Identification and characterization of the major proteins of malignant catarrhal fever virus

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Malignant catarrhal fever virus (MCFV), a gammaherpesvirus, causes a severe inflammatory and lymphoproliferative disease of cattle and other susceptible ruminants. Polyclonal antisera and monoclonal antibodies (MAbs) to the Minnesota isolate of MCFV were produced and used to examine the characteristics of the viral proteins. Immunoprecipitation of antigens of the Minnesota isolate of MCFV with polyclonal antisera revealed at least 11 proteins with molecular masses ranging from 17 kDa to 145 kDa. Among 279 candidate anti-MCFV hybridomas, 14 were selected and clustered into six groups on the basis of the patterns of reactivity to viral proteins in immunoprecipitation and immunoblot. The group I MAbs exhibited strong neutralizing activity and recognized a glycosylation-dependent conformational epitope on a 110 kDa protein. The MAbs in group II bound a non-neutralizing conformational epitope on a 130 kDa non-glycosylated protein. A glycosylated protein complex of 115/110/105/78/45 kDa moieties was identified by the MAbs in group III. The MAbs in groups IV, V and VI reacted with non-glycosylated proteins of 36/34 kDa, 24 kDa and 17 kDa, respectively. Comparison of three MCFV isolates [the Minnesota isolate, the Austrian isolate (Au-732) and the African prototype isolate (WC-11)] revealed no apparent differences in immunoprecipitation patterns with the single exception that the 110 kDa protein of WC-11 was slightly smaller than its counterpart in the Minnesota isolate.

Introduction

Malignant catarrhal fever (MCF), a severe lymphoproliferative and inflammatory syndrome of domestic cattle, deer and certain other susceptible ruminants, is caused by a gammaherpesvirus (Plowright, 1990). The virus, its epidemiology, and the pathogenesis of the disease it causes in susceptible hosts, are poorly characterized at present. The prototype virus, alcelaphine herpesvirus 1 (AHV-1) is indigenous in wildebeest, in which there is inapparent infection (Plowright et al., 1960; Plowright, 1968). Thus classical MCF due to AHV-1 is referred to as wildebeest-associated MCF (WA-MCF). The term sheep-associated MCF (SA-MCF) is used in deference to the presumed source of the virus (i.e. domestic sheep) in most cases of MCF in domestic cattle (Rossiter, 1982; Heuschele et al., 1984; Harkness, 1985; Plowright, 1990). Although the SA-MCF virus has never been successfully isolated, substantial epidemiological and serological evidence indicates that a virus closely related to AHV-1 exists in sheep and is transmitted, often at lambing, to cattle, deer or other susceptible ruminants (Pierson et al., 1973; Reid et al., 1979).

Characterization of the SA-MCF agent has languished due to the lack of availability of an isolated virus. Even for the prototype AHV-1, only limited information concerning the viral structures is available. Adams & Hutt-Fletcher (1990) identified a major envelope protein complex of AHV-1 that carried neutralizing epitopes. Additional characterization of the components of MCFV is needed to serve as the basis for development of diagnostic and prophylactic reagents, and for dissecting the complicated pathogenesis of the disease.

In the present study a panel of monoclonal antibodies (MAbs) was produced against the Minnesota isolate of MCFV. Using these MAbs we have identified and partially characterized the major proteins of MCFV, compared the protein patterns of three MCFV isolates, and identified a dominant neutralizing epitope.

