JOURNAL OF VIROLOGY, Apr. 1995, p. 2664–2666 Vol. 69, No. 4
0022-538X/95/$04.00+0

Antibodies to the Vitronectin Receptor (Integrin $\alpha_V\beta_3$) Inhibit Binding and Infection of Foot-and-Mouth Disease Virus to Cultured Cells

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Received 4 August 1994/Accepted 10 January 1995

The amino acid sequence Arg-Gly-Asp (RGD) is highly conserved on the VP1 proteins of different serotypes and subtypes of foot-and-mouth disease virus (FMDV) and is essential for cell attachment. This sequence is also found in certain extracellular matrix proteins that bind to a family of cell surface receptors called integrins. Within the *Picornaviridae* family, human enterovirus coxsackievirus A9 also has an RGD motif on its VP1 capsid protein and has recently been shown to utilize the vitronectin receptor integrin $\alpha_V\beta_3$ as a receptor on monkey kidney cells. Competition binding experiments between type A_{12} FMDV and coxsackievirus A9 using BHK-21 and LLC-MK2 cells revealed shared receptor specificity between these two viruses. Polyclonal antiserum to the vitronectin receptor and a monoclonal antibody to the α_V subunit inhibited both FMDV binding and plaque formation, while a monoclonal antibody to the β_3 subunit inhibited virus binding. In contrast, antibodies to the fibronectin receptor ($\alpha_5\beta_1$) or to the integrin ($\alpha_V\beta_5$) had no effect on either binding or plaque formation. These data demonstrate that the $\alpha_V\beta_3$ vitronectin receptor can function as a receptor for FMDV.

Foot-and-mouth disease virus (FMDV), the only member of the genus *Aphthovirus* in the family *Picornaviridae* (1), is composed of seven serotypes and more than 67 subtypes (19). The virus particle consists of a single-stranded RNA genome enclosed in a capsid made up of 60 copies each of four different proteins, VP1 to VP4. The major immunodominant site on the virion surface resides on the βG-βH loop of VP1 (G-H loop) which includes a highly conserved Arg-Gly-Asp (RGD) sequence (31).

This sequence is also found in a number of cell matrix and adhesion proteins which bind to a family of cell surface receptors called integrins (14, 25). Integrins are heterodimers composed of noncovalently associated α and β subunits (14, 24). There are 14 known α and 8 β subunits, forming at least 21 different heterodimers, which recognize distinct ligands. The first integrin recognition site to be defined was the RGD sequence, present in fibronectin, vitronectin, and a variety of other adhesive proteins (20).

The conservation of the RGD sequence on the G-H loop in addition to the inhibitory effect of RGD peptides on the binding of FMDV to susceptible cells suggested that integrins might act as cellular receptors for FMDV (3, 10). In support of this hypothesis, X-ray diffraction studies of the G-H loop of type O_1 virus have shown structural similarities between the RGD regions of the loop and the integrin-binding protein γII-crystallin (15), and mutations introduced into the RGD sequence via an infectious cDNA clone from type A_{12} resulted in the production of noninfectious viral particles which could not interact with the cellular receptor (17).

Several pathogens are known to interact with cells by an RGD sequence. Binding of *Borrelia burgdorferi*, the causative agent of Lyme disease, is mediated by the RGD-recognizing integrin, $\alpha_{IIb}\beta_3$ (9), and the integrins $\alpha_V\beta_5$ and $\alpha_V\beta_3$ play a role in the attachment and/or internalization of adenoviruses (2, 4, 33). Another member of the integrin superfamily, VLA-2 ($\alpha_2\beta_1$), has been reported to function as a receptor for echovirus types 1 and 8 (5, 6).

The human enterovirus coxsackievirus A9 (CAV-9) has an insertion of 17 amino acids at the C-terminal end of its VP1 capsid protein which includes an RGD sequence (7, 8) that has been shown to be essential for attachment of virus to cells (22). Recently, the integrin $\alpha_V\beta_3$, also known as the vitronectin receptor (VNR), was found to be a receptor for CAV-9 on monkey kidney cells (23), although there is evidence that this virus may also utilize a non-RGD-dependent alternative receptor (22).

