Antigenic Relationship Between the Herpesviruses of Infectious Bovine Rhinotracheitis, Marek's Disease, and Burkitt's Lymphoma

D. L. EVANS, J. W. BARNETT, J. M. BOWEN, AND L. DMOCHOWSKI

Department of Virology, The University of Texas at Houston, M. D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston, Texas 77025

Received for publication 28 March 1972

Common herpesvirus (HV) antigens in infectious bovine rhinotracheitis (IBR), Marek's disease (MDV), and Burkitt's lymphoma (EBV) were found. Immunodiffusion tests in 0.7% agarose demonstrated a line of identity with the HV preparations by using specific antisera prepared against, IBR, MDV, and EBV. These common antigens were found to consist of multiple components: i.e., at least two MDV antigens were identical to IBR and EBV components when subjected to immunoelectrophoresis in 0.7% agarose. Indirect immunofluorescence testing of EBV strain P₃HR-1 and IBR-infected embryonic bovine kidney cells, with antisera prepared against partially purified IBR, MDV, and EBV antigens, revealed identical activity of the three antisera as demonstrated by brilliant nuclear fluorescence (perinuclear clumping) in P₃HR-1 cells and evenly distributed cytoplasmic activity in 18-hr IBR-infected bovine kidney cell cultures. Initial physical-chemical studies of the partially purified antigens were carried out by differential centrifugation cycles (6,000, 25,000 and 100,000 $\times g$), rate zonal centrifugation in 5 to 20% sucrose density gradients, and analysis by disc electrophoresis in 5 and 7% polyacrylamide gels. These studies revealed similar molecular weight ($>1,000,000$) and size characteristics and similar electrophoretic mobilities among the three partially purified HV antigens.

Herpes-type viruses comprise a group of ubiquitous agents; some of these have been linked with neoplasia in man, simians, rabbits, chickens, and amphibians. Incriminating evidence has been provided primarily by morphological data. However, some immunological relationships have been tentatively established.

Although group-specific antigens of some mammalian leukemia viruses (4, 5) have been demonstrated by immunoprecipitation reactions, similar studies on antigenic components in herpesviruses associated with oncogenesis in animals and man have not shown conclusive results.

A herpes viral antigen associated with Lucké renal adenocarcinoma (3) of the frog has been reported as identical to an antigen of the herpesvirus (HV) associated with the African Burkitt's lymphoma of man, Epstein-Barr Virus (EBV). S. Kato et al. (*In Oncogenesis and herpes-type viruses*, 20-25 June, Cambridge, England, p. 73) and others (8, 10) have shown that at least one antigen of the HV associated with Marek's disease (MDV) is identical to an EBV antigen obtained from a Burkitt's lymphoma cell line

(P₃HR-1). In this report, however, normal human sera also gave lines of identity when tested against these antigen (MDV and EBV) preparations. Indirect and direct immunofluorescence tests (9) using sera from MDV-infected chickens, "normal humans", and patients with Burkitt's lymphoma or nasopharyngeal carcinoma have shown reactions in varying degrees with MDV-infected duck and quail embryo fibroblasts and with EBV-containing P₃HR-1 cells. Other investigations, however, (11) have not confirmed these results.

Group- and type-specific antigens of some herpesviruses have been reported (J. Kirkwood et al. *In Oncogenesis and herpes-type viruses*, 20-25 June, 1971, Cambridge, England, p. 70). Group-specific antigens were found in the herpesviruses of Lucké renal adenocarcinoma, Burkitt's lymphoma, and *Herpes simplex*. Preparations of Lucké adenocarcinoma, MDV, *H. simplex*, and cytomegaloviruses gave reactions of identity in immunodiffusion (ID) tests. These group-specific antigens are sedimented after 1-hr at 80,000 $\times g$ and are excluded from 2% agar in ID tests. Naturally occurring antibodies to

the above agents are widespread, however. Type-specific antigens were found in association with EBV, MDV, *H. simplex*, and cytomegalovirus preparations.

Infectious bovine rhinotracheitis (IBR) is caused by a HV which is antigenically related to, or identical with, the HV which causes infectious bovine keratoconjunctivitis (1). The antigenic relatedness of the IBR virus to other herpes-viruses has not been widely investigated.

The present study presents data on the isolation and partial characterization of common antigens in IBR, Burkitt's lymphoma (EBV), and MDV. Additional evidence is provided by demonstration of cross-reactivity in indirect immunofluorescence tests, by using specific antisera prepared against these antigens, and in immunodiffusion and immunoelectrophoresis determinations.

