

Integrin $\alpha_v\beta_3$ (vitronectin receptor) is a candidate receptor for the virulent echovirus 9 strain Barty

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The enterovirus echovirus 9 strain Barty (E9/Barty) is pathogenic for newborn mice as well as for humans. In contrast to the apathogenic prototype strain Hill, strain Barty encodes an RGD motif in the C-terminal part of the structural protein VP1. Data are presented that show that E9/Barty binds its target cells via contact of the RGD motif to the $\alpha_v\beta_3$ integrin (vitronectin receptor), whereas prototype Hill uses a different, still unidentified receptor site. Furthermore, virus titres of murine muscle tissue were compared after infection of newborn and 1-, 2-, 3- and 12-week-old mice. The replication capacity of the virus decreased dramatically with age of the infected mice. Since E9/Barty does not replicate or replicates only poorly in mice older than about 5 days, and expression of the vitronectin receptor is reported to be down-regulated in striated muscle tissue during development, it is suggested that susceptibility of mice to this echovirus infection is controlled by the availability of $\alpha_v\beta_3$ integrin.

Echoviruses are members of the genus *Enterovirus* within the family *Picornaviridae*. In humans, echoviruses may cause acute disease such as myocarditis, pericarditis, myositis, meningitis and encephalitis (Melnick, 1996; Morens *et al.*, 1991). Echoviruses were originally distinguished from coxsackieviruses by the lack of pathogenicity for newborn mice, e.g. echovirus 9 prototype Hill (E9/Hill) (Ramos-Alvarez & Sabin, 1954). However, in contrast, the antigenically related echovirus 9 strain Barty (E9/Barty), isolated from the cerebrospinal fluid of a child suffering from aseptic meningitis (Eggers & Sabin, 1959), causes paralysis in newborn mice.

In earlier studies, it has been proposed that echovirus 9 strains E9/Hill and E9/Barty vary in their capsid surfaces, since

they adsorb to GMK cells with different kinetics and strain-induced antibodies exhibit quantitatively different neutralizing capacities (Rosenwirth & Eggers, 1982). This hypothesis was supported by our finding that the main difference between the strains is an insertion in the C-terminal part of VP1 of the virulent strain E9/Barty, including an arginine–glycine–aspartic acid (RGD) motif (Zimmermann *et al.*, 1995, 1996). It has already been shown that three members of the *Picornaviridae*, foot-and-mouth disease virus, coxsackievirus A9 and echovirus 22, interact with their target cells by an RGD– $\alpha_v\beta_3$ or RGD– $\alpha_v\beta_1$ contact (Neff *et al.*, 1998; Pulli *et al.*, 1997; Roivainen *et al.*, 1994). In the case of E9/Barty, a 310 amino acid segment that includes the RGD motif is responsible for cell attachment and mouse pathogenicity and, furthermore, cell attachment of E9/Barty but not E9/Hill is inhibited specifically by RGD-containing peptides (Zimmermann *et al.*, 1997). These results allow the presumption that E9/Barty may utilize the RGD motif as its receptor-binding site.

In this communication, we present evidence that E9/Barty binds via the RGD motif to the $\alpha_v\beta_3$ integrin (vitronectin

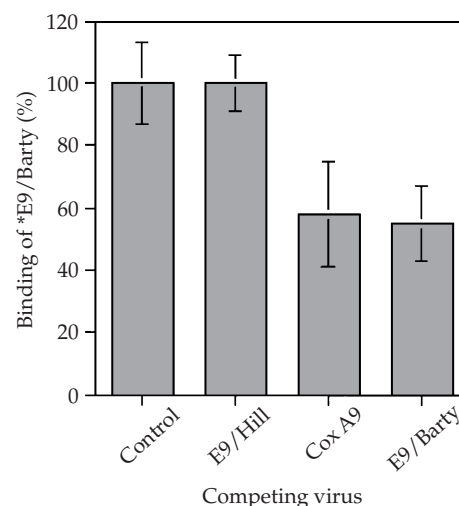


Fig. 1. Competition binding experiments with radiolabelled E9/Barty (*E9/Barty). GMK cell monolayers were incubated with *E9/Barty (10^7 p.f.u.) in the presence of unlabelled competing virus (10^7 p.f.u.) or without a second virus (control) for 30 min at room temperature. After washing, cell-bound radioactivity was determined. Mean values of two experiments are shown and standard deviations are indicated. Results are given as percentages, with the values for the control taken as 100%. Cox A9, Coxsackievirus A9.