Even though CAV-9 is a human picornavirus and FMDV is a livestock pathogen, we decided to examine whether these two viruses could use the same cellular receptor site. As a first step, we tested the abilities of type A_{12} FMDV and CAV-9 to replicate in several different cell lines to determine which cell types could be used to probe receptor specificity. Both viruses replicated well in LLC-MK2, a rhesus monkey kidney cell line, and FMDV replicated to high titers in BHK-21 cells, whereas CAV-9 replicated poorly in these cells (Table 1). Only FMDV could grow in a bovine kidney cell line (BK-LF) and HeLa cells, while neither virus replicated in CHO cells (Table 1). The attachment of radiolabeled FMDV and CAV-9 to these cell lines was also examined (Table 1). The results of these experiments showed that FMDV was able to attach to all cell lines it replicated in, although the extent of binding varied. The virus also attached to CHO cells at low levels, a result noted previously (16). CAV-9 however, adsorbed only to LLC-MK2 and BHK-21 cells, although the higher binding to BHK-21 cells did not correlate with more efficient replication in this cell line.

To investigate the possibility that FMDV and CAV-9 could utilize the same receptor site on LLC-MK2 and BHK-21 cells, competition binding experiments were performed. The attach-

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TABLE 1. Binding and replication of FMDV and CAV-9 in different cell lines

Cell line	Replication[a]		Binding[b] (%)	
	FMDV	CAV-9	FMDV	CAV-9
BHK-21	10^3	10^1	81	71
LLC-MK2	10^2	10^3	37	25
CHO	NI[c]	NI	11	2.6
HeLa	10^2	NI	15	2.6
BK-LF	10^2	NI	29	2.3

[a] Each cell line was infected with either FMDV, derived from the infectious cDNA clone $pRMC_{35}$ (21), or CAV-9 at a multiplicity of infection of 10 PFU per cell. The results are expressed as the increase in titer between 1.5 and 24 h postinfection.

[b] Cells, in suspension at 2.5×10^7 cells per ml in phosphate-buffered saline with 1% calf serum, were incubated with either [^{3}H]uridine-labeled type A_{12} FMDV (1×10^3 to 2×10^3 cpm) or [^{35}S]methionine-labeled CAV-9 (3×10^3 to 4×10^3 CPM) at 10^3 particles per cell for 2 h at room temperature. The results are expressed as the percentage of total counts bound to cells.

[c] NI, no increase in titer between 1.5 and 24 h postinfection.

TABLE 2. Effect of antibodies to integrin subunits on binding and plaque formation of FMDV and CAV-9 in LLC-MK2 cells

Antibody[a] and dilution or concn	% Reduction			
	Binding[b]		Plaques[c]	
	FMDV	CAV-9	FMDV	CAV-9
a-VNR (a-$\alpha_V\beta_3/\beta_5$) (1:10)	74	69	55	76
a-FNR (a-$\alpha_5\beta_1$) (1:10)	<10	<10	<10	52
1C12 (a-α_V) (1:20)	39	43	79	72
3F12 (a-α_V) (200 μg/ml)	15	<10	<10	<10
7G2 (a-β_3) (200 μg/ml)	53	26	<10	<10
P1F6 (a-$\alpha_V\beta_5$) (200 μg/ml)	<10	<10	<10	<10

[a] a-VNR and a-FNR were whole sera, MAb 1C12 was an ascitic fluid, and MAbs 3F12, 7G2, and P1F6 were purified immunoglobulin G.

[b] LLC-MK2 cells, in suspension at 2.5×10^7 cells per ml in phosphate-buffered saline with 1% calf serum, were incubated with antibodies, at the indicated concentrations for 1 h at room temperature. [^{35}S]methionine-labeled type A_{12} FMDV (1×10^3 to 2×10^3 cpm) or CAV-9 (3×10^3 to 4×10^3 cpm) was added, and the cells were incubated for an additional 1.5 to 2 h at 4°C. Results are expressed as the percent reduction of binding compared with control cells incubated with either buffer, normal rabbit sera, or normal mouse ascitic fluid.

