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Use of a Group-Reactive and Other Monoclonal Antibodies in an Enzyme Immunodot Assay for Identification and Presumptive Serotyping of Aquatic Birnaviruses†

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A panel of monoclonal antibodies (MAbs) was prepared and used to develop an enzyme immunodot assay for the rapid identification and presumptive serotyping of aquatic birnaviruses. Comparison of the reaction patterns of these MAbs with representative virus isolates indicated that one MAb recognizes a serogroup-reactive epitope and can therefore be used for identification of all serogroup A aquatic birnaviruses, the predominant serotype worldwide. Other MAbs exhibited more restrictive specificities, permitting the presumptive serotyping of viruses of the three recognized serotypes and the identification of some individual strains. This assay, in which MAbs are used, is more efficient in terms of time, cost, and ease of performance and provides significant advantages in specificity and standardization compared with currently used tests.

The *Birnaviridae* are a family of viruses characterized by a bisegmented, double-stranded RNA genome and an unenveloped, single-layer, icosahedral capsid approximately 60 nm in diameter (5). Members of the family *Birnaviridae* include the aquatic birnaviruses such as infectious pancreatic necrosis virus (IPNV) and other related viruses from fish and shellfish, the avian infectious bursal disease virus, and the insect *Drosophila* X virus. Aquatic birnaviruses are the etiological agents of diseases in a variety of economically important fish species used worldwide in aquaculture and fish farming, such as infectious pancreatic necrosis in salmonids and branchionephritis in eels (19, 27). Also, related aquatic birnaviruses have been isolated from a variety of other apparently healthy fish species and marine molluscs (2, 7, 8, 18, 20–22, 25, 26).

Previous serological studies of IPNV and other aquatic birnaviruses have shown that most of these viruses are antigenically related. On the basis of reciprocal neutralization titers with rabbit antisera, three major serotypes are currently recognized (12, 16). However, Hill and Way (B. J. Hill and K. Way, Abstr. First Int. Conf. Eur. Assoc. Fish Pathol. 1983, p. 10) have presented evidence for six additional serotypes, which, together with the original three, would constitute a major serotype (serogroup A). Four other isolates have been identified that apparently represent a minor, unrelated serogroup (serogroup B). The three well-defined serotypes of serogroup A originally were designated Sp, Ab, and VR299, but Hill and Way (Abstr. First Int. Conf. Eur. Assoc. Fish Pathol.) and other investigators (3) have designated this last serotype as West Buxton (WB). The six recently proposed serotypes have been tentatively designated Hecht (He), Tellina (Te), Jasper (Ja), Canada 1 (C1), Canada 2 (C2), and Canada 3 (C3). Virtually all aquatic birnavirus isolates from the United States belong to the WB serotype, whereas four serotypes (Ja, C1, C2, and C3) have been reported to occur in Canada and four (Sp, Ab, He, and Te) are found in Europe. To date, most isolates from Asia appear to belong to either the WB or Ab serotype.

Specific and accurate identification of these viruses is required for effective control and an understanding of the epidemiology of the diseases that they cause. Various rapid serological tests with polyclonal antisera have been developed for the general identification of one or more aquatic birnaviruses (4, 9, 13–15, 17, 23). Recently, monoclonal antibodies (MAbs) have been developed and used in the analysis of birnavirus epitopes and the comparison of different isolates and in enzyme-linked immunosorbent assays (ELISAs) for the identification of some strains of IPNV (3, 28). However, most of these rapid assays identify only certain strains of virus, and none have been shown to be universally applicable in identifying all aquatic birnaviruses from a variety of species and geographical areas. Also, the serotype of a birnavirus isolate usually is determined by virus neutralization tests with polyclonal antisera. In this report, we describe the use of a group-reactive MAb in an enzyme immunodot assay for general virus identification and a panel of MAbs with various specificities for presumptive serotyping of the aquatic birnaviruses.

