Use of Herpesvirus Papio 2 as an Alternative Antigen in Immunoblotting Assay for B Virus Diagnosis

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ABSTRACT. Herpesvirus papio 2 (HVP2), which infects baboons, is much more closely related genetically and antigenically to monkey B virus (BV) than to human herpes simplex virus 1 (HSV1) and other related herpes viruses. The usefulness of HVP2 as an alternative test antigen in immunoblotting assays to detect BV-antibody in macaque monkey sera was assessed. Six HVP2 proteins reacted with BV-positive sera in immunoblotting. No specific bands could be detected with BV-negative sera. These results show the usefulness of HVP2 antigen as an alternative and safer antigen than authentic BV antigen in detecting BV antibody in immunoblotting.

KEY WORDS: B virus, diagnosis, Herpesvirus papio 2, immunoblotting.

Monkeys are important as laboratory animals in biomedical research, especially as alternative model animals for humans. Hence, many species of macaques (Macaca spp.) have been utilized in various medical research fields. It is necessary to consider the possibility of zoonotic infections that may be derived from macaques in some experiments. Several pathogens from macaques are known to infect humans. Herpesvirus simiae (Cercopithecine herpesvirus 1, monkey B virus; BV) [4, 13, 27] is the most serious of them.

BV is a member of the α herpesviruses family. Macaques incur BV infection mainly through the oral pathway or sexual contact [9, 13, 27–29]. Up to 90% of adult animals in any given macaque population will be naturally infected [14, 26, 30]. Following primary infection of mucous membranes, the virus infects the nervous system and then establishes a latent infection in sensory ganglia [28, 29]. The latent virus can often be reactivated by some stimulus such as stress. The infectious virus is excreted and becomes the agent of further infection [2, 17, 24].

While macaques suffer no serious problems due to BV infection, other species, including humans, show severe symptoms that usually proceed to fatal consequences [4, 20, 27]. Animal caretakers, veterinary staff and researchers may suffer BV infection, usually due to monkey bites or scratches, sometimes due to contact with various body secretions from monkeys shedding an infectious virus [3, 8, 21]. One means of preventing such deadly BV infection is to develop specific-pathogen-free macaque colonies [25]. This requires an accurate system to diagnose monkeys infected with BV.

It is quite dangerous and difficult to obtain authentic BV antigen because BV is categorized as a biosafety level 4 pathogen [1] and special biological containment facilities are required for it. There are only a few facilities in the world where tests for BV with authentic BV antigen can be performed. BV is very similar to other herpesviruses which infect humans or other primates. Herpes simplex virus 1 of humans (HSV1) has been widely used as an alternative test antigen to detect BV-positive sera, relying on antigenic cross-reactivity between HSV1 and BV [2]. SA8 isolated from African green monkeys [15, 16] is also used for the same purpose in cynomolgus monkeys [23].

Ohsawa et al. showed that Herpesvirus papio 2 (HVP2), which is closer to BV than HSV1 or SA8 both genetically and antigenically [5, 6], has equal sensitivity and specificity to BV as an ELISA antigen for detection of BV-positive macaque sera [18]. This virus is classified as a biosafety level 2 pathogen [1] and therefore can be handled in P2 level laboratories.

Hilliard et al. tested several possible methods for BV surveillance [10] and showed that ELISA, competitive ELISA, immunoblotting and PCR could all be used to diagnose BV infection. While we now have an alternative ELISA system based on those findings, other methods are necessary to confirm a diagnosis of BV.

For this reason and to examine the possibility of using HVP2 as alternative antigen, here we assessed the usefulness of HVP2 as a test antigen in immunoblotting assays to detect BV-positive macaque sera.

MATERIALS AND METHODS

Cells and viruses: HVP2 strain OU1-76 was originally isolated from oral ulcerations of an infant baboon at the Oklahoma University Health Sciences Center, Oklahoma City [7]. The virus was grown in Vero cells and titrated. Cells were grown in Eagle’s minimum essential medium (MEM) containing 5% fetal bovine serum, 1.5% NaHCO3, and 0.1% each of penicillin G potassium, streptomycin sulphate, and kanamycin sulphate.