Methods

Viruses, cell culture and antisera. The Minnesota isolate of MCFV (MN-MCFV) originally derived in Minnesota, USA, from a clinical case of MCF in a cow enclosed along with sheep (Hamdy et al., 1978),
WC-11 (cell culture passaged strain of AHV-1), fetal mouflon sheep kidney (FMSK) cells and sera from wildebeest naturally infected with AHV-1 were kindly provided by W. Heuschele, Center for Reproduction of Endangered Species, Zoological Society of San Diego, San Diego, Calif., USA. A putative SA-MCFV isolate from Austria (Schuller et al., 1990) (designated herein as Au-732) and bovine antiserum to this isolate were kindly provided by J. Pearson, National Veterinary Service Laboratory, USDA Animal and Plant Inspection Service, Ames, Iowa, USA. The rabbit antiserum against the WC-11 strain of AHV-1 was obtained from a commercial source (Cytimmune). Antisera to MN-MCFV were raised in rabbits by immunization with MN-MCFV antigens prepared by discontinuous sucrose gradient centrifugation as described previously (Li et al., 1991). Antigens for immunoblot were prepared in the same way. Serum from sheep experimentally infected with a US isolate of AHV-1 (Castro et al., 1982; Wan, 1986) was kindly provided by A. Castro (Pennsylvania State University, State College, PA, USA). Bovine herpesvirus 1 (BHV-1) (LA strain, ATCC VR-188) and bovine herpesvirus 4 (BHV-4) (DN-599, ATCC VR-631) were obtained from the American Type Culture Collection. All MCFV isolates were propagated on FMSK cells grown in high glucose Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum. Viral infectivity was measured by a plaque assay in which dilutions of virus were inoculated onto FMSK monolayers in six-well plates and overlayed with DMEM containing 2 % methylcellulose. After 5 days, plaques were stained with crystal violet solution and counted microscopically.

Monoclonal antibodies. Adult BALB/c mice were each injected subcutaneously with 20 μg/mouse of MN-MCFV antigens, emulsified in 0.1 ml of Ribi MPL + TDM Emulsion (Ribi Immunochern Research). Hybridomas were produced by previously described methods (Davis, 1988; Li et al., 1991). Hybridomas were screened for reactivity to MCFV antigens in an enzyme-linked immunosorbent assay (ELISA) and an indirect immunofluorescence assay, and further characterized by immunoprecipitation and immunoblot. Ascitic fluid was produced and immunoglobulins were quantified as described previously (Li et al., 1991).

Viral neutralization. All assays were performed in triplicate. Volumes of medium containing 200 p.f.u. MN-MCFV, which was titrated on FMSK monolayers as described above, were mixed with 100 μl of MAb (20 μg/ml) and incubated at 37 °C for 1 h. The reaction mixture was then added to the monolayer of FMSK cells in six-well plates. After 2 h adsorption, 3 ml of methylcellulose overlay-medium was added and incubation continued at 37 °C for 5 days. After fixing and staining, plaques were counted microscopically and the percentage plaque reduction relative to controls without antibody was calculated. Any mean percentage plaque reduction, calculated from triplicate assays, which exceeded the upper limit of the 99 % confidence interval of control wells (n = 7), was considered significant neutralization.

Protein labelling, tunicamycin treatment and immunoprecipitation. FMSK cells were infected with one of the three MCFV isolates (MN-MCFV, Au-732 or WC-11) at an m.o.i. of 3. At 3 h post-infection, monolayers were washed and equilibrated with methionine-free DMEM for 2 h and incubated in the presence of 40 μCi/ml [35S]methionine or [3H]glucosamine (New England Nuclear). The cultures were harvested when 90 % of cells showed cytopathic effects, about 96 h post-infection. Following harvest, cells were disrupted by treatment with 1 % Nonidet P-40 at 4 °C for 30 min, and sonication for 30 s (Shen et al., 1991). The supernatant containing labelled proteins was clarified by centrifugation at 50 000 × g for 120 min and stored at −70 °C.

The procedure for tunicamycin experiment was described previously (Li et al., 1991). Briefly, the cells were infected as described above. Three hours after infection the monolayers were rinsed and incubated in methionine-free DMEM containing 2.5 μg/ml tunicamycin (Boehringer Mannheim) and 40 μCi/ml [35S]methionine. The labelled proteins were prepared as described above.

