunoassay.

PCR assay. Viral DNA was extracted by the method described previously with some modifications (Sirivan *et al*., 1998). To design specific primers for the detection of GPV, 7 complete GPV genome sequences were retrieved from the GenBank (Acc. Nos. AY506547, EU088101, EU088102, EU088102, EU583389, EU583392, U25749) and aligned with biosoftware DNAman (Lynnon BioSoft). A set of two primers, forward primer 5'-GGGTGCCGATGGAGTGGG-3', reverse primer 5'-CAGCCTGTCTAAGTCCTGTGAATG-3' at the position of 3074–3091 nts and 3734–3711 nts, respectively, in the genome of B strain was designed. The amplified fragment was 689 bp long located in the conserved region of VP3 of GPV genomic DNA. Amplification was carried out in the reaction volume of 25 μl with a standard procedure. An aliquot of 5 μl of the PCR product was analyzed in a 1% agarose gel to visualize the amplicons after staining with ethidium bromide. Specificity and reliability of the method was validated previously in our laboratory (unpublished data).

Immunoassay. The cell suspensions were smeared on the poly-L-lysine (Sigma) coated slides, fixed, and permeabilized with methanol supplemented with 0.3% H_2O_2. The cells were blocked using 2% BSA in PBS containing 0.05% Tween 20 (PBST) at 37°C for 1hr. Then the cells were incubated at 37°C for 1 hr with mouse anti-GPV monoclonal antibody (MAb) GPA_1 diluted at 1:1000 in the blocking reagent. After washing with PBST the bound MAb was detected by the incubation for 1hr with HRP-conjugated goat anti-mouse secondary antibody (Boster) diluted at 1:1000 in the blocking reagent (Dong *et al*., 1996). After washing, the cells were stained by 3-amino-9-ethylcarbazole (AEC) substrate and examined under the light microscopy, where GPV-infected cells appeared as red ones.

Flow cytometry assay. Spleen cells in amount of 2×10^6 from the GPV-infected or non-infected goslings were fixed with 2% paraformaldehyde in PBS and permeabilized by 0.1% Triton X-100 (Shanghai Sangon Biological Engineering Technology and Service). Then, the cells resuspended in MAb GPA1 solution (1/1500) were incubated at room temperature for 1 hr, washed twice with PBST, stained with FITC-conjugated goat anti-mouse IgG (Sigma), incubated at room temperature for 1 hr, washed twice and analyzed directly by FACS (FACScalibur, Becton-Dickinson).

Table 1. Detection of the virus in spleen cells of GPV-infected goslings by immunoassay and PCR

Animal No.	Group	Time p.i. (hrs)	Animal status	Immunoassay[1]	PCR[2]
1	Infected	76	Dead	++	+
2		70	Dead	+	+
3		81	Dead	+++	+
4		74	Dead	+	+
5		77	Dead	+	+
6		72	Dead	++	+
7*	Non-infected	120	Symptomatic	+	+
8*		120	Symptomatic	+	+
9		120	Healthy	–	–
10		120	Healthy	–	–
11		120	Healthy	–	–
12		120	Healthy	–	–

*Non-infected animals kept together with the infected ones. [1]Weakly (+), moderately (++), and strongly positive (+++).[2]Positive (+), negative (–).

Results

Virus detection in immune system organs of GPV-infected goslings

Three days p.i. some goslings showed classical symptoms of the GPV infection such as nasal and ocular discharge with head shaking, diarrhea with blood, or they died. To confirm that the symptoms were caused by the virus infection, we used the method of PCR to detect the viral DNA. It could be detected in the collected organs of GPV-infected goslings including spleen, bone marrow, thymus, bursa of Fabricius, and blood. We did not detect the virus in the blood (Fig. 1) and other tissues and organs (not shown in Fig. 1) of non-infected goslings as control birds. It indicated that the birds were infected with GPV successfully and the virus was distributed in all examined immune system organs. Thus, the organs were suitable for further investigation of the natural target cells and evaluation of the suitable immunoassay for virus detection.

Then, the target cells in the immune organs and tissues were investigated with the immunoassay. As a result, the GPV-infected cells were observed in the two immune system organs, specifically bone marrow and spleen, but they

were not observed in the thymus, bursa of Fabricius, and blood (Fig. 2).

To confirm the results of immunoassay that the spleen contained virus-replicating cells, another accurate and reliable method was employed. The spleen cells from goslings either non-infected or GPV-infected were subjected to FACS. The results showed that the spleen of GPV-infected animals contained virus- replicating cells (Fig. 3). Thus, the fact that some natural target cells for the GPV infection were located in the spleen was reliably confirmed.