[c] LLC-MK2 cells in tissue culture dishes were incubated with the antibodies, diluted to the indicated concentrations in phosphate-buffered saline with 1% calf serum for 45 min at room temperature. They were then infected with approximately 100 PFU of either type A_{12} FMDV or CAV-9 in the presence of antibody. After a 30-min adsorption period at room temperature, the virus-antibody mixture was removed and the cells were overlaid with 0.6% gum tragacanth. Cells were stained with crystal violet-formalin at either 48 h (FMDV) or 72 h (CAV-9), and plaques were counted. The results are expressed as the reduction in plaque number compared with cells infected in the presence of either buffer, normal rabbit sera, or normal mouse ascitic fluid.

ment of radiolabeled FMDV and CAV-9 to LLC-MK2 and BHK-21 cells was analyzed in the presence of unlabeled homologous or heterologous virus (Fig. 1). The binding of FMDV and CAV-9 to BHK-21 cells could be inhibited by either unlabeled virus (Fig. 1b). In LLC-MK2 cells, however, the binding of CAV-9 was inhibited by both viruses, although FMDV was inhibited only by itself (Fig. 1a). Analyses of binding affinity using Scatchard plots showed that FMDV had a greater affinity for LLC-MK2 cells than CAV-9 did (not shown), so the lack of inhibition of FMDV binding by CAV-9 may be due to the dissociation of CAV-9 from the cells in the presence of FMDV. For a control, we also analyzed the binding of poliovirus type 1 (PV1) to LLC-MK2 cells. PV1 does not

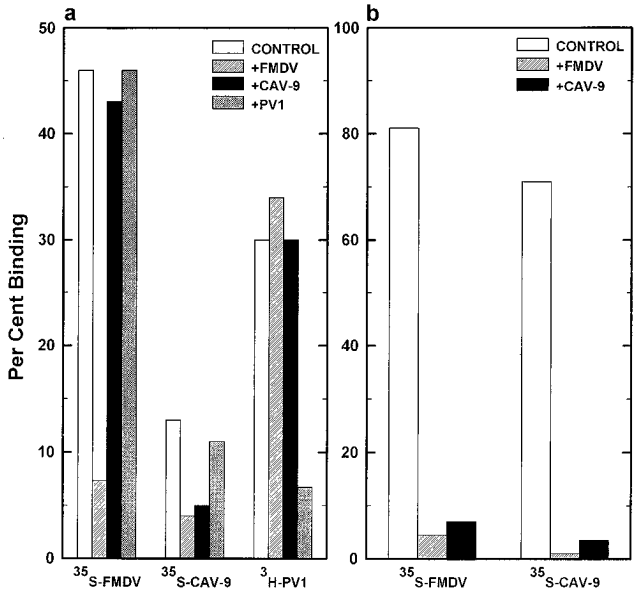

FIG. 1. LLC-MK2 (a) or BHK-21 (b) cells, in suspension at 2.5×10^7 cells per ml in phosphate-buffered saline with 1% calf serum, were incubated with purified unlabeled type A_{12} FMDV or CAV-9 at a multiplicity of 2.5×10^4 (a) or 1×10^5 (b) particles per cell, for 1 h at room temperature. ^{35}S-labeled FMDV (2×10^3 to 3×10^3 cpm), ^{35}S-labeled CAV-9 (3×10^3 to 4×10^3 cpm), or ^{3}H-labeled PV1 (1.5×10^3 cpm) was added, at a multiplicity of 10^3 particles per cell, and the cells were incubated for an additional 2 h at room temperature. The results are expressed as the percentage of total counts bound to cells.

contain an external RGD sequence and uses a receptor which is a member of the immunoglobulin superfamily (18). The results in Fig. 1a show that PV1 inhibits only its own binding, and neither FMDV nor CAV-9 can inhibit PV1 binding to LLC-MK2 cells. These results confirm our previous findings that FMDV and PV2 do not share receptor sites on HeLa cells (27). We have also shown previously that FMDV and encephalomyocarditis virus, a cardiovirus, used different receptor sites on BHK-21 cells (27). Taken together, these results indicate that FMDV and CAV-9 share binding sites on the surfaces of cultured cell lines that are not bound by other picornaviruses.