Antigens and sera: BV antigen for SDS-PAGE analysis, the positive and negative control pooled sera and some BV-
positive Rhesus serum specimens for immunoblotting analysis were gifts from Dr. Julia Hilliard and Dr. David Katz in the B Virus Research and Resource Laboratory, Georgia State University, Atlanta, U.S.A. BV-positive Japanese monkey serum specimens were gifts from Dr. Ryuzo Torii of the Research Center for Animal Life Science, Shiga University of Medical Science, Japan. BV-negative Japanese monkey serum specimens were obtained from our stocks which were harvested from quarantined animals.

**SDS-PAGE analysis:** Vero cells were infected with virus at a multiplicity of infection (MOI) of 5 PFU per cell, and extracts of the cells were prepared at 72 hr postinfection. Protein dissociation buffer (10% β-mercaptoethanol, 10% sodium dodecyl sulfate [SDS], 25% glycerol, 10 mM Tris-HCl [pH 6.8], 0.02% bromophenol blue) was added to each cell extract and the mixtures were heated to 100°C for 5 min. Proteins were resolved by SDS-10% polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie brilliant blue (Phast Gel blue-R & G, Pharmacia).

**Immunoblotting analysis:** Proteins resolved by SDS-PAGE were electroblotted on to an Immobilon membrane (ATTO). The membrane was blocked with blocking buffer (5% skim milk in phosphate-buffered saline [PBS]) for 30 min at 37°C. The membrane was incubated with serum samples (diluted 1:100 in PBS) for 1 hr at room temperature. The membrane was set in a screener blotter (Cosmo bio Co., Ltd) for analysis of a number of serum samples. After four 5-min washes in washing buffer (0.05% Tween 20 in PBS), bound antibody was detected with peroxidase-conjugated anti-human antibodies (1:1,000 in PBS, Cosmo bio Co., Ltd.). The ECL (enhanced chemiluminescence) system (Amersham Lifescience) was used to detect attached conjugate.

**BV ELISA:** Anti-BV antibody detection by ELISA was performed by The Corporation for Production and Research of Laboratory Primates, Tsukuba, Japan.

**RESULTS**

**Analysis of HVP2 protein:** Lysates of Vero cells infected with HVP2 or BV were resolved with 10% SDS-PAGE. Some HVP2-specific proteins could be detected thereby. The major stained HVP2 proteins were near those of BV antigen in SDS-PAGE, but did not have exactly the same migration (data not shown).

**Immunoblotting analysis employing staining with pooled BV serum:** Membranes onto which HVP2 proteins resolved by SDS-PAGE had been blotted were analyzed by staining with BV-positive serum. Some HVP2-specific proteins were detected thereby, and their molecular weights were generally similar to those of BV antigens, though not identical (data not shown). Six specific bands were thus distinguished and their molecular weights were estimated as follows: one band at 121.8 kd, one band at 103.0 kd, one

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**Fig. 1.** Lysate of Vero cells that were infected with HVP2 or not infected were resolved by electrophoresis in an SDS-10% polyacrylamide gel. BV-infected cell lysate was used as a positive control. Proteins were electroblotted from the gel to a membrane and the membrane was stained with BV-positive Rhesus serum samples (Fig. A, lanes 3, 4, 5 and 6) or BV-positive Japanese monkey serum samples (Fig. B, lanes 7, 8, 9 and 10). Lane 1: BV positive pooled serum. Lane 2: BV negative pooled serum. The bound antibodies were detected with peroxidase-conjugated anti-human antibodies and the ECL system. Open triangles show the positions of specific stained bands. Japanese monkey sera were obtained from Shiga University, Japan, and Rhesus sera were obtained from a BV lab in Atlanta, U.S.A. Arrows show the positions of specific stained bands. The sizes of molecular weight markers are indicated in kilodaltons.
band at 63.5 kd, one band at 53.5 kd, one band at 49.8 kd and one band at 44.8 kd (Fig. 1, lane 1). No specific band was detected by staining with BV-negative control serum (Fig. 1, lane 2).