MATERIALS AND METHODS

Cell cultures and viruses. EBV was obtained from P₃HR-1 cells maintained as a continuous herpes-type virus-producing cell line (The John L. Smith Memorial for Cancer Research, Chas. Pfizer and Co., Inc., Maywood, N.J.). IBR virus (O. M. Franklin Serum Co., Amarillo, Texas) was grown in embryonic bovine kidney cells (BKC) and virus was harvested 18-hr postinoculation of each passage. Marek's disease antigen was obtained from MDV-infectious whole blood (UA-1 strain, *unpublished data*) and from MDV-infected duck embryo fibroblasts (USDA, East Lansing, Michigan).

Antigen preparation. All antigens were prepared in a similar fashion. Virus-containing cells and MDV-infectious whole blood were disrupted by three cycles of freezing and thawing or by sonic oscillation (Sonifer, Branson Instruments Inc., Stanford, Conn.). Suspensions were subjected to differential centrifugation at $6,000 \times g$ (20 min), $25,000 \times g$ (1 hr), and $100,000 \times g$ (2.5 hr). Each pellet and the supernatant fluid samples of each cycle were tested for the presence of the HV antigens. The pellets centrifuged at $100,000 \times g$ were suspended in phosphate-buffered saline (PBS), pH 7.2.

Immunodiffusion tests. All immunodiffusion reactions were conducted by using either 2.0 or 0.7% agarose in barbital buffer (pH 8.6) containing 0.87 M NaCl and 2 ml of a 10% solution of sodium azide. Control reagents for all tests consisted of noninfected bovine kidney cells, whole human embryo and human kidney cell concentrates, and normal, human, buffy coat cells.

Immunoelectrophoresis. Electrophoresis was carried out (Buchler Instruments; d-c power supply) by using 0.7% agarose in a barbital buffer (pH 8.6; ionic strength 0.01) for 100 min at 5 ma per slide.

Immunofluorescence. P₃HR-1 cells and IBR-infected and noninfected BKC were air dried in the cold, fixed in cold acetone for 30 min, washed with PBS (five times), and air dried at room temperature.

Cells were flooded with each antiserum and incu-

bated for 30 min at 37 C. After two 5-min washings with PBS the slides were air dried. Fluorescein-conjugated goat antirabbit-IgG globulin (Microbiological Associates, Inc.) was then added to the cells, and the mixture was incubated at 37 C. After 30 min, the slides were washed several times with PBS and examined. All antisera were absorbed with lyophilized bovine serum, lyophilized chicken serum, and guinea pig kidney powder (1 mg/ml in each case).

Antisera production. The partially purified antigen preparations ($100,000 \times g$ pellets) were injected into three different (8-week-old) rabbits over a 120-day period of time. Freund's adjuvant (complete) was used in 1:1 proportions for the first subcutaneous inoculations and for the nine subsequent weekly injections. The last two inoculations were given intravenously at 10-day intervals.

Disc electrophoresis. Each $100,000 \times g$ pellet was subjected to disc electrophoresis (Canalco model 300B, Rockville, Md.) in 5 and 7% polyacrylamide gels. Standard reagents and procedures were utilized (28).

Rate zonal density gradient centrifugation. The $100,000 \times g$ resuspended pellets were centrifuged in 5 to 20% (w/w) sucrose in 0.002 M tris(hydroxymethyl)aminomethane and 0.002 M ethylenediaminetetraacetate (pH 7.0) at 24,000 rev/min for 2.5 hr. Each fraction (0.5 ml) was tested for the presence of antigen by ID tests.

RESULTS

Differential centrifugation of the virus preparations followed by rabbit inoculation of the high-speed pellets ($100,000 \times g$) resulted in the production of antisera which contained antibody to virus-related antigens.

The $6,000 \times g$ and $25,000 \times g$ centrifugation cycles did not sediment the virus antigens, and activity was observed only in the resuspended high-speed pellets ($100,000 \times g$) and in the supernatant fluids from the two low-speed cycles. The $100,000 \times g$ supernatant fluids did not contain detectable antigen.