MATERIALS AND METHODS

Viruses. The viruses used are listed in Table 1. The type strains of the WB, Ab, and Sp birnavirus serotypes, various WB and Sp representative strains, and AS virus were from stock preparations maintained in our laboratory for many years. The C1, C2, C3, Ja, He, and Te type strains were provided by B. Hill, MAFF, Fish Diseases Laboratory, Weymouth, England. Representative isolates of the Ab serotype were obtained from J. L. Wu and Y. L. Hsu, Academia Sinica, and C. F. Lo, Fu-Jen University, Taipei, Republic of China.

Each virus was cloned by terminal dilution or plaque purification and subsequently propagated in CHSE-214 cells, an established cell line originated from Chinook salmon embryos (10). All viruses were purified as previously described (3). Briefly, virus-infected cells were removed from cell culture supernatants by low-speed centrifugation, suspended in TNE buffer (0.01 M Tris hydrochloride, 0.1 M

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TABLE 1. Aquatic birnaviruses

Virus	Country of origin[a]	Animal of origin	Serotype
West Buxton (WB) (type strain)	USA	Trout	WB
Dry Mills	USA	Trout	WB
Gilbert	USA	Trout	WB
VR299	USA	Trout	WB
Berlin	USA	Trout	WB
Buhl	USA	Trout	WB
Reno	USA	Trout	WB
Taiwan	Taiwan, ROC	Trout	WB
Menhaden	USA	Menhaden	WB
Striped bass	USA	Striped bass	WB
Sp (type strain)	Denmark	Trout	Sp
Bonnamy	France	Trout	Sp
d'Honninchton	France	Trout	Sp
OV-2	England	Oyster	Sp
OV-7	England	Oyster	Sp
Ab (type strain)	Denmark	Trout	Ab
EEV	Japan	Eel	Ab
E1S	Taiwan, ROC	Eel	Ab
PV	Taiwan, ROC	Perch	Ab
CV-1	Taiwan, ROC	Clam	Ab
CV-HB-1	Taiwan, ROC	Clam	Ab
CV-TS-1	Taiwan, ROC	Clam	Ab
Hecht (type strain)	FRG		He
Tellina (type strain)	England	Tellina	Te
Canada 1 (type strain)	Canada	Trout	C1
AS	Canada	Atlantic salmon	C1
Canada 2 (type strain)	Canada	Trout	C2
Canada 3 (type strain)	Canada	Arctic char	C3
Jasper (type strain)	Canada	Trout	Ja

[a] Abbreviations: USA, United States of America; ROC, Republic of China; FRG, Federal Republic of Germany.

NaCl, 1 mM EDTA [pH 7.5]), and extracted with an equal volume of Freon 113 (Du Pont Co., Wilmington, Del.) to recover cell-associated virus. This was combined with released virus, which had been precipitated from the cell-free supernatants with polyethylene glycol (molecular weight, 20,000). Virus was then layered over a step gradient consisting of 20% (wt/wt) sucrose, 30% (wt/vol) CsCl, and 40% (wt/vol) CsCl and centrifuged for 2 h at 143,000 × *g*. Fractions containing virus then were banded a second time by isopycnic centrifugation on a self-forming CsCl (initial density, 1.33 g/cm^3) gradient for 16 h at 113,000 × *g*. Fractions containing virus were dialyzed in buffer, filtered through a filter membrane (pore size, 0.22 μm), and stored frozen in liquid nitrogen.

