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Rapid Subtyping of Equine Herpesvirus 1 with Monoclonal Antibodies†

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Two antigenically similar subtypes of equine herpesvirus 1 (EHV-1) cause disease in horses. A procedure for rapid differentiation of the two EHV-1 subtypes with monoclonal antibodies was developed. Subtype-specific pools of monoclonal antibodies were constructed, characterized, and used in enzyme immunofiltration and indirect immunofluorescence assays to subtype 50 epizootiologically unrelated field isolates of EHV-1. Both assays allowed accurate subtype identification of each EHV-1 isolate with the monoclonal antibody pools. The subtyping procedures were simple and amenable to typing many isolates at one time and permitted unambiguous EHV-1 subtype identification within 3 h after isolation of the virus in tissue culture.

Infection of horses by equine herpesvirus 1 (EHV-1) is a leading cause of both morbidity and mortality in horse populations and a source of serious economic loss to the horse breeding industry (3, 6). EHV-1 infections may result in epizootics of respiratory tract disease, abortion, and a paralytic disorder of the central nervous system (3, 5, 7). Although all EHV-1 isolates are antigenically similar (3), several workers have reported genetic and serological differences among strains of EHV-1 and have distinguished two molecular subtypes (1, 4, 13–16). The two subtypes of EHV-1 can be differentiated by serological tests and by major differences in their restriction endonuclease cleavage patterns. However, subtyping techniques based on polyclonal antisera are hampered by the extensive cross-reactivity of the antisera, and the DNA fingerprinting technique requires time, specialized equipment, and technical expertise. The development of alternative laboratory methods that are simple, rapid, and unambiguous for the determination of EHV-1 subtype would therefore be desirable.

Other closely related herpesviruses have been differentiated by rapid serological analyses with monoclonal antibodies (2, 10–12). The objective of the investigation described in this report was to construct subtype-specific monoclonal antibodies to EHV-1 for use in the rapid subtyping of clinical isolates from field outbreaks of EHV-1-related disease.

MATERIALS AND METHODS

Cells and viruses. Equine dermal fibroblast (KyED) cells were used to propagate EHV-1 isolates. The origin of the virus strains used in this study has been described previously (1, 16). NS-1 myeloma cells (a generous gift from L. O. Arthur) and subsequent hybrid cells were grown in Dulbecco modified Eagle medium with 20% fetal bovine serum and 10% NCTC 109.

Production of hybrid cell lines secreting EVH-1 subtype-specific monoclonal antibodies. EHV-1 subtype 1- and subtype 2-specific monoclonal antibodies were produced by immunizing mice with Army 183 and Kentucky T2 strains of EHV-1, respectively. EHV-1 virions were obtained as described earlier (1) and resuspended in phosphate-buffered saline (PBS). The protocol used for producing immunoglobulin-secreting hybrid cell lines was that outlined by Nowinski et al. (9). Briefly, BALB/c mice were primed and boosted 2 weeks later with an intraperitoneal injection of approximately 0.35 mg of virion protein in 0W3 adjuvant (Fort Dodge Laboratories, Fort Dodge, Iowa). Ten days after the boost, the mice were injected intraperitoneally with 0.7 mg of virus in PBS. After 3 days, the spleen cells from three immunized mice were fused to NS-1 myeloma cells in a 10:1 ratio. For screening viable hybrid cells for production of the desired antibody, an enzyme-linked immunosorbent assay was performed as described previously (L. W. Turtinen, Ph.D. thesis, University of Kentucky, 1983). Mouse ascites preparations of monoclonal antibodies were made as described by Nowinski et al. (9).