With the antisera to each of the HV antigen preparations, the antigenic similarities between IBR, EBV, and MDV were examined. Cross-reactivities were observed when ID tests were conducted. In Fig. 1 the peripheral well arrangement in 0.7% agarose is EBV antigen, 1; IBR antigen, 2; bovine serum, 3; chicken serum, 4; whole human embryo cell concentrate, 5; and MDV antigen, 6. The center well contains antiserum prepared against the partially purified IBR virus preparation. One line of identity is seen with the bovine serum contaminant extending from wells 3, 2, and 1. The common antigens in the IBR, EBV, and MDV preparations (arrow) are demonstrated by a line of identity extending from wells 2, 1, and 6. Also, there is an additional precipitation line between well 2

FIG. 1. Immunodiffusion reaction of IBR antisera in 0.7% agarose (center well) against: 1, EBV; 2, IBR; 3, bovine serum; 4, chicken serum; 5, whole human embryo; and 6, MDV antigen preparations. Arrow indicates line of identity of HV antigens.

and the center well and between well 6 and the center well. A similar reaction is seen when antiserum prepared against the partially purified EBV antigen is in the center well (Fig. 2) with the same peripheral well arrangement as in Fig. 1. Again there is a bovine serum contaminant precipitation line connecting wells 3, 2, and 1. The common HV antigens are demonstrated by the line of identity between wells 6, 1, and 2. One additional faint line is seen between well 1 and the center well. In tests using the MDV antisera, similar ID results were obtained, except no bovine serum contaminant was observed. All above described results were seen only in 0.7% agarose; when 0.2% agarose was used no reactions were observed with the antigen preparations.

Immunoelectrophoresis of the partially purified antigen preparations further demonstrated antigenic similarities. In Fig. 3, electrophoresis was carried out on EBV (upper well—U) and IBR (lower well—L) antigen preparations with EBV antisera in the center trough. The arrows indicate the main virus antigens which are identical. Electrophoretic migration is from right (cathode) to left (anode). The other precipitation arcs represent bovine serum contaminants (controls consisted of substitution of bovine serum for one of the antigen preparations, followed by electrophoresis, etc.). Figure 4 shows at least two MDV antigens (upper well—U) in common with EBV (lower well—L; arrows) when reacted with EBV antisera. The MDV

preparations did not contain any bovine serum or any other contaminant (within the detection limitations of this test) so all arcs observed represent virus-specific components. The MDV preparations contain two or three antigenic components in common with EBV. In Fig. 5 at least two or more MDV antigens (upper well—U) again are identical with IBR (lower well—L) in the presence of EBV antisera. Antisera prepared against IBR reacted against EBV (upper well—U) and IBR (lower well—L; Fig. 6), MDV (upper—U) and EBV (lower—L; Fig. 7), and MDV (upper—U) and IBR (lower—U; Fig. 8) virus preparations. Again there is at least one common component in these antigen preparations (bovine serum again served as a control). Common multiple components are not seen when this antiserum is used, except for the MDV preparation where at least two common antigens can be observed.

Immunofluorescence tests were conducted using antisera prepared against each of the partially purified antigens. P₃HR-1 cells producing EBV and IBR-infected and noninfected BKC were examined. P₃HR-1 cells labeled with antisera to IBR (Fig. 9), EBV (Fig. 10), and MDV (Fig. 11) all show a similar type of fluorescence. Nuclear fluorescence was observed in approximately 75% of the cells. Perinuclear clumps of brightly fluorescing material are characteristic.

In IBR-infected embryonic BKC, evenly distributed cytoplasmic fluorescence was observed

FIG. 2. Immunodiffusion test of EBV antisera (center well) in 0.7% agarose against: 1, EBV; 2, IBR; 3, bovine serum; 4, chicken serum; 5, whole human embryo; and 6, MDV antigen preparations. Arrow indicates line of HV antigen identity.

FIG. 3. *Immunoelectrophoresis of EBV (upper) and IBR (lower) antigens against EBV antisera (trough). Arrow indicates common antigen.*

FIG. 4. *Immunoelectrophoresis of MDV (upper) and EBV (lower) antigens against EBV antisera (trough). Arrows indicate common HV antigens.*

FIG. 5. *Immunoelectrophoresis of MDV (upper) and IBR (lower) against EBV antisera. Arrows indicate common HV antigens.*

FIG. 6. Immunoelectrophoresis of EBV (upper) and IBR (lower) antigen preparations against IBR antisera (trough). Arrows indicate common HV components.

FIG. 7. Immunoelectrophoresis of MDV (upper) and EBV (lower) antigens against IBR antisera (trough). Arrows indicate common HV components.