Production of MAbs. The production and characterization of MAbs W1 to W5 against WB IPNV have been described previously (3). MAbs E1, E2, E3, E5, and E6 were prepared against European eel virus, a representative of the Ab serotype, and MAb AS-1 was prepared against AS IPNV, a representative of the C1 serotype, by the procedures of Caswell-Reno et al. (3). Briefly, 8-week-old BALB/c mice were injected intraperitoneally with purified virus (5×10^8 50% tissue culture infective doses [$TCID_{50}$]; approximately 0.8 μg of protein) in complete Freund adjuvant and then 5 weeks later intraperitoneally and intravenously with boosting doses of purified virus (1×10^9 $TCID_{50}$; approximately 1.6 μg of protein each) without adjuvant. Spleen cells were harvested 4 days later from mice with high antibody titers and fused with Sp2/0-Ag-14 mouse myeloma cells at a ratio of 10:1 by using polyethylene glycol. Each well of 96-well plates was seeded with approximately 2.7×10^5 total cells in 0.1 ml of HAT growth medium (GM-HAT) consisting of Dulbecco modified Eagle medium with D-glucose (4.5 mg/ml), NCTC 109 (10%, vol/vol), bovine pancreatic insulin (0.2 μg/ml), oxalacetic acid (1 mM), pyruvic acid (0.45 mM), fetal bovine serum (15%, vol/vol), hypoxanthine (0.1 mM), aminopterin (4×10^{-4} mM), and thymidine (1.6×10^{-2} mM) and incubated at 37°C in a humidified 10% CO_2 atmosphere. Hybridomas producing virus-specific antibody were adapted to HAT-free GM, expanded, cloned at least twice, and stored frozen in liquid nitrogen.

Immunodot assay. The dot immunobinding assay was adapted from the method of Hawkes et al. (6). All virus preparations consisted of infected-cell culture supernatants that had been clarified by centrifugation at 1,000 rpm (100 × *g*) for 10 min. Aliquots of virus (2 μl) at a minimum concentration of 10^7 $TCID_{50}$/ml were dotted onto nitrocellulose strips (Bio-Rad Laboratories, Richmond, Calif.) and air dried at room temperature. In some cases, the use of virus concentrations up to 10^{10} $TCID_{50}$/ml was tested, with no difference in reaction patterns observed in comparison with those with the minimum concentration. After the strips had been washed four times with Tris-buffered saline (TBS) (pH 7.4), the remaining nonspecific protein-binding sites were blocked with 3% (wt/vol) bovine serum albumin and 10% (vol/vol) calf serum. Individual strips were then incubated for 3 h at room temperature in each MAb (hybridoma cell culture supernatants) with titers two to four times the minimum previously determined to give positive results with homologous virus. After being washed and subjected to the blocking procedure as above, the strips were incubated for 2 h with goat anti-mouse immunoglobulin conjugated with horseradish peroxidase, washed, and incubated for 15 min in substrate (0.5 mg of 4-chloronaphthol and 10 μl of 3% H_2O_2 in 1 ml of TBS). A positive reaction was indicated by a visible purple developing in 2 to 15 min. Occasionally, with some isolates exhibiting weakly positive reactions with some MAbs, the contrast of the colored dot with the background appeared less distinct after drying. Therefore, for maximum sensitivity in such cases, results were read immediately.

To confirm the specificity of positive reactions, we used two types of negative controls (data not shown). The antigen controls were negative reactions with unrelated antigens applied as dots to the strips. These included (i) uninfected CHSE-214 cell culture supernatant; (ii) equivalent concentrations of infectious hematopoietic necrosis virus, a fish rhabdovirus; and (iii) equivalent concentrations of infectious bursal disease virus, an unrelated birnavirus. In addition, the specificity of these MAbs for aquatic birnaviruses previously had been demonstrated by the lack of reaction in an ELISA with equivalent amounts of a purified double-stranded RNA mycovirus (Rhs 717) from *Rhizoctonia solani* (24) and purified Rhs 717 mycovirus double-stranded RNA. The antibody control was the lack of reaction with virus dots of a known birnavirus-negative MAb (E0), used at dilutions comparable to those of birnavirus-specific MAbs.