For immunoprecipitation, the labelled proteins (106 c.p.m. of trichloroacetic acid-precipitable material) were mixed with antisera or MAbs and antigen–antibody complexes precipitated with recombinant protein G beads (GENEX). The precipitates were washed, solubilized and subjected to SDS-PAGE (Li et al., 1991).

Immunoblot. Separated viral protein bands were transferred from polyacrylamide gels to nitrocellulose filters. After blocking with 5 % non-fat dried milk in phosphate-buffered saline (PBS), the filters were exposed to dilutions of antisera or MAbs. Immunoglobulins bound to the protein bands on the filters were visualized by anti-Ig-peroxidase conjugate using chemiluminescence (Amersham).

Results

Proteins identified by polyclonal antisera

Lysates from [35S]methionine-labelled FMSK cells infected with MN-MCFV and mock-infected cells were immunoprecipitated with polyclonal antisera from a wildebeest naturally infected with AHV-1 or from a sheep experimentally inoculated with AHV-1, and the precipitates were analysed by SDS-PAGE. As shown in Fig. 1(a), 11 major bands were identified in immunoprecipitation, with molecular masses of 17, 24, 36, 45, 78, 105, 110, 115, 130 and 145 kDa. Non-labelled antigens from these same cells were immunoblottedted with rabbit antiserum to the WC-11 strain of virus. Only seven of the 11 major proteins identified by immunoprecipitation were detectable in immunoblot using this antiserum (Fig. 1b).

Proteins identified by monoclonal antibodies

The predominant proteins of MCFV were divided into six major groups (Table 1, Fig. 2), based on their reactivities with MAbs. Group I, represented by MAb 169-A, immunoprecipitated a 110 kDa protein that labelled with both [35S]methionine and [3H]glucosamine (Fig. 2a, Fig. 3). Group II, represented by MAb N1-A, immunoprecipitated a 130 kDa protein that labelled with [35S]methionine, but not with [3H]glucosamine (Fig. 2a, Fig. 3). Neither group I nor group II proteins were detected by the MAbs in immunoblot. Group III, represented by MAb 152-A, immunoprecipitated a protein complex containing five bands of 115/110/105/78/45 kDa, which was labelled with both [35S]methionine and [3H]glucosamine. However, only the 45 kDa protein of the complex was detected in immunoblot. In group IV and V, the MAbs (N55-A and 36-A) reacted with a 36/34 kDa protein dimer and a 24 kDa protein respectively, but only in immunoblot (Fig. 2b). In group VI, MAb N10-A both immunoprecipitated and immunoblotted a 17 kDa protein (Fig. 2b) which was not glycosylated (not shown).
Fig. 1. (a) Immunoprecipitation and immunoblotting of [35S]methionine-labelled proteins of MN-MCFV infected cell lysate (V) or uninfected cell lysate (C) with antisera from a wildebeest naturally infected with MCFV (1) and a sheep experimentally infected with a US isolate of AHV-1 (2). (b) Immunoblotting of purified MN-MCFV antigens (V) and FMSK cell antigens (C) with antiserum from a rabbit immunized with WC-11.

Fig. 2. (a) Immunoprecipitation of [35S]methionine-labelled proteins of MN-MCFV infected cell lysate (V) and uninfected cell lysate (C) with MAbs 169-A, N1-A and 152-A. (b) Immunoblotting of purified MN-MCFV antigens (V) and cell antigens (C) with MAbs N55-A, 36-A and N10-A.