Enzyme immunofiltration assay. The assay was similar to that of Richman et al. (12) with minor modifications. It was performed with a vacuum filtration holder and 96-well filtration plates (Millititer filtration system; Millipore Corp., Bedford, Mass.), which allowed the simultaneous processing of 96 individual samples. All incubations with reagents were done in the wells of the plate, and unbound reagents and rinse buffers were removed by vacuum filtration through the membrane (pore size, 5 μm) of the filtration plates. Before assay, the plate membranes were conditioned by rinsing with RS buffer (PBS containing 5% rabbit serum, 0.3% gelatin, and 0.01% Merthiolate). EHV-1-infected or uninfected KyED cells (10^5) were then added to duplicate wells; each well was incubated in succession for 1 h at 37°C with 50 μl of each of the following monoclonal antibody, with peroxidase-conjugated rabbit anti-mouse immunoglobulin G (IgG) antibody (diluted 1:5,000 in RS buffer), and with peroxidase substrate solution (*o*-phenylenediamine–hydrogen peroxide). Each well was rinsed with 1 ml of gelatin buffer (PBS containing 0.3% gelatin and 0.01% Merthiolate) between incubations with each of the reagents. The absorbance at 490 nm was measured on acidified samples removed from each well. Optimal antigen concentration for the immunofiltration assay was determined by block titrations of EHV-1-infected and uninfected KyED cells against serial dilutions of a monoclonal antibody. The cell concentration which allowed the highest signal/noise ratio (10^5 cells per well) was used.

Indirect immunofluorescence. EHV-1-infected or uninfected KyED cells were collected from monolayer cultures, washed in PBS containing 0.3% gelatin, and air dried onto

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eight-chambered microscope slides (Miles Scientific, Naperville, Ill.). After fixation with methanol, the cells were incubated in succession with 5% normal goat serum, monoclonal antibody, and fluorescein-conjugated goat anti-mouse IgG antibody (Cappel Laboratories, Cochranville, Pa.) as described by Goding (8). Thorough rinsing with PBS was performed between incubations with the reagents. The slides were then mounted with cover slips by using a solution of 50% glycerol in PBS and examined by epifluorescence microscopy with a Leitz Ortholux microscope.

RESULTS

Production of hybridomas secreting antibodies to EHV-1. Hybrids resulting from the fusion experiment with spleens from subtype 1-immunized mice were screened only against the subtype 1 Army 183 strain of EHV-1 used for mouse immunization. No attempt was made to screen for antibodies which reacted solely with this subtype. Of 576 wells that were planted, 34% were positive for antibody directed against the immunizing strain of EHV-1. Virtually every well that was planted with the fusion mixture exhibited macroscopic hybrid growth by day 10 postfusion. Culture fluids from 24 of the original 199 positive wells contained high-titer antibodies. Cells from these wells were cloned by limiting dilution (9), and 14 stable antibody-secreting hybrid cell lines were obtained. Four of the clones secreted monoclonal antibodies that were subtype 1 specific, as determined by a negative enzyme-linked immunosorbent assay result when tested against a single subtype 2 strain of EHV-1 (Kentucky T2).

For the production of hybrid cell lines secreting subtype 2-specific monoclonal antibodies, the supernatant fluids from growing colonies were screened in an enzyme-linked immunosorbent assay against virus isolates (Army 183 and Kentucky T2) representing both subtypes of EHV-1. Of 341 wells that were planted, 29 of the wells contained antibodies to both subtype strains, whereas 40 wells contained subtype 2-specific antibodies. Culture fluids from 10 of the 40 wells contained high levels of antibody against the subtype 2 strain, and these hybrids were cloned by limiting dilution (9). A total of eight stable hybrid cell lines which secreted subtype 2-specific monoclonal antibodies were isolated.

Screening anti-EHV-1 monoclonal antibodies for subtype specificity. An enzyme immunofiltration assay against 20 EHV-1 isolates was used to determine which of the four subtype 1- and eight subtype 2-specific monoclonal antibodies would be suitable for typing field isolates of EHV-1. The EHV-1 isolates chosen for this initial screening with the subtype-specific monoclonal antibodies were unrelated and diverse with regard to year, farm where isolated, and DNA fingerprint pattern (1). Table 1 shows the immunofiltration titers of the six antibodies that were suitable for subtyping EHV-1 isolates. The other six antibodies exhibited some cross-reaction with one or more virus strains of the heterologous subtype (data not shown). The six selected monoclonal antibodies exhibited little or no binding to cells infected with any of the 10 EHV-1 isolates of the heterologous subtype. The titers of each monoclonal antibody to homologous isolates were not correlated with the DNA fingerprint pattern of the EHV-1 isolate.