RESULTS

A set of five aquatic birnavirus MAbs (W1 to W5) with different epitope specificities have been developed and char-

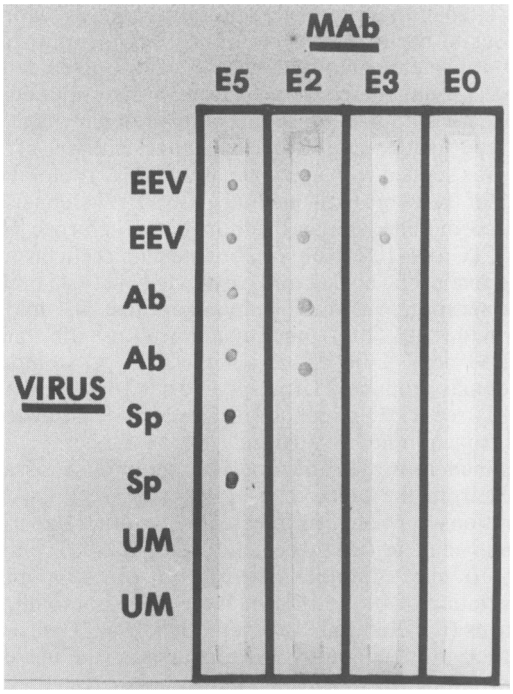

FIG. 1. Typical reactions of representative aquatic birnaviruses with several MAbs in the immunodot assay. Abbreviations: EEV, European eel virus; Ab, Ab IPNV; Sp, Sp-IPNV; UM, culture supernatants from uninfected cell monolayer; E5, E2, and E3, birnavirus-specific MAbs; E0, birnavirus-negative hybridoma cell culture supernatant.

acterized previously (3). To enlarge the available library of such antibodies, we developed additional MAbs in this study. Six of these new MAbs were selected for use in developing enzyme immunoassays. All six new MAbs were immunoglobulin G (IgG) with κ light chains and represented the following isotypes: E1, IgG2a; E2, IgG2a; E3, IgG2b; E5, IgG1; E6, IgG1; and AS-1, IgG1. Also, all the new MAbs were shown to recognize different epitopes by differences in reaction patterns with various isolates and/or radioimmune precipitation of individual viral proteins (V. Lipipun, Ph.D. thesis, University of Maine, Orono, 1988). A simple immunodot test involving the use of the five previously available and six new MAbs was developed for the rapid identification of aquatic birnaviruses from infected-cell culture supernatants. The test protocol was designed by determining the optimal concentrations of reagents and reaction times for each homologous virus-MAb pair. Preliminary studies of the sensitivity of this assay with a more limited selection of MAbs and viruses demonstrated that supernatants from infected-cell cultures exhibiting extensive cytopathic effect and a virus titer of $\geq 10^4$ $TCID_{50}/\mu l$ gave clearly visible positive results (11). The appearance of a typical assay is shown in Fig. 1. In this case three related birnaviruses were distinguished by different reaction patterns with three MAbs.

This immunodot assay was used to compare the reactivity of the panel of 11 MAbs with the type strains and a variety of other representative isolates of the three widely recognized aquatic birnavirus serotypes (WB, Sp, and Ab). The results are shown in Table 2. Several MAbs had a wide range of cross-reactivity, whereas others reacted only with certain serotypes or isolates. Epitopes recognized by MAbs AS-1, E1, and E5 appear to be highly conserved among these viruses; these epitopes were present on all birnaviruses tested from the three established serotypes, including isolates from North America, Europe, and Asia. Other MAbs exhibited more specific patterns of cross-reactions. Several reacted only with representatives of a single serotype; MAbs W4 and W5 reacted with all serotype WB viruses, and MAb E2 reacted with all serotype Ab viruses. Several MAbs reacted with some or all isolates from two of the three serotypes. MAb E3 reacted with all isolates of serotype Ab except Ab virus itself, the type strain for this serotype. All of the Sp serotype viruses exhibited identical reaction patterns, whereas at least two distinct strains were apparently represented within each of the WB and Ab serotypes. Overall, the serotype of the viruses tested belonging to the three established serotypes (WB, Sp, and Ab) of serogroup A could be determined presumptively by the distinctive reaction patterns of these viruses with the entire panel of MAbs. A summary scheme for presumptive serotyping of these viruses is illustrated in Fig. 2A.