Table 1. Characteristics of monoclonal antibodies to malignant catarrhal fever virus

<table>
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<th>MAb</th>
<th>Group</th>
<th>Isotype</th>
<th>GP*</th>
<th>Glycosylation-Dependent</th>
<th>Conformational</th>
<th>Immunoprecipitation (kDa)</th>
<th>Immunoblot (kDa)</th>
<th>Neutralization test‡</th>
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* GP, The protein(s) recognized by MAbs were glycosylated.
† Numbers indicate the percentage plaque reduction; the upper limit of the 99% confidence interval calculated from control wells (n = 7) was 42% plaque reduction; NS, no significant neutralizing activity; ND, not done.
Neutralization activity of MAbs

The virus-neutralizing activities of the MAbs were assessed using a plaque reduction assay. As shown in Table 1, all group I MAbs neutralized strongly (over 80% plaque reduction). Of the three MAbs in group III tested, one neutralized significantly, but at a lower level (53%) reduction. The MAbs against the other viral proteins did not significantly neutralize MN-MCFV activity (Table 1).

Comparison of immunoprecipitation patterns between MCFV isolates

The only difference observed in immunoprecipitation patterns between MN-MCFV and AHV-1 (WC-11) was that the single 110 kDa protein of AHV-1 (WC-11) was about 1 kDa smaller than its counterpart in MN-MCFV (Fig. 6). No differences were observed between MN-MCFV and Au-732 when immunoprecipitated with polyclonal antisera (data not shown).

Analysis of viral proteins under non-reducing conditions

Disulphide-linked glycoprotein complexes have been documented in several herpesviruses (Grose et al., 1984; Britt & Auger, 1986; Shen et al., 1991) including AHV-1 (Adams & Hutt-Fletcher, 1990). To confirm whether the complex in MN-MCFV had disulphide linkages similar to AHV-1, immunoprecipitation patterns were compared under reducing and non-reducing conditions (with or without 2-mercaptoethanol). The results were consistent with a previous report (Adams & Hutt-Fletcher, 1990). Under reducing conditions five bands were detected, including the 78/45 kDa bands. Under non-reducing conditions, these latter two bands dis-
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appeared and a new 120 kDa band appeared (Fig. 4), suggesting the existence of disulphide bond(s) between the 78 and 45 kDa proteins. Moreover, under non-reducing conditions the apparent size of the 110 kDa glycoprotein precipitated by MAb 169-A was approximately 2 kDa larger than under reducing conditions, indicating that disulphide bond(s) were involved in stabilization of its secondary structure.

Inhibition of glycosylation

To determine whether any of the epitopes were glycosylation-dependent, virus-infected cells grown in the presence of [\textsuperscript{35}S]methionine were treated with tunicamycin to inhibit glycosylation. The single 110 kDa protein from tunicamycin-treated cells was not recognized by its MAbs (Fig. 5), indicating that their epitopes were glycosylation-dependent. Similarly, the epitopes located on the 115/110/105/78/45 kDa protein complex (group III MAbs) were shown to be glycosylation-dependent (data not shown). In contrast, immunoprecipitation of the 130 kDa (Fig. 5) and 17 kDa (data not shown) proteins was not affected by tunicamycin treatment.

Discussion

Immunoprecipitation of [\textsuperscript{35}S]methionine-labelled proteins of MN-MCFV with antisera to three different isolates of MCFV yielded similar polypeptide patterns. At least 11 viral proteins were identified, with molecular masses ranging from 17 kDa to 145 kDa. Antigenic studies with MAbs revealed that the 11 proteins constituted six major antigenic groups. The protein complex of 115/110/105/78/45 kDa identified herein is consistent with a previous report on MCFV (Adams & Hutt-Fletcher, 1990). Five additional virus proteins with molecular masses of 130, 110, 36/34, 26 and 17 kDa were identified with MAbs in this study. Two antigenically distinct proteins of the virus co-migrated at 110 kDa. Evidence for the existence of a second single 110 kDa protein was the finding of a MAb that reacted with a unique epitope which was not present on the 110 kDa moiety of the 115/110/105/78/45 kDa complex. In addition, the size of the single 110 kDa band was decreased by about 1 to 2 kDa in WC-11, whereas the 110 kDa band in the protein complex remained unchanged (Fig. 6).

