ON ENTEROPATHOGENIC BOVINE CORONAVIRUS

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INTRODUCTION

Coronaviruses cause severe and economically important intestinal infections in newborn calves which become manifest as enteritis with diarrhea and additional clinical consequences. Studies concerning the pathogenesis of enteropathogenic bovine coronavirus (EBC-virus) enteritis have led to a better understanding of morphological alterations of the intestinal tract as well as of the individual cells, helping to explain the functional disorders observed with this disease (for references see Storz and Rott, 1980). Relatively little is known, however, about structural details of the virus and about the biochemical events of EBC-virus replication, principally a result of the difficulties encountered in growing the virus in tissue culture. After we succeeded in adapting EBC-virus strain L-9 isolated by Mebus et al. (1973) to grow in bovine fetal thyroid or brain cells in reasonable amounts it became possible to study some of the many unanswered questions. As a beginning, efforts were made to determine optimal growth conditions in order to provide an in vitro source for viral antigen. In addition, biochemical analysis of the virus was undertaken.

DISTRIBUTION OF EBC-VIRUS IN HUMANS AND CALVES

In a sero-epidemiological survey in certain regions of Germany we used immunodiffusion with an alkali extract of infected bovine fetal thyroid cells or the serum neutralization test for the assay of antibodies.

TABLE 1

Frequency of Antibodies against Enteropathogenic Bovine Coronavirus in Man and Cattle

Serum group	Number positive [*] per total tested	Percent of positive sera
Cattle from Bavaria	62/97	67
Cattle from Hesse	113/192	61
Cattle Mü 1978	8/12	66
Cattle Mü 1979	0/7	0
Cattle Ma	3/5	60
Cattle HKR	2/8	25
Calf (for tissue culture)	2/3	
Fetal Calf	0/5	
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Human (blood bank)	7/100	7
Human (hospital)	9/36	25
Students (from Colorado)	8/26	31
Laboratory personnel	4/8	50

* tested by immunodiffusion

The results are demonstrated in Table 1. About 60% of randomly selected bovine sera were positive for EBC-virus. Antibodies were also found in two out of three batches of calf serum purchased for tissue culture purposes. Fetal calf serum never contained antibodies to EBC-virus (Storz and Rott, 1980).

EBC-virus-specific antibodies could also be demonstrated in human sera. This was true for blood samples obtained from the local blood bank and in particular for sera from the clinics of the university of Giessen where respectively 7% or 25% sera gave positive reactions in the immunodiffusion test. Lines of identity were formed when the positive human sera were alternated with positive bovine sera. All positive serum samples had a very high antibody content with neutralization indices of 3 and more. The results with human antibodies were confirmed by immunofluorescence and immune electron microscopy (Storz and Rott, 1981). Studies with fluorescent antibodies already implied an antigenic relationship between the human coronavirus strain OC43 and a bovine strain (Pedersen et al., 1978).

These findings clearly demonstrate that infections with EBC-virus or a closely related virus are wide spread in cattle and in humans. The high titers in human sera indicate that the virus must

be capable of replicating in human. One should therefore be aware of the possibility that the infection is of clinical importance. Whether the same virus strain infect man as well as cattle or whether the demonstration of antibodies is merely due to similarities in the antigenic structure in two different species-specific virus strains is not yet known. There is, however, evidence that EBC-virus can be transmitted from calves to man with ensuing gastroenteritis (Storz et al., 1981). The high content of neutralizing antibodies and the electron microscopic findings that the antibodies bind to the viral peplomers suggest that the reactive antigen is a glycoprotein of the viral envelope. The general experience that viral glycoproteins are strain-specific antigens could support the assumption that the same virus strain, which has a stable antigenic structure, infects either species.

GROWTH AND STRUCTURE OF EBC-VIRUS

In bovine fetal thyroid and bovine fetal brain cells EBC-virus induces only mild cytopathic changes. As in other coronavirus infections, virus titers begin to increase after a lag period of about 4 hours to reach a maximum of about 10^6 PFU/ml at 20 - 25 hours after infection. Cell-associated virus titers rise faster than released virus titers. Principally 5 size classes of viral polypeptide species were observed in virus particles and infected cells analyzed by SDS polyacrylamide gel electrophoresis: gp 23, p50, gp65, gp90 and gp180.