Comparison of immunoassay with PCR in detecting the virus

Since the spleen contained natural target cells for the GPV infection and it was easy to obtain the large amount of spleen cells, we employed these cells to evaluate the immunoassay for the detection of virus. About 72 hrs p.i. six GPV-infected goslings died and before dying, the classical symptoms of GPV infection were observed. Furthermore, two non-infected birds kept together with the infected ones were still alive after 90 hrs p.i., but they showed the signs of GPV infection. The non-infected birds kept separately did not show any symptom of the virus infection. Six birds from the infected group (Nos. 1–6) showed the positive results in both immunoassay and PCR. Two birds (No. 7 and 8) from the control group, but kept together with the infected ones also showed positive results detected with both methods. Negative results were obtained from the four goslings (Nos. 9–12) of the control group. These results were consistent with the clinical diagnosis and observed symptoms (Table 1). The results of immunoassay completely matched those of PCR analysis. In another aspect, these results further confirmed

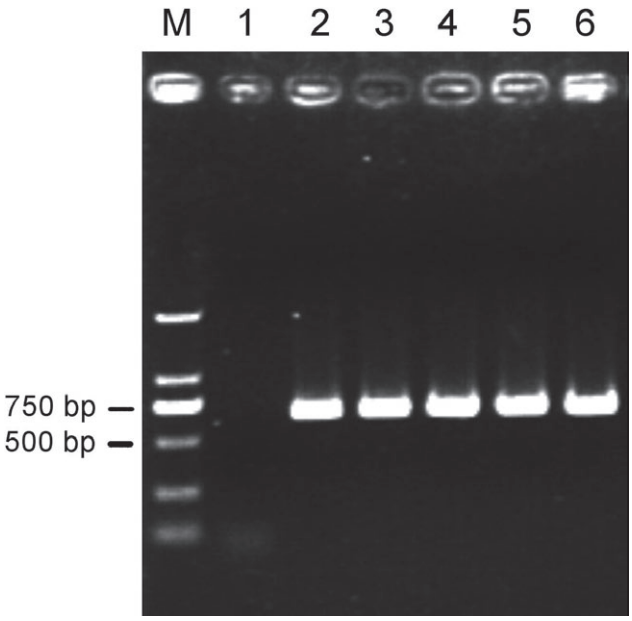

Fig. 1

Detection of the virus in immune system organs of GPV-infected goslings by PCR

DNA size marker (lane M), negative control with the non-infected gosling blood (lane 1), spleen (lane 2), bone marrow (lane 3), thymus (lane 4), bursa of Fabricius (lane 5), and blood (lane 6).

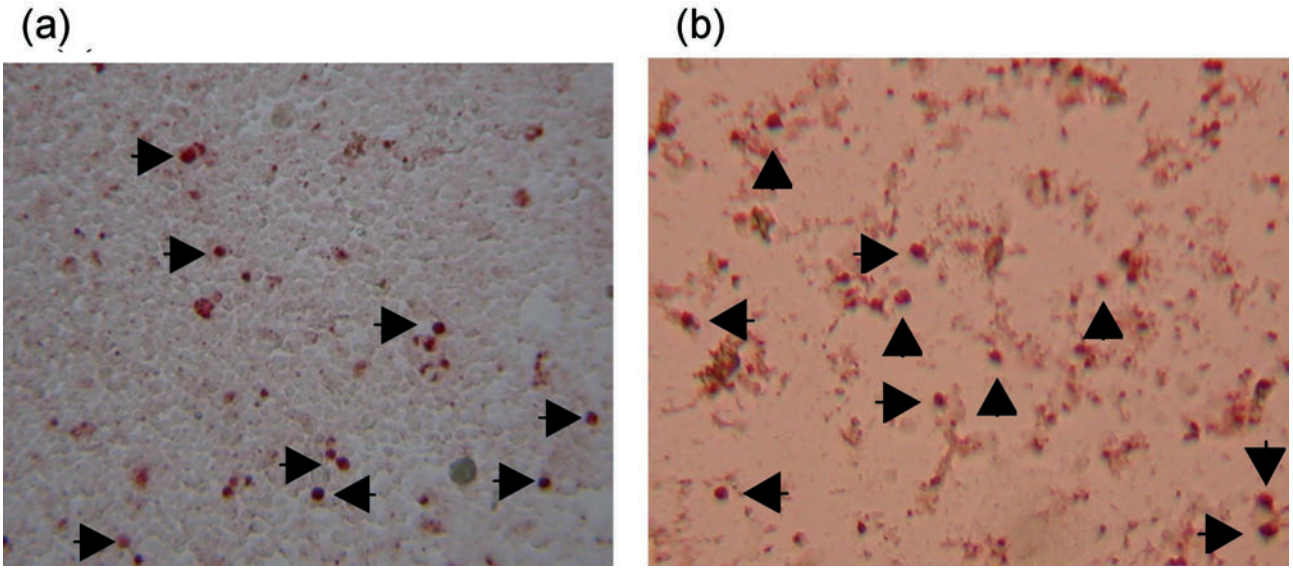

Fig. 2

Detection of the viral antigen in bone marrow (a) and spleen (b) of GPV-infected goslings by immunoassay

GPV-infected cells are indicated by arrows.