The fact that tunicamycin treatment abrogated the
binding of the MAbs in group I indicates that their epitopes are glycosylation-dependent. Failure of the MAbs to react with the 110 kDa protein in immunoblots suggests that the epitope was also conformation-dependent.

The epitope on the single 130 kDa non-glycosylated protein was also conformation-dependent. The reactivity of the epitope was abolished by heating at 95 °C for 3 min. Immunoprecipitation with polyclonal antisera from several different animal species infected or immunized with MCFV revealed a prominent 130 kDa band. This protein is non-glycosylated, immunodominant, and carries no neutralizing epitopes. It may represent a major structural component of the virion such as a capsid or tegument protein. While Adams & Hutt-Fletcher (1990) reported a glycosylated protein of 130 kDa precipitated by polyclonal antiserum to AHV-1, we could not confirm this observation. Since there is a possibility of two co-migrating 130 kDa proteins, further clarification is needed. Since the 130 kDa protein was prominently precipitated by a wide range of anti-MCFV sera, and was present in all MCFV isolates tested, this protein should be a potential candidate for diagnostic tests.

Neutralizing MAbs to MCFV have been previously reported (Adams & Hutt-Fletcher, 1990). Since the single 110 kDa protein was not identified in that study, the presence of neutralizing epitopes on this protein was not examined. In the present study the MAbs in group I to the single 110 kDa glycoprotein exhibited strong neutralizing activity (80 to 94% plaque reduction), whereas only one of the three group III MAbs tested, which bind to the glycoprotein complex, neutralized significantly (53% plaque reduction). This suggests the predominant neutralizing epitope(s) are located on the single 110 kDa protein.

Serological cross-reactivities between MCFV and other bovine herpesviruses (Heuschele, 1982; Dubuisson et al., 1989) are of considerable concern. None of the MAbs in this report reacted with BHV-1 or BHV-4 in indirect immunofluorescence assay. However, the N10-A MAb against the 17 kDa MCFV protein reacted with the 71 kDa protein of BHV-1 in immunoblot only (data not shown). Conversely, polyclonal antiserum to BHV-1 did not react with the 17 kDa MCFV protein in immunoblot. This observation suggests that the epitope for MAb N10-A on the 71 kDa protein of BHV-1 becomes exposed only after protein denaturation.

Previous comparison of MN-MCFV with AHV-1 (WC-11) by restriction fragment profiles and Southern blot hybridization revealed no significant differences between the two isolates (W.P. Heuschele, personal communication). In the present study, all 279 hybridomas reacted similarly with MN-MCFV and AHV-1 (WC-11) in indirect immunofluorescence assay and immunoprecipitation with the exception of a single protein. The 110 kDa glycoprotein of WC-11 was slightly smaller, suggesting that the gene for this protein has been truncated or alteration of the apparent mass is due to the difference in glycosylation by passage in different cell lines. A similar effect has been noted with BHV-1 (Shen et al., 1991). Though MN-MCFV and Au-732 viruses were isolated from cases reported to be associated with sheep (Hamdy et al., 1978; Schuller et al., 1990), the sheep origin of these isolates has not been confirmed. The marked similarity to each other and to AHV-1 (WC-11) suggests that all may in fact be different isolates of AHV-1.

A confirmed SA-MCFV is not currently available. The strategy of identifying viral components conserved between SA-MCFV and WA-MCFV is therefore being used in this laboratory to develop specific assays to detect the agent in infected sheep and other ruminants. Sera from a variety of ruminants naturally or experimentally infected with MCF viruses of both sheep and wildebeest origin react with several major proteins of MCFV, and compete with MAbs against these proteins. These observations have supported the recent development of a competitive-inhibition ELISA for antibody against all strains of MCFV examined to date (Li et al., 1994).

We thank Dongyue Zhuang and Soonhee Kown for excellent technical assistance.

References


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(Received 20 June 1994; Accepted 6 September 1994)