Growth of rotaviruses in continuous human and monkey cell lines that vary in their expression of integrins

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Rotavirus replication occurs in vivo in intestinal epithelial cells. Cell lines fully permissive to rotavirus include kidney epithelial (MA104), colonic (Caco-2) and hepatic (HepG2) types. Previously, it has been shown that cellular integrins α2β1, α4β1 and αXβ2 are involved in rotavirus cell entry. As receptor usage is a major determinant of virus tropism, the levels of cell surface expression of these integrins have now been investigated by flow cytometry on cell lines of human (Caco-2, HepG2, RD, K562) and monkey (MA104, COS-7) origin in relation to cellular susceptibility to infection with monkey and human rotaviruses. Cells supporting any replication of human rotaviruses (RD, HepG2, Caco-2, COS-7 and MA104) expressed α2β1 and (when tested) αXβ2, whereas the non-permissive K562 cells did not express α2β1, α4β1 or αXβ2. Only RD cells expressed α4β1. Although SA11 grew to higher titres in RD, HepG2, Caco-2, COS-7 and MA104 cells, this virus still replicated at a low level in K562 cells. In all cell lines tested, SA11 replicated to higher titres than did human strains, consistent with the ability of SA11 to use sialic acids as alternative receptors. Levels of cell surface α2 integrin correlated with levels of rotavirus growth. The α2 integrin relative linear median fluorescence intensity on K562, RD, COS-7, MA104 and Caco-2 cells correlated linearly with the titre of SA11 produced in these cells at 20 h after infection at a multiplicity of 0.1, and the data best fitted a sigmoidal dose–response curve (r2 = 0.99, P = 0.005). Thus, growth of rotaviruses in these cell lines correlates with their surface expression of α2β1 integrin and is consistent with their expression of αXβ2 and α4β1 integrins.

Introduction

Rotaviruses are double-stranded RNA viruses in the family Reoviridae and are the major cause of infantile gastroenteritis in most mammalian species. Primary replication of rotaviruses takes place in the mature enterocytes of the intestine. The outermost layer of the non-enveloped, icosahedral virion is composed of the 37 kDa glycoprotein VP7 and spikes of the 88 kDa protein VP4, as dimers (Prasad et al., 1990), which extend about 12 nm above the VP7 surface (Prasad et al., 1988; Yeager et al., 1994). Both VP4 and VP7 independently elicit neutralizing, protective antibodies (Hoshino et al., 1995; Offit et al., 1986). VP4 is an important determinant of host cell tropism (Kalica et al., 1983; Ramig & Galle, 1990), virulence (Hoshino et al., 1995), receptor binding and cell penetration (Kirkwood et al., 1998; Ludert et al., 1996). Proteolytic cleavage of VP4 into two subunits, VP8* (28 kDa) and VP5* (60 kDa) (Espejo et al., 1981), results in increased infectivity (Clark et al., 1981; Estes et al., 1981) and rapid internalization of virus. VP7 may have a minor role in host cell entry (Ludert et al., 1996).

A minority of animal rotaviruses, including the simian strain SA11 and the rhesus rotavirus RRV, bind to cell surface sialic acid (Fukudome et al., 1989) via VP4 (Mackow et al., 1989), but this binding does not appear to be essential for
infectivity of these viruses, as sialic acid-independent mutants retain their infectivity (Mendez et al., 1993). Human rotaviruses do not utilize sialic acid for cellular attachment (Ciarlet & Estes, 1999; Fukudome et al., 1989). Cell lines fully permissive to human and monkey rotaviruses are monkey kidney epithelial (MA104) and human colonic adenocarcinoma (Caco-2, HT-29) types. However, HepG2 cells have been shown to support growth of hepatotropic rotaviruses (Raming & Galle, 1990).

Recently, we have shown that integrins are involved in rotavirus cell attachment and entry (Coulson et al., 1997; Hewish et al., 2000). Integrins are \( \alpha \beta \) heterodimeric, transmembrane glycoproteins that are important in cell adhesion and signalling. Most human and animal rotaviruses (87%), including SA11, contain the amino acid sequence DGE at positions 308–310 of VP5. The peptide DGE(A) has been reported to act as a ligand in type I collagen for the \( \alpha 2\beta 1 \) integrin (Staatz et al., 1991). The VP7 of 43-7% of rotaviruses, including SA11 and RRV, contains the sequence LDV at aa 237–239. The related sequences LDI and IDI are present in all mammalian rotaviruses at aa 269–271 (Coulson et al., 1997).