Analysis of BV-positive serum samples by immunoblotting with HVP2 antigen: Eight BV-positive monkey serum samples which were obtained from the B Virus Research and Resource Laboratory (BV lab), Georgia State University, Atlanta, U.S.A. and the Research Center for Animal Life Science, Shiga Medical University, Japan, were analyzed in immunoblotting with HVP2 antigen. These BV-positive monkey serum samples were diagnosed BV positive by BV ELISA (data not shown). Some HVP2-specific proteins were detected with those serum samples; but none of these sera could stain all six specific proteins (Fig. 1).

Analysis of BV-negative monkey serum by immunoblotting with HVP2 antigen: Twenty-eight BV-negative Japanese monkey serum samples in our stock were analyzed by immunoblotting with HVP2 antigen. As shown in Fig. 2, some bands were stained (shown by closed triangle), but none of them was of the same molecular size as any virus-specific bands. Serum No. 14, which was not judged as positive or negative by BV or HVP2 ELISA, stained one specific band whose molecular weight was 53.5 kd.

Analysis of BV-positive or negative monkey serum by immunoblotting with Vero antigen: The BV-positive or negative monkey serum samples were analyzed by immunoblotting with Vero antigen without infecting HVP2. No bands were stained by these samples (data not shown).

DISCUSSION

The pattern of electrophoretically resolved proteins of HVP2 was quite similar to that of BV in analyses with SDS-PAGE and immunoblotting stained with BV-positive pooled serum (data not shown). HVP2 has 98% homology with BV, as shown by genetic analysis [19]. Our results were in accord with this fact, and support the possibility of using HVP2 as an alternative antigen in BV diagnosis.

We utilized HVP2 antigen alternatively to analyze BV positive pooled serum in immunoblotting and identified six specifically staining bands thereby (Fig. 1). Some of them were observed in other BV-positive serum samples. Pooled serum was harvested from 500 monkeys, and probably includes all the possible patterns in immunoblotting. Thus, all field samples obtained to date should be able to stain some of these six bands which were seen in the pooled serum. Although we analyzed only 10 positive serum samples in these experiments, HVP2 immunoblotting appears to be a useful method for BV diagnosis because its use resulted in the staining of some of the same specific bands stained with BV antigen used as BV-positive pooled serum.

There are additional bands in lanes 3 and 4 in Fig. 1. These bands could not be identified as positive or negative by BV or HVP2 ELISA. Therefore these bands should not be utilized for diagnostic purposes.

There was one sample in our stock which could not be identified as positive or negative by BV or HVP2 ELISA. We analyzed it with HVP2 immunoblotting and detected some specific bands. This means that this sample was positive. The monkey from which this serum was harvested had already been euthanized and it was therefore impossible to verify BV infection in this monkey but this result shows the usefulness of HVP2 immunoblotting as a supplemental diagnostic method after ELISA screening.

Lane 12 in Fig. 2 could not be seen the presence of the bands because the entire membrane was stained. It is diffi-
cull to diagnose such samples by immunoblotting. We should use ELISA or other diagnostic methods to analyze such samples.

We sometimes use Japanese macaques. Though no BV strains have been isolated from Japanese macaques yet, many Japanese macaques have been shown to have anti-BV antibody in their sera [22]. It is possible that the BV which infects Japanese macaques is different from the BV in rhesus monkeys. Some researchers have tried to analyze the origin of BV in Japan by using molecular techniques [11]. Such trials may clarify whether Japanese macaques are infected with unique BV strains. The present report showed that it was possible to detect BV-positive serum specimens with HVP2 antigen but we need to accumulate more serological data to fully establish this diagnostic system.

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