A variety of isolates representing the six newly proposed aquatic birnavirus serotypes (Hill and Way, Abstr. First Int. Conf. Eur. Assoc. Fish Pathol.) were not available; however, the type strains of each of these serotypes as well as one other C1 serotype virus (AS virus) were tested for reactivity with the panel of MAbs (Table 2). As with the three well-established serotypes, MAbs AS-1 and E5 reacted with all six serotypes, whereas other MAbs discriminated among viruses. Interestingly, MAb E1, which reacted with all representatives of the three recognized serotypes, reacted with only three of the newly proposed serotypes. The type strains of four (Ja, Te, He, and C1) of these proposed serotypes not only could be distinguished from each other and from the other two by their unique patterns of MAb cross-reactivity but also were different from the three original serotypes. Of these, only the serotype pair of C2 and C3 could not be distinguished from each other in this assay. The reaction patterns of these six serotypes are summarized in Fig. 2B as a presumptive serotyping scheme.

DISCUSSION

The results presented in this report describe a simple immunodot assay in which a panel of MAbs is used for the rapid identification of aquatic birnaviruses from infected-cell culture supernatants. MAb AS-1 reacted with all isolates from serogroup A tested in this study, indicating that this MAb recognizes a group-reactive epitope. This is supported by results of preliminary investigations showing that this MAb also reacts with all viruses from a diverse group of untyped birnaviruses recently isolated from a variety of fish and mollusc species in Asia (Lipipun, Ph.D. thesis). Therefore, general identification of serogroup A aquatic birnaviruses can be made in a few hours by using this assay with MAb AS-1. This is particularly significant, since all but 4 of almost 200 aquatic birnavirus isolates worldwide belong to serogroup A (Hill and Way, Abstr. First Int. Conf. Eur. Assoc. Fish Pathol.). Previously developed rapid identification tests used antibodies that were applicable only to specific serotypes or even individual strains (3, 9, 13, 14, 17, 23, 28). Use of MAb AS-1 would probably make these other assays more universally applicable. MAb E5 also reacted with all virus isolates tested in this study; however, it does not react with two recently isolated, uncharacterized aquatic birnaviruses from Asia (Lipipun, Ph.D. thesis). That these two new isolates are serogroup A aquatic birnaviruses is indicated by their reaction with MAb AS-1. Therefore, MAb

TABLE 2. Reaction of MAbs with representative aquatic birnaviruses

Virus	Reaction of following MAb[a]:										
	AS-1	W1	W2	W3	W4	W5	E1	E2	E3	E5	E6
Established serotypes											
West Buxton (WB)											
WB (type strain)	+	+	+	+	+	+	+	−	−	+	+
Dry Mills	+	+	+	+	+	+	+	−	−	+	+
Taiwan	+	+	+	+	+	+	+	−	−	+	+
Gilbert	+	+	+	+	+	+	+	−	−	+	±
VR 299	+	−	−	+	+	+	+	−	−	+	+
Berlin	+	−	−	+	+	+	+	−	−	+	+
Menhaden	+	−	−	+	+	+	+	−	−	+	+
Striped bass	+	−	−	+	+	+	+	−	−	+	+
Buhl	+	−	−	±	±	+	+	−	−	+	+
Reno	+	−	−	±	+	+	+	−	−	+	+
Sp											
Sp (type strain)	+	−	−	+	−	−	+	−	−	+	−
OV-2	+	−	−	+	−	−	+	−	−	+	−
OV-7	+	−	−	+	−	−	+	−	−	+	−
Bonnamy	+	−	−	±	−	−	+	−	−	+	−
d'Honninchton	+	−	−	+	−	−	+	−	−	+	−
Ab											
Ab (type strain)	+	+	+	−	−	−	+	+	−	+	+
EEV	+	+	+	−	−	−	+	+	+	+	+
E1S	+	+	+	−	−	−	+	+	+	+	+
CV-1	+	+	+	−	−	−	+	+	+	+	+
CV-HB-1	+	+	+	−	−	−	+	+	+	+	+
CV-TS-1	+	+	+	−	−	−	+	+	+	+	+
Proposed serotypes											
Hecht (He)											
He (type strain)	+	−	−	−	−	−	−	−	−	+	−
Tellina (Te)											
Te (type strain)	+	±	−	−	−	−	+	+	−	+	+
Canada 1 (C1)											
C1 (type strain)	+	−	−	−	−	−	+	+	−	+	+
AS	+	−	−	−	−	−	+	+	−	+	+
Canada 2 (C2)											
C2 (type strain)	+	−	−	−	−	−	−	−	−	+	+
Canada 3 (C3)											
C3 (type strain)	+	−	−	−	−	−	−	−	−	+	+
Jasper (Ja)											
Ja (type strain)	+	+	+	+	+	+	+	−	−	+	−