FIG. 8. Immunoelectrophoresis of MDV (upper) and IBR (lower) HV antigens against antisera prepared against IBR virus. Arrows indicate common antigens.

FIG. 9. Immunofluorescence test of *P₃HR-1* cells labeled with conjugated antisera to IBR.

FIG. 10. Immunofluorescence test of *P₃HR-1* cells labeled with conjugated antisera to EBV antigen preparations.

in 75% or more of the cells when antisera to IBR (Fig. 12), EBV (Fig. 13), and MDV (Fig. 14) were tested with these cells. No nuclear fluorescence was observed. Noninfected BKC

showed only a small amount of autofluorescence when tested with specific antisera (Fig. 15). Additional tests using infected BKC and *P₃HR-1* cells with normal rabbit sera showed no reactivity.

The HV partially purified antigens prepared by differential centrifugation ($100,000 \times g$ pellets) were further analyzed by rate zonal centrifugation on sucrose density gradients. Gradient fractions of 0.5 ml each were tested by ID against antisera to MDV, EBV, and IBR antigens. As can be seen from the data in Table 1, the three antisera detected antigens in similar fractions. Positive fractions were pooled, concentrated by dialysis against 10% polyethylene glycol, and analyzed by disc electrophoresis.

Disc electrophoresis in 7 and 5% polyacrylamide gels of the three antigens, as prepared by density gradient centrifugation, revealed similar electrophoretic and molecular-size characteristics among the three HV antigens (Fig. 16). In Fig. 16, the arrow indicates the position of the common antigens in gel 1 (IBR), 2 (EBV), and 3 (MDV) preparations after electrophoresis on 5% polyacrylamide gels. The polyacrylamide gels were imbedded in 0.7% agarose immediately after electrophoresis. Antisera were placed in troughs (Fig. 17) which allowed a precipitation reaction (arrow) to occur in the area where the HV antigen had migrated. This area coincided with the location of the common IBR,

FIG. 11. Immunofluorescence test of *P₃HR-1* cells labeled with conjugated antisera to MDV virus preparation.

FIG. 12. *Immunofluorescence test of IBR-infected bovine kidney cells labeled with conjugated antisera prepared against IBR virus.*

FIG. 13. *Immunofluorescence test of IBR-infected bovine kidney cells labeled with conjugated antisera against EBV preparations.*

FIG. 14. *Immunofluorescence tests of IBR-infected bovine kidney cells labeled with antisera against MDV antigen preparations.*

FIG. 15. *Immunofluorescence test of noninfected bovine kidney cells against specific labeled antisera prepared against IBR virus preparations. Control test showing no nonspecific fluorescence.*

TABLE 1. *Immunodiffusion tests of density gradient fractions from partially purified preparations of Burkitt's lymphoma (EBV), Marek's disease (MDV), and infectious bovine rhinotracheitis (IBR) virus antigens*

Antigen	Fractions which gave precipitin lines with antisera to:		
	IBR	EBV	MDV
MDV	15-19	15-19	16-19
IBR	13-19	13-19	14-19
EBV	12-19	13-19	13-19

EBV, and MDV components as determined by staining of the polyacrylamide gels. When these same preparations were electrophoresed in 7% polyacrylamide gels, they traveled a distance of only 1 to 2 mm.

DISCUSSION

The role of the herpesviruses in some neoplastic disorders remains an unanswered question. There is a growing body of evidence implicating the herpesviruses seen in EBV and MDV, for example, as the causative agents in these lymphoproliferative diseases. The herpes-type virus has been observed in divergent types of tissues from many different kinds of animals. Most of these findings have not coincided with a particular disease entity so that etiological relationships have been difficult to establish or remain undetermined. In addition, a fairly large population of "naturally" occurring antibodies to some of these HV (i.e., EBV) may be seen in up to 90% of a population of healthy chimpanzees (6), normal adults and children, and in patients with divergent types of malignancies. Some investigators (J. Kirkwood et al., as cited previously) have proposed that the presence of type-specific antibodies is related to infection or malignancy caused by a particular HV, whereas group-specific antigens are shared by HV generally.

In the present study, HV antigens partially purified from IBR, MDV, and EBV preparations were shown to be related. The EBV and MDV components had similar sedimentation characteristics in differential centrifugation cycles like those that have been previously described (J. Kirkwood et al., as cited previously). IBR antigen exhibited similar properties in terms of the centrifugation cycle fractions which were reactive in ID tests with the three antisera. In addition, these antigens (IBR, EBV, and MDV) did not migrate through 2% agarose, but required 0.7% agarose in order to be observed as lines of identity in ID tests.