Since CAV-9 has recently been shown to use the VNR, $\alpha_V\beta_3$, as a cellular receptor (23), we tested several antiintegrin antibodies for their effect on virus adsorption and plaque formation of FMDV and CAV-9. The results of these studies are shown in Table 2. A polyclonal anti-VNR ($\alpha_V\beta_3/\beta_5$) serum (Calbiochem) inhibited the binding of both FMDV and CAV-9 to LLC-MK2 cells by about 70%. The antiserum also caused a reduction in plaque formation by both viruses, indicating that the inhibition of binding interfered with the infection. Polyclonal anti-fibronectin receptor (FNR) ($\alpha_5\beta_1$) serum (Calbiochem) did not inhibit the binding of either virus to LLC-MK2 cells, but this serum inhibited plaque formation of CAV-9, suggesting that the FNR may play some role in the infectious process of CAV-9 subsequent to adsorption. In a related cell line, GMK cells, this antibody did not inhibit plaque formation of CAV-9 (23), suggesting some difference in expression of the quality or quantity of the $\alpha_5\beta_1$ integrin on these two cell lines. A monoclonal antibody (MAb) to the $\alpha_V\beta_5$ integrin complex, P1F6 (32), had no effect on either virus (Table 2). Two MAbs prepared against the human α_V subunit, 1C12 (6a) and 3F12 (11, 26), were tested. Only 1C12 was able to inhibit both binding and plaque formation by FMDV and CAV-9. This result can be related to the higher affinity of the 1C12 antibody for the α_V subunit (6a). A MAb against the β_3 subunit (7G2;

11, 26) inhibited adsorption of both viruses to LLC-MK2 cells, but in contrast to the effect of the other antibodies, had no effect on plaque formation. None of the antibodies had any effect on binding or replication of FMDV in BHK-21 cells, nor did they react with proteins extracted from these cells on Western blots (immunoblots) (not shown), which is not surprising since they were generated with human antigens.

Members of either the immunoglobulin or integrin superfamily are known to function as receptors for several picornaviruses. The poliovirus receptor (18), the major group human rhinovirus receptor (intracellular adhesion molecule 1) (12, 29, 30), and the receptor for the D variant of encephalomyocarditis virus (vascular cell adhesion molecule 1) (13) are all members of the immunoglobulin superfamily. The receptor for echovirus types 1 and 8 has been identified as VLA-2 (5, 6), and VNR has been identified as a receptor for CAV-9 (23). These latter two receptors belong to the integrin superfamily.

The RGD motif is recognized by several integrins. Specifically, VNR ($\alpha_V\beta_3$) can also recognize ligands other than vitronectin which contain the RGD sequence (14, 28). In the case of FMDV and CAV-9 infection of LLC-MK2 cells, $\alpha_V\beta_3$ binds virus and may be involved in steps subsequent to adsorption, since some of the antibodies could inhibit plaque formation as well as binding. This RGD sequence, however, is essential for FMDV infectivity, since any alteration of this sequence results in the production of noninfectious viral particles which have lost the ability to bind to cells (17). Although VNR could be used to bind both FMDV and CAV-9 to cells, the results in Table 1 suggest that FMDV may use alternative RGD-dependent integrins, since it can bind and replicate in some cells that CAV-9 cannot. The results presented in this report, however, constitute the first direct evidence that an integrin can function as the receptor for FMDV.

We express our gratitude to Eric J. Brown and Scott D. Blystone, Washington University School of Medicine, St. Louis, Mo., for providing MAbs 1C12, 3F12, and 7G2 and for their advice and encouragement and to Dean Sheppard, University of California at San Francisco, for providing MAb P1F6. We also thank A. J. Franke for excellent technical assistance.

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