[a] Results from at least three experiments. Symbols: +, positive reaction; ±, weakly positive reaction; −, no reaction.

E5, although not universally applicable, may also be useful in general screening assays, particularly for virus isolates from North America and Europe.

Furthermore, this assay can be used for presumptive serotyping of members of the three established serotypes (WB, Sp, and Ab) of birnavirus serogroup A by their unique patterns of reaction with the entire MAb panel (Fig. 2A). Currently, these viruses are usually serotyped by performing neutralization tests of each isolate with polyclonal antisera and reference viruses for each serotype. The results also indicate that this panel of MAbs may be useful in the presumptive serotyping of isolates form the six newly proposed serotypes. The type strains of four (JA, Te, He, and C1) of these new serotypes could be distinguished from each other as well as from the other two (C2 and C3) and the three original, well-defined serotypes. The type strains of serotypes C2 and C3 reacted with only three MAbs and could not be distinguished from each other with these MAbs; however, the reaction patterns of these two viruses were distinct from those of each of the other seven serotypes. The development of additional MAbs specific for one or both of these viruses should enable the specific identification of these two serotypes. Whether the patterns of cross-reactions of these type strains are representative of the serotype must be determined by comparison with other representative isolates, as was done with WB, Sp, and Ab.

Current general screening assays for identifying fish virus isolates most often use virus neutralization tests with unstandardized polyclonal antisera, although the use of ELISA and other rapid assays is becoming more widespread. This immunodot assay with MAbs provides distinct advantages over these other assays in ease of performance, cost, and/or standardization. Both the assay system itself and the use of MAbs in place of polyclonal antisera contribute to these advantages. For instance, the use of a standardized amount of a broadly reacting MAb such as AS-1 will identify all known serotypes of the major aquatic birnavirus serogroup more effectively than will the use of unstandardized polyclonal antibody mixtures which exhibit different reactivities for viruses of different serotypes. In serotyping viruses, the

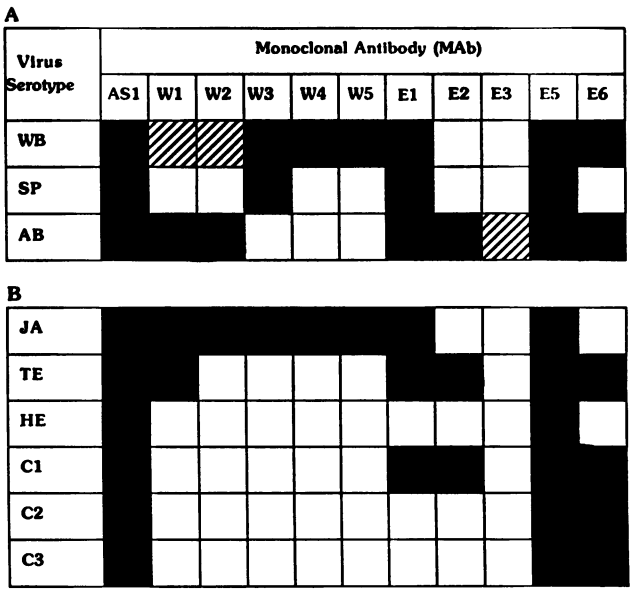

FIG. 2. Scheme for presumptive serotype identification of aquatic birnaviruses in the immunodot assay with MAbs. (A) Recognized serotypes. Reaction patterns based on tests of representatives of each serotype previously serotyped by reciprocal virus neutralization tests with polyclonal antisera. Symbols: ■, always positive; □, always negative; ▨, variable reaction with different viruses. (B) Proposed new serotypes. The reaction pattern is based on a test with type strains only.