All six subtype specific monoclonal antibodies contained kappa light chains. Three antibodies (13B2, 19B8, and 20F3) were IgG2b; IgG2a was the isotype of 16C12, 16H9, and 21C5. No antibody bound more than one isotype of heavy or light chain. Antibody isotype and EHV-1 subtype specificity were not correlated.

No neutralization of homologous or heterologous virus was observed for any of the six monoclonal antibodies in the presence or absence of guinea pig complement.

Use of monoclonal antibodies for subtyping EHV-1 isolates. Two subtype-specific pools of monoclonal antibodies were created by combining ascites preparations of three monoclonal antibodies for each pool. The proportions of individual antibodies composing each pool were based on the immunofiltration titers of each ascites preparation. The endpoint titers of subtype-specific monoclonal antibodies to homologous virus-infected cells were as follows: 13B2, 30,000; 16C12, 24,000; 16H9, 32,000; 19B8, 192,000; 20F3, 192,000; 21C5, 383,000. Titers to heterologous virus-infected cells in all cases were less than 1,500. Normal ascites had endpoint titers of less than 1,000 to both subtypes of target cells.

Table 2 shows the results of subtyping 50 EHV-1 isolates with the two pools of subtype-specific monoclonal antibodies. Both antibody pools gave significantly higher absorbance readings with homologous EHV-1 isolates than with isolates of the heterologous subtype. The variation between duplicate assay samples was not significant (F test result, $\alpha = 0.152$) in three of the four data groups. However, the difference between duplicate assay samples (32.56 versus 28.40, sum of replicates 1 and 2) for subtype 1-specific monoclonal antibody pool and subtype 1 EHV-1 isolates was significant (F test result, $\alpha = 0.0005$). Although significant, the magnitude of the difference between duplicates was small (1.15) as compared with an 8.13-fold difference be-

TABLE 1. Antibody titers of monoclonal antibodies against EHV-1 isolates by enzyme immunofiltration assay

EHV-1 isolate[a]	Titer of the following monoclonal antibody[b]					
	13B2	16C12	16H9	19B8	20F3	21C5
Subtype 1						
T5	80	320	120	<10	<10	48
T19	120	320	240	<10	<10	<10
T93	80	240	60	<10	<10	<10
T186	80	240	80	<10	<10	<10
T203	120	480	80	<10	<10	<10
T246	65	320	120	<10	<10	<10
T391	120	240	80	<10	<10	20
T426	120	440	160	<10	<10	<10
T453	120	320	240	<10	<10	20
T495	120	320	160	<10	<10	<10
A183	480	2,560	320	ND[c]	ND	ND
Subtype 2						
T3	<10	<10	<10	320	480	240
T45	<10	<10	<10	320	480	480
T85	<10	<10	<10	480	640	640
T161	<10	<10	<10	240	640	320
T200	<10	<10	<10	240	480	640
T213	<10	<10	<10	120	480	1,280
T288	<10	<10	<10	240	560	240
T369	<10	<10	<10	320	480	960
T463	<10	<10	<10	320	800	640
T2	ND	ND	ND	240	320	1,280

[a] The virus isolates were recovered from 21 epizootiologically unrelated outbreaks of EHV-1 abortion or respiratory tract disease that occurred in U.S. horses between 1941 and 1983. The isolates were subtyped by restriction endonuclease analysis as described previously (1).

[b] Each value represents the mean of two replicates expressed as the reciprocal of the highest dilution of hybrid cell culture fluids resulting in an A_{490} of 0.3 or more in an enzyme immunofiltration assay with virus-infected KyED cells as antigen.

[c] ND, Not determined.