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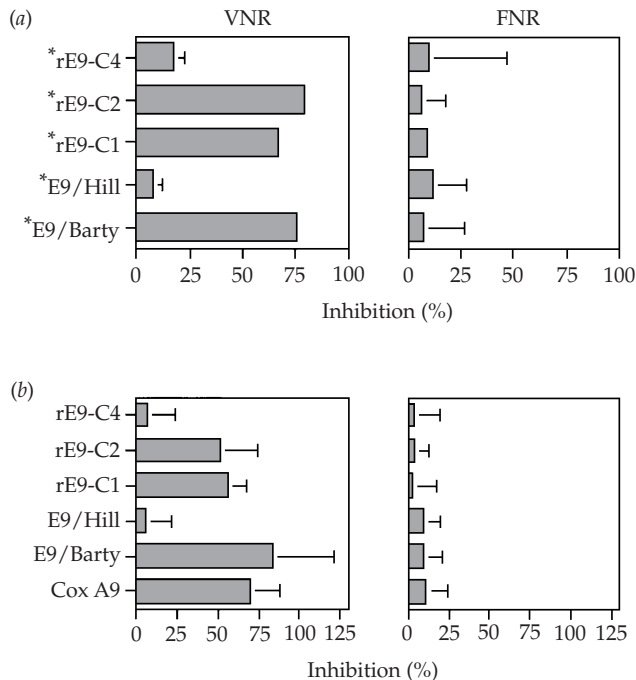


Fig. 2. Effect of polyclonal antibodies against VNR or FNR on attachment (a) and plaque formation (b) of recombinant echovirus 9 strains rE9-C1, -C2 and -C4, described previously (Zimmermann *et al.*, 1997), as well as the original isolates E9/Barty and E9/Hill. Standard deviations calculated from two experiments are included. (a) [³⁵S]Methionine-labelled recombinant echovirus 9 strains E9/Barty and E9/Hill (*virus) were used to infect GMK cells in the presence of the indicated antibody. Viruses were allowed to attach for 15 min, free virus was then washed off and the remaining, cell-bound radioactivity was determined. Cell-bound radioactivity is given as a percentage, with 100% being determined by infection without antibody. (b) GMK cell monolayers were pre-incubated with VNR or FNR antibodies before the indicated virus strains were added. After 15 min attachment, the virus-antibody mixture was removed and a plaque test was performed. The results are expressed as the percentage reduction in plaque number as compared with controls without preceding antibody treatment. Cox A9, Coxsackievirus A9.

receptor; VNR). We demonstrate firstly that E9/Barty and coxsackievirus A9, which is known to bind to VNR (see above), share receptor specificities. Furthermore, we confirm that antiserum to VNR inhibits attachment and plaque formation of E9/Barty and related recombinant constructs. Both shared receptor specificity and inhibition by the antiserum are not true for the apathogenic prototype E9/Hill. It is hypothesized that susceptibility of murine muscle tissue to infection with E9/Barty depends on the developmental stage of the cells.

We performed competition binding experiments and analysed cell attachment of radiolabelled E9/Barty in the presence of unlabelled homologous or heterologous viruses. For that purpose, GMK cell monolayers were simultaneously infected with standard amounts of ³⁵S-labelled E9/Barty and competing virus. After 30 min incubation at room temperature, the supernatant was removed and cell-bound radioactivity was determined. Binding of radiolabelled E9/Barty to GMK cells was reduced to about 50% by either unlabelled E9/Barty or

coxsackievirus A9, whereas E9/Hill did not affect binding of ³⁵S-labelled E9/Barty (Fig. 1). These results confirm the hypothesis that E9/Barty utilizes the same cellular receptor as coxsackievirus A9 on GMK cells, i.e. VNR, whereas the apathogenic E9/Hill uses another site.

Next, we tested the effect of antibodies against VNR (Gibco BRL) and, as a negative control, against the fibronectin receptor (FNR) (Gibco BRL) on cell attachment and plaque formation of E9/Hill, E9/Barty and three recombinant virus variants (rE9-C2, rE9-C1 and rE9-C4) described previously (Zimmermann *et al.*, 1997). The genomes of these recombinant viruses are composed of 1880, 2981 and 3880 nucleotides, respectively, of the 5'-E9/Hill sequence in front of the appropriate 3' part of E9/Barty. Hence, rE9-C2 and rE9-C1 contain the RGD motif, whereas VP1 of rE9-C4 consists exclusively of the E9/Hill sequence.