In spite of similar growth characteristics in these two cell types, analyses by polyacrylamide gel electrophoresis revealed differences in virus structure. These differences mainly concern the high molecular weight glycoproteins gp 180 and gp 90 (Fig. 1). After multiplication in fetal brain cells gp 180 is the dominant glycoprotein while in EBC-virus grown in fetal thyroid cells gp 90 is much more prominent. A precursor relationship between gp 180 and gp 90 may exist, but it remains to be seen whether the differences in the proportion of these two structural components has any biological significance. The small differences in the molecular weights of gp 65 might be due to host cell specific variations in the size of the oligosaccharide side chains. The smallest glycoprotein, the gp 23, apparently corresponds to the glycoprotein E1 of the murine coronavirus A 59. In contrast to gp 180, gp 90 and gp 65, which could be metabolically labeled with glucosamine, galactose, mannose and fucose, gp 23 was only labeled with radioactive glucosamine and galactose (Fig. 2). It therefore represents a new type of viral glycoproteins which appears to be common in several coronaviruses (see Niemann and Klenk, this volume).

Fig. 1. Polyacrylamide gel electrophoresis of enteropathogenic bovine coronavirus strain L-9 grown in bovine fetal brain (upper panel) or thyroid cells (lower panel).

Virus was labeled with a mixture of tritiated leucine and valine and harvested 30 hours after infection. Clarified infectious culture medium (200 μl) was mixed at 4° C with a bovine convalescent serum (5 μl); 12 hours later an antbovine IgG serum from rabbits was added, 4 hours later the precipitates were washed and then subjected to 10% polyacrylamide gels. Markers were ^{14}C -labeled Semliki Forest virus proteins (upper panel) or Vesicular stomatitis virus proteins (lower panel) extracted from infected cells.

Fig. 2. Incorporation of radioactive markers into bovine coronavirus strain L-9 polypeptides.

Coronavirus L-9 was grown in primary bovine fetal thyroid cells in the presence of ^{14}C -amino acids (panel A) or radio-labeled sugars as indicated (panels B to E). Virus was harvested 30 hours after infection and analyzed on 5 to 15% SDS-polyacrylamide slab gels.

Whereas gp 180 and gp 90 and gp 65 could be labeled with glucosamine, galactose, mannose, and fucose, gp 23 was only labeled with galactose and glucosamine (for details see Niemann and Klenk, this volume).

ACTIVATION OF BIOLOGICAL PROPERTIES OF EBC-VIRUS BY TRYPSIN TREATMENT

In analogy to our experiences with myxoviruses (for review see Klenk and Rott, 1980) the enhancement effect of trypsin on virus replication was tested (Storz et al., 1981). As can be seen in Fig. 3 the presence of 10 μ g of trypsin/ml in the culture medium indeed enhanced virus yield in bovine fetal thyroid cells by about 1 log unit. This is also the case when virus has been grown in bovine fetal brain cells. The hemagglutinin released into the culture medium appeared earlier and the concentration was increased by several factors in trypsin-treated, infected cell culture.

Fig. 3. Growth curve of bovine coronavirus strain L-9 in bovine fetal thyroid cells in the absence or presence of 10 μ g trypsin per ml culture medium.

Under these conditions, syncytium formation could be observed 12 - 18 hours after infection. The number of polykaryons, which usually contained up to 20 nuclei each, increased with incubation time, however, their total size remained relatively small. Not all

cells of a monolayer were recruited into polykaryons in trypsin-treated cell cultures. In the absence of the enzyme syncytia are not formed (Fig. 4).

Fig. 4. Cell fusion in L-9 infected bovine fetal brain cells in the presence of 10 μ g trypsin per ml culture medium.

A further consequence of trypsin treatment of infected cell cultures was the appearance of clearly visible plaques 3 days after plating, while in the absence of trypsin only turbid and tiny plaques could be observed, reaching a measurable size of about 1 mm in diameter 6 days after infection. In contrast, in the presence of 10 μ g trypsin/ml overlay the maximal plaque size of 5 mm was reached already on day 4 (Fig. 5). The plaque number depended directly on the virus dilution.

If virus was pretreated with trypsin or trypsin was present only during viral adsorption, plaque enhancement and cell fusion was not observed. This could mean that a change in the structure of the newly synthesized glycoproteins rather than a change on the cell surface is responsible for the trypsin effect. Formation of syncytia in the presence of a protease is also reminiscent of the situation in ortho- and paramyxoviruses where the viral fusion factors must be activated by proteolytic cleavage (for review see

Klenk and Rott, 1980). However, the precise site of proteolytic attack in EBC-virus infection remains to be detected.

Fig. 5. Plaques induced by enteropathogenic coronavirus strain L-9 in bovine fetal thyroid cells 6 days after plating in the absence (without) or 4 days after plating in the presence (with) of 10 μ g trypsin per ml of overlay.

The enhancing effect of trypsin on EBC-virus replication in vitro mimics the situation in vivo where in the intestinal tract ample amounts of trypsin and other proteases are present and may be an essential factor for massive virus multiplication and the clinical expression of cell destruction.

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