TABLE 2. Subtyping of EHV-1 isolates with monoclonal antibodies by enzyme immunofiltration and indirect immunofluorescence assays

Subtype of EHV-1 isolates	Results of tests with the following ascites pool[a]			
	Anti-subtype 1		Anti-subtype 2	
	Filtration	Fluorescence	Filtration	Fluorescence
1	1.22 ± 0.29 (0.66–1.97)	+	0.12 ± 0.02 (0.07–0.17)	−
2	0.15 ± 0.05 (0.09–0.31)	−	0.96 ± 0.50 (0.35–1.96)	+

[a] Filtration is expressed as the mean immunofiltration A_{490} ± standard deviation of the color development resulting from testing duplicates of 25 EHV-1 isolates with each ascites pool in an enzyme immunofiltration assay. Values in parentheses represent the range of absorbance values of each group of 25 isolates. Controls included uninfected KyED cells reacted with each hybridoma ascites pool, and normal ascites reacted at 1:10,000 or 1:3,000 with each of the 50 EHV-1 isolates; all controls had a mean absorbance of less than 0.3. The difference between the absorbance means of each group of 25 EHV-1 isolates when reacted with the homologous or heterologous ascites pool was significant ($P < 0.01$ by Student's t test). Fluorescence is expressed as positive (+) or negative (−) fluorescent staining of methanol-fixed virus-infected KyED cells after incubation with monoclonal antibody pool and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Cappel Laboratories). Uninfected KyED cells exhibited no fluorescence with either monoclonal antibody pool.

tween subtype 1 and 2 EHV-1 isolates when reacted with subtype 1-specific monoclonal antibody pool. There was no overlap in the range of absorbance values between either EHV-1 subtype when reacted with the two subtype-specific monoclonal antibody pools (Table 2). The EHV-1 isolates listed in Table 2 originated from epizootiologically unrelated outbreaks of EHV-1 abortion and respiratory tract disease that occurred in U.S. horses during the past 20 years (1). All 50 EHV-1 isolates were correctly typed with the immunofiltration assay.

The immunofluorescence assay also accurately indicated the subtype of EHV-1 infecting KyED cells (Table 2). Both pools gave a cytoplasmic fluorescence that can be described as a pinpoint dot pattern. This pattern of immunofluorescence has also been reported for monoclonal antibodies reacting to HSV-2-infected cells (11). The subtype 2 pool of monoclonal antibodies showed weak fluorescence with many subtype 2 strains of EHV-1 when used at a 1:10,000 dilution. The intensity of fluorescence was increased when a lower dilution of monoclonal antibody (1:3,000) was applied.

DISCUSSION

This report describes the production and use of subtype-specific monoclonal antibody pools to type field isolates of EHV-1. The use of a pool rather than single monoclonal antibodies diminishes the chance that an isolate will escape immune recognition as a result of a loss or alteration in the antigenic determinant that is recognized by one particular antibody.

The enzyme immunofiltration and indirect immunofluorescence assays were used to rapidly subtype EHV-1 isolates. In both assays, the two EHV-1 subtype-specific monoclonal antibody pools exhibited a distinct difference in the strength of their reaction with EHV-1 isolates of the homologous subtype as compared with that exhibited by isolates belonging to the heterologous subtype. Inspection of the range and mean values exhibited in the immunofiltration assay (Table 2) showed that the EHV-1 subtype 2-specific monoclonal antibody pool reacted more weakly and with greater variation with homologous strains of EHV-1 than did the subtype 1-specific antibody pool. A possible explanation for this phenomenon is that EHV-1 subtype 2-infected cells may contain a lower concentration of the monoclonal target antigen on their cell surface, thus reducing the available antibody-binding sites. In support of this hypothesis is the demonstration by a Western blotting technique that the two antibody pools react with different target antigens of the virus (unpublished data). Although several individual EHV-1 subtype 2 isolates exhibited immunofiltration values for the homologous reactions that were low and close to the highest value (0.31) observed for the heterologous reaction, the difference between the homologous and heterologous reactions for all but one individual subtype 2 specimen was never less than 0.20 (a twofold difference), thus allowing clear identification of subtype.