Series of experiments were carried out using an inoculum of labelled echovirus 9 strains E9/Hill, E9/Barty, rE9-C1, rE9-C2 and rE9-C4. [³⁵S]Methionine-labelled virus was used to infect GMK cell monolayers that had been pre-incubated for 45 min with the indicated antiserum (1:1000 dilution of the stock solution from Gibco BRL). After an additional 15 min incubation in the presence of labelled virus, the supernatant was removed and cell-bound radioactivity was measured. Cell attachment of E9/Barty, rE9-C1 and rE9-C2 was inhibited markedly by antiserum against human VNR (Fig. 2a). On the other hand, only background inhibition was measurable for E9/Hill and rE9-C4.

In addition, the influence of VNR or FNR antiserum on plaque formation was determined. GMK cell monolayers were again pre-incubated with the respective antibodies for 45 min at room temperature. Subsequently, the indicated viruses (approx. 100 p.f.u.) were added and allowed to adsorb for 15 min at room temperature. Afterwards, the virus-antibody mixture was removed and cells were overlaid with 0.75% agarose in DMEM. After incubation for 48 h at 37 °C, plaques were counted. Again, the inhibitory effect of antiserum against human VNR was limited to strains E9/Barty, rE9-C1 and rE9-C2 (Fig. 2b). Polyclonal anti-FNR serum inhibited neither cell attachment nor plaque formation of either virus tested (Fig. 2a, b).

These results strongly suggest (i) that VNR is a candidate receptor for E9/Barty, but not for the prototype E9/Hill, and (ii) that the virus-cell interaction takes place via the RGD motif, since only recombinant viruses encoding this motif were inhibited significantly by antibodies against VNR.

The fact that E9/Barty and coxsackievirus A9 recognize the same receptor and, therefore, infect the same cells may help to understand why clinical symptoms induced by these two viruses in newborn mice are indistinguishable. On the other hand, the different receptor specificities of E9/Hill and E9/Barty may explain their different pathogenicity for newborn mice (Eggers & Sabin, 1959; Zimmermann *et al.*, 1997). In further studies, the possible importance of a functional RGD

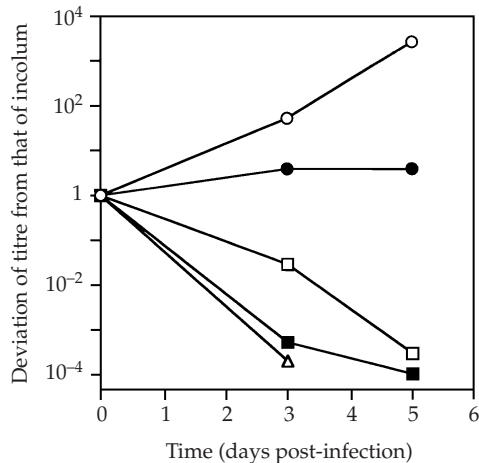


Fig. 3. Replication of E9/Barty in newborn mice (○) or 1- (●), 2- (□), 3- (■) or 12- (△) week-old mice. The animals were infected intraperitoneally with 2×10^6 p.f.u. of the virus. At indicated times, two mice were harvested and carcasses (whole mouse without head, skin and viscera) were ground and suspended as described previously (Zimmermann *et al.*, 1995). The amount of infective virus was determined by plaque assay. Five days after inoculation of 12-week-old mice, no remaining virus was detectable. The fold deviation from the titre of the inoculum (2×10^6 p.f.u.) of the titre determined in the mouse muscle tissue is given.

motif for pathogenicity of several isolates of echovirus 9 from humans has been demonstrated (Nelsen-Salz *et al.*, 1999).

Integrins are supposed to be involved in terminal muscle cell differentiation (Menko & Boettiger, 1987); for example, the expression of integrin $\alpha_v\beta_3$ is down-regulated as part of the myogenic differentiation programme of human skeletal muscle *in vitro* (Blaschuk *et al.*, 1997). It has already been described for coxsackievirus A13 that productive infection of muscle cells cultured from tissues of foetal mice is limited to the stage of differentiation characterized by rapid cell fusion (Goldberg & Crowell, 1971). These data and our findings concerning the receptor-binding site are consistent with the fact that clinical symptoms are clearly decreased after infection of suckling mice with E9/Barty 2, 4 or 6 days after birth, as compared with severe disease after infection of newborn mice (Bültmann *et al.*, 1983). Moreover, the rate of virus replication clearly depends on the age of the infected animal (Fig. 3). In 1-week-old mice, we observed only a limited increase in infectious titre in the muscle tissue. No virus replication took place in 2-, 3- or 12-week-old mice. This allows the suggestion that the reduced pathogenicity of E9/Barty in older mice is not only due to the increased mass of compensatory muscle tissue, but is also a consequence of the down-regulation of $\alpha_v\beta_3$ integrin.

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