use of a panel of MAbs offers obvious advantages in specificity over the use of polyclonal antisera. Also, the immunodot assay described in this report has distinct advantages over the available MAb-based ELISA (3) for identifying aquatic birnaviruses. For maximum sensitivity and specificity, the ELISA uses either polyclonal antisera as an initial capture antibody system to remove virus from infected-cell culture supernatants or purified virus bound directly to the solid phase. This immunodot assay, however, is performed by using clarified supernatants taken directly from infected-cell cultures. The two assays have comparable levels of sensitivity.

In addition to identification and presumptive serotyping, these MAbs provide some interesting information about the antigenic characteristics of these aquatic birnaviruses. The viruses tested within each of the three recognized serotypes (WB, Sp, and Ab) were relatively homogeneous in antigenic characteristics. All representatives of the Sp serotype exhibited identical patterns of reaction with these MAbs. Two distinct sets of WB serotype isolates were apparent from their reaction or lack of reaction with two MAbs (W1 and W2). The Ab serotype viruses also showed two reaction patterns. A previous report (11) demonstrated that the Ab type strain differed from at least one other Ab serotype virus (European eel virus) by its failure to bind MAb E3, as is also shown here. In the present study, failure to react with this single MAb was the only feature distinguishing the Ab type strain virus from European eel virus, even with this larger panel of MAbs. Interestingly, all of the other Ab serotype viruses tested were identical to European eel virus. It should be noted that MAb W2 exhibited a positive reaction with Ab virus in this immunodot assay, although in a previous study it failed to bind to this virus in an ELISA (3). The exact reason for this apparent anomaly is not clear; however, it is well known that the manner in which epitopes are presented (e.g., attachment of antigen to polystyrene versus nitrocellulose substrates) can markedly affect the binding of certain MAbs. Such conformational changes in a virus when bound to plastic have been demonstrated for tobacco mosaic virus (1). Moreover, it has been shown that certain aquatic birnavirus MAbs which gave marginal or equivocal results in an ELISA when the virus was adsorbed directly to plastic reacted much more strongly when the virus was attached by an intermediary layer of capture antibody (3).

The MAb reaction pattern of Jasper virus, the type strain for the proposed Ja serotype, was particularly interesting. This virus appeared to be closely related antigenically to viruses of the WB serotype with this panel of MAbs. Indeed, Jasper virus could be distinguished from four of the WB serotype viruses only by its failure to react with MAb E6. It should be noted that although most of the other recognized and proposed serotypes are represented by numerous isolates and/or strains from diverse host species and geographical areas, the proposed Ja serotype is represented only by this single isolate. Perhaps, therefore, the antigenic characteristics of this virus and the establishment of a separate Ja serotype also should be investigated in more detail.

Also interesting was the fact that several pairs of viruses isolated from different species and widely separated geographical areas were distinguished only by the presence or absence of one or two epitopes defined by these MAbs. For example, Canada 1 virus from salmonid fish in Canada was distinguished from Tellina virus from a bivalve mollusc in Europe only by the failure of the former to bind MAb W1. Therefore, these MAbs also may be useful probes for investigating the function(s) of individual epitopes in virus infectivity, host specificity, and epidemiology.

In summary, we report the development of a panel of MAbs, including one recognizing a group-reactive epitope, and the use of these MAbs in a simple and rapid immunodot assay for the general identification and presumptive serotyping of aquatic birnaviruses. The assay requires about 2 h of hands-on time, and results can be obtained without specialized equipment in approximately 6 h. The specificity and availability of these MAbs, combined with the simplicity of the immunodot test, should make this an important assay system in aquatic animal disease diagnosis and research.

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