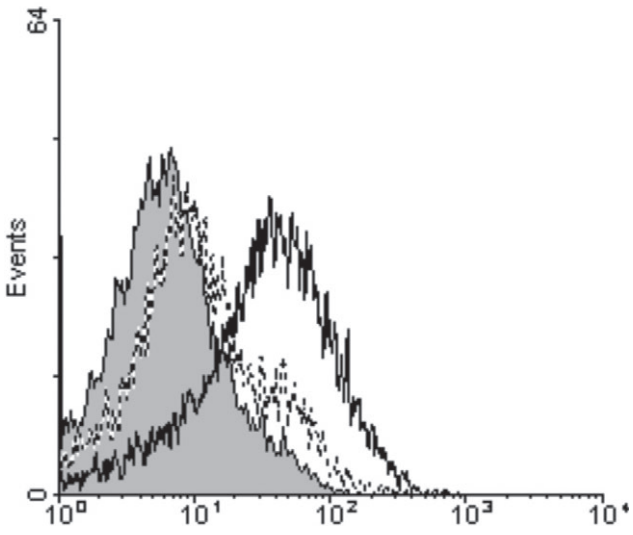

Fig. 3

Detection of GPV-infected spleen cells of goslings by FACS

GPV-infected (solid line, clear area), non-infected (broken line, clear area), and autofluorescence, negative control (solid line, shaded area).

the reliability of conclusion that spleen contained natural target cells for the GPV infection.

Discussion

Until now, the target cells for GPV infection *in vivo* have not been well elucidated. Available data demonstrated that the virus genome could be detected by PCR in all the tissues from Muscovy duckling, which is another natural host for the GPV (Limn *et al*., 1996). With fluorescence quantitative PCR assay Bi *et al*. (2008) found that the viral genome could be detected in all the tissues from the virus-infected goslings, but the spleen showed the highest copy numbers of the viral genome. Roszkowski *et al*. (1982) showed that the virus could replicate only in hepatic cells, but not in the cells from spleen and heart of the infected goslings as detected with immunoperoxidase technique. However, the other parvoviruses showed cell tropism for some immunocytes. GPV is a member of the family *Parvoviridae* and whether it has a tropism for the immune system organs or immunocytes has to be confirmed with a proper method.

Several methods including virus isolation, antigen-capture ELISA (Kardi and Szegletes, 1996), immunoperoxidase technique (Roszkowski *et al*., 1982), immunofluorescence staining (Schettler, 1973), PCR, and fluorescence quantitative PCR assay have been developed for the GPV detection in cell culture or tissues of the goslings (Limn *et al*., 1996; Sirivan *et al*., 1998; Bi *et al*., 2008). Though each method has some drawbacks, they are widely used for a routine diagnosis. Here, we presented a simple method for acquiring cells of the immune system organs and performing appropriate immunoassay to identify intracellular virus with the specific MAb. It was convenient to obtain the spleen cells from a dead gosling and quickly prepare the cell suspensions. So, this method is different from the immunochemical assays that are tedious and time-consuming. The method for the detection of GPV antigen was validated with 12 samples and showed the same accuracy as the PCR. Though, a larger amount of clinical samples would be needed for further evaluation of the method that showed potential usefulness for the clinical diagnosis of GPV infection. On the other hand, the accuracy of immunoassay ensured the reliability of results.

In this study, PCR analysis demonstrated that the virus was distributed in all the collected tissues of GPV-infected animals, what was consistent with the previous reports (Limn *et al*., 1996; Bi *et al*., 2008). However, the immunoassay showed that virus replicated only in the cells of spleen and bone marrow. It seemed that the results of immunoassay were not consistent with those of PCR analysis. However, we explained that PCR could detect both intracellular virus and extracellular virus in the blood, while immunoassay was specific only for the detection of intracellular virus. Usually, the newly formed virus is released from the infected cells into the blood and transported to the other tissues. Accordingly, the virus was detected in all the tissues with PCR or even with the virus isolation method. This is the reason we adopt the method of immunoassay to identify the target cells for GPV infection. The method used by us is similar to the immunoperoxidase technique used by Roszkowski *et al*. (1982).

The intracellular virus in the spleen cells was confirmed by another reliable and accurate method of FACS. However, we could not get sufficient amount of cells from the bone marrow and use them in this method. Consequently, the presence of intracellular virus of bone marrow cells could not be confirmed with FACS. Though, we located the target cells for GPV infection in the spleen and bone marrow and due to the missing MAb against goose CD molecules, we could not determine the specific cell type.

Our study demonstrated that some natural target cell populations for GPV infection were located in the spleen and bone marrow. This work may shed light on the pathogenesis of GPV infection and provide a simple method for the detection of GPV.

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