Previous studies have demonstrated an antigenic relationship between EBV and MDV in ID tests (8), but no studies have shown a common antigenic component in EBV and IBR and IBR and MDV preparations. These relationships have clearly been shown by ID tests in this study. These components, however, were found to consist of multiple antigens when the same virus antigen preparations were subjected to immunoelectrophoresis. The EBV and MDV virus preparations had two or more antigens in common. This was also the case for the IBR and MDV comparisons. Multiple, common, specific lines were not readily found when IBR and EBV antigens were subjected to electrophoresis. The group-specific antigens of some herpesviruses consist of several components instead of just one as has previously been suggested (J. Kirkwood et al., as cited previously). One must assume, therefore, that the original criteria for group specificity are incomplete, and the number and kinds of shared components

FIG. 16. *Disc electrophoresis of IBR (1), EBV (2), and MDV (3) partially purified common antigens (arrow) in 5% polyacrylamide gels.*

FIG. 17. Disc electrophoresis of the IBR antigen preparation followed by embedding the gel in 0.7% agarose. Trough contains IBR antisera and arrow indicates arc of specific precipitation.

by any given herpesvirus depend on the methods of antigen preparation and the sensitivities of the tests for each particular system.

Further evidence for the partial purity of these antigen preparations was provided by sedimentation velocity in density gradient centrifugation. The similar fractions which contained antigenic activity exhibited similar electrophoretic mobilities and molecular size characteristics in 5% polyacrylamide gel electrophoresis. The inability of the antigens to enter 7% polyacrylamide gels of more than 1 to 2 mm indicated a molecular-weight range greater than one million. The exact molecular weights have not, at present, been determined.

Indirect immunofluorescence tests using antisera against each of the partially purified antigens gave strong supportive evidence for the common HV components. Earlier results, (7, 13) from human sera and P₃HR-1 cells, revealed strong fluorescence in the cells with the appearance of cellular aggregation of fluorescing material, granular staining, and the presence of "inclusion" body formations in some cells. Sim-

ilar results were reported (8) when sera from MDV-infected chickens were added to P₃HR-1 cells. In the present study, P₃HR-1 cells showed brilliant nuclear fluorescence very similar to that previously described (8). EBV, IBR, and MDV antisera all produced similar results in these tests, showing granularity and nuclear clumping of brightly fluorescent material. These particular cells (P₃HR-1) have previously been shown to produce herpesviruses.

When similar tests were conducted with IBR-infected BKC cultures very strong cytoplasmic fluorescence was observed in the presence of the antisera to IBR, EBV, and MDV. Intense cytoplasmic fluorescence has been observed in 12- to 18-hr IBR-infected BKC cultures by specific antisera to IBR (12). No nuclear fluorescence was seen, and the overall appearance of the cells was very much like that seen in the present study. Again, it was previously demonstrated that the BKC cultures used in the present study contained herpesviruses (J. M. Bowen et al., *Bacteriol. Proc.*, p. 158, 1967).

Although immunofluorescence tests were not

carried out on MDV-infected cells, the identical fluorescent activity of the MDV antisera on each of the two different cell lines (P₃HR-1 and BKC) provided ample evidence of antigenic identity between MDV, IBR, and EBV preparations.

The addition of IBR to the growing number of herpesviruses (EBV, MDV, Lucké, *H. simplex*, and cytomegaloviruses) which share common components adds additional evidence to support the occurrence of HV group-related antigens in diverse species of animals.

However, because of the ubiquitous occurrences of herpesviruses, careful interpretations must be presented to account for the widespread finding of "naturally" occurring EBV antibodies, the appearance of "herpesvirus" antigens in nasopharyngeal carcinoma and cervical carcinoma, and the finding of HV particles in various apparently normal tissues from a wide variety of animals.

The biological significance of all of the above discussed phenomena remains unanswered and can be established only after more is known about the relationship between herpesviruses and disease processes.

ACKNOWLEDGMENTS

This study was supported by Public Health Service contract PH43-NCI-E-65-604 and by grants CA-05831 and RR-05511 from the National Cancer Institute.

Marek's disease virus and antisera were kindly supplied by L. T. Patterson and J. N. Beasley (UA-1 strain; Univ. of Arkansas, Fayetteville, Ark.) and by B. R. Burmester (US Agricultural Research Service, East Lansing, Mich.).

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