The enzyme immunofiltration and indirect immunofluorescence assays accurately indicated the subtype of 50 independent isolates of EHV-1. Because of the diverse and independent origins of these isolates that had been correctly typed, one may speculate that virtually any field isolate of EHV-1 should be recognized by one of the pools of monoclonal antibodies. Previously, the lengthy and technically specialized restriction endonuclease fingerprinting technique was the only unambiguous method for typing isolates of EHV-1 (1). The immunofiltration and immunofluorescence assays are rapid and can be executed in 3 h after viral isolation in tissue culture. They require small quantities of reagents and are amenable to the simultaneous characterization of many EHV-1 specimens.

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LITERATURE CITED

1. **Allen, G. P., M. R. Yeargan, and L. W. Turtinen.** 1983. Molecular epizootiologic studies of equine herpesvirus 1 infections by restriction endonuclease fingerprinting of viral DNA. Am. J. Vet. Res. **44**:263–271.
2. **Balachandran, N., B. Frame, M. Chernesky, E. Kraiselburd, Y. Kouri, D. Garcia, C. Lavery, and W. E. Rawls.** 1982. Identification and typing of herpes simplex viruses with monoclonal antibodies. J. Clin. Microbiol. **16**:205–208.
3. **Bryans, J.** 1980. Herpesviral diseases affecting reproduction in the horse. Vet. Clin. North Am. Large Anim. Pract. **2**:303–312.
4. **Burrows, R., and D. Goodridge.** 1975. Experimental studies on equine herpesvirus type 1 infections. J. Reprod. Fert. **23**(Suppl.):611–615.
5. **Campbell, T., and M. Studdert.** 1983. Equine herpesvirus type-1 (EHV-1). Vet. Bull. **53**:135–146.
6. **Doll, E., and J. Bryans.** 1963. Epizootiology of equine viral rhinopneumonitis. J. Am. Vet. Med. Assoc. **142**:31–37.
7. **Doll, E., and J. Bryans.** 1963. A planned infection program for immunizing mares against viral rhinopneumonitis. Cornell Vet. **53**:249–262.
8. **Goding, J. W.** 1983. Monoclonal antibodies: principles and practice. Academic Press, Inc. Ltd., London, England.
9. **Nowinski, R. C., M. E. Lostrom, M. R. Tam, M. R. Stone, and W. M. Burnette.** 1979. The isolation of hybrid cell lines producing monoclonal antibodies against the p15(E) protein of murine leukemia virus. Virology **93**:111–126.
10. **Pereira, L., D. V. Dondero, D. Gallo, V. Devlin, and J. D. Woodie.** 1982. Serological analysis of herpes simplex virus types

1 and 2 with monoclonal antibodies. Infect. Immun. **35**:363–367.
11. **Peterson, E., O. W. Schmidt, L. C. Goldstein, R. C. Nowinski, and L. Corey.** 1983. Typing of clinical herpes simplex virus isolates with mouse monoclonal antibodies to herpes simplex virus types 1 and 2: comparison with type-specific rabbit antisera and restriction endonuclease analysis of viral DNA. J. Clin. Microbiol. **17**:92–96.
12. **Richman, D. D., P. H. Cleveland, and M. N. Oxman.** 1982. A rapid enzyme immunofiltration technique using monoclonal antibodies to serotype herpes simplex virus. J. Med. Virol. **9**:299–305.
13. **Sabin, M., G. Robertson, and J. Whalley.** 1981. Differentiation of subtypes of equine herpesvirus 1 by restriction endonuclease analysis. Aust. Vet. J. **57**:148–149.
14. **Shimizu, T., R. Ishizaki, S. Ishis, Y. Kawakami, K. Sugimura, and M. Matumoto.** 1959. Isolation of equine abortion virus from natural cases of equine abortion in horse kidney cell culture. Jpn. J. Exp. Med. **29**:643–649.
15. **Studdert, M., T. Simpson, and B. Roizman.** 1981. Differentiation of respiratory and abortigenic isolates of equine herpesvirus 1 by restriction endonucleases. Science **214**:562–564.
16. **Turtinen, L., G. Allen, R. Darlington, and J. Bryans.** 1981. Serologic and molecular comparisons of several equine herpesvirus 1 strains. Am. J. Vet. Res. **42**:2099–2104.