Also, SA11, RRV and some human rotaviruses contain the sequence IDA at aa 538–540 of VP4 (Hewish et al., 2000). In the first connecting segment of the independently spliced IIICS domain of fibronectin, LDV is the minimal essential sequence for a major site of adhesion of fibronectin to the \( \alpha 4\beta 1 \) and \( \alpha 4\beta 7 \) integrins on a range of cell types (Komorita et al., 1991), and IDA is an \( \alpha 4 \) integrin ligand sequence in the C-terminal HepII domain of fibronectin. In addition, at aa 253–255 in VP7, all mammalian rotaviruses contain the sequence GPR, which is a ligand for the \( \alpha X\beta 2 \) integrin in the N-terminal domain of fibrinogen (Loike et al., 1991). In our experiments, peptides RDGE and GPRP and monoclonal antibodies (MAbs) directed to \( \alpha 2 \), \( \alpha 4 \), \( \alpha X \), \( \beta 1 \) and \( \beta 2 \) integrin subunits blocked rotavirus infection specifically in an additive and dose-dependent manner (Coulson, 1997; Coulson et al., 1997; Hewish et al., 2000). The ligand sequence in VP7 for \( \alpha X\beta 2 \) integrin, GPRP, is likely to be functional, since the GPRP peptide blocked SA11 and human rotavirus RV-5 infection of MA104 and Caco-2 cells (Coulson et al., 1997). The role of the LDV and LDV-like sequences in VP7 is less clear, as VP4 of some rotavirus strains also contains the \( \alpha 4 \) integrin ligand sequence (IDA, see above), and the LDV-containing peptide has not yet been tested for blocking of virus binding or infection in cells expressing detectable \( \alpha 4 \) integrin. MAbs directed to \( \alpha X \), \( \alpha 5 \), \( \alpha 6 \), \( \alpha L \), \( \alpha M \) and \( \beta 4 \) and RGD-containing peptides did not block SA11 rotavirus infection (Coulson, 1997; Coulson et al., 1997).

Most recently, we have shown that \( \alpha 2\beta 1 \) and \( \alpha 4\beta 1 \) integrins can act as cellular receptors for SA11, by studying SA11 attachment to and replication in K562 cells expressing \( \alpha 2\beta 1 \) (\( \alpha X-K562 \), \( \alpha 3\beta 1 \) (\( \alpha 3-K562 \)) or \( \alpha 4\beta 1 \) (\( \alpha 4-K562 \)) integrins on their surface as a result of transfection with integrin subunit cDNA (Hewish et al., 2000). Levels of virus binding and infection in \( \alpha X-K562 \) and \( \alpha X-K562 \) cells were increased specifically over levels in \( \alpha 3-K562 \) and K562 cells. Additionally, phorbol ester treatment of K562 parent and transfected cells induced endogenous gene expression of \( \alpha 2\beta 1 \) integrin, which correlated with further increases in the level of SA11 virus growth. Virus growth in \( \alpha X-K562 \) cells that had also been induced to express \( \alpha 2\beta 1 \) integrin with phorbol ester was to a level approaching that in MA104 cells (Hewish et al., 2000).

One explanation for the restrictions on rotavirus replication in vitro is that expression of virus receptors, including \( \alpha 2\beta 1 \) and \( \alpha 4\beta 1 \), varies between cell lines. In order to examine this question, we have determined the levels of expression of integrins that have been demonstrated to be capable of acting as rotavirus receptors (\( \alpha 2\beta 1 \) and \( \alpha 4\beta 1 \)) or implicated in rotavirus cell entry (\( \alpha X\beta 2 \)) on a range of cell lines of human and monkey origin and correlated these levels with virus titres produced after infection with monkey and human rotavirus strains.

**Methods**

- **Cell lines.** Caco-2 cells were obtained from the ATCC. Human rhomboendosarcoma (RD) and hepatoma (HepG2) cells were respectively provided by C. Birch and H. Siow of the Macfarlane Burnet Centre for Medical Research, Fairfield, Victoria, Australia. COS-7 cells were derived originally from the African green monkey kidney epithelial cell line CV-1 (Gluzman, 1981) and were provided by M. Sandrin, The Austin Research Institute, Austin and Repatriation Medical Centre, Heidelberg, Victoria, Australia. African green monkey epithelial (MA104), COS-7, RD and HepG2 cells were grown in Dulbecco's modification of Eagles' medium including 2 mM L-glutamine (Gibco), 20 mM HEPES (Boehringer Mannheim), 26 μg/ml gentamicin (CIDomycin; Roussel) and 2 μg/ml Fungizone (Gibco) (DMEM), supplemented with 10% (v/v) heat-inactivated FCS (Commonwealth Serum Laboratories, Parkville, Victoria, Australia). Caco-2 cells were grown in modified DMEM (Trace Biosciences), which contained 0.075% (w/v) sodium bicarbonate and 20% (v/v) FCS. Human non-adherent myelogenous leukaemic cells (K562) were provided by I. Bertoncocco, Peter McCallum Cancer Research Institute, Melbourne, Victoria, Australia, and grown in DMEM containing 20% (v/v) FCS.

- **Viruses.** The origins of monkey rotavirus strains now designated as serotype P5B[2], G3 (SA11 and RRV), and human rotaviruses designated as serotypes P1A[8], G1 (RV-4, Wa), P3A, G1 (K8), P1B[4], G2 (RV-5), and P2A[6], G4 (ST-3), have been described previously (Coulson, 1993; Coulson et al., 1985). Following activation of infectivity with 10 μg/ml porcine trypsin (Sigma) for 20 min at 37 °C, viruses were propagated in MA104 cells in the absence of serum and the presence of 1 μg/ml porcine trypsin as described previously (Hewish et al., 2000; Sato et al., 1981). Virus-cell lysates were clarified by low-speed centrifugation and then stored at −70 °C. Titres of infectious virus were determined by indirect immunofluorescent staining of infected cells in MA104 cell monolayers inoculated with serial dilutions of the stocks (Coulson et al., 1985).

- **MAbs.** Mouse MAbs to human integrin subunits used in flow cytometry were as follows: AK7 and RMAC11, directed against the \( \alpha \) chain (CD49b) of \( \alpha 2\beta 1 \) integrin (Gamble et al., 1993; O’Connell et al., 1991), from M. Berndt (Baker Medical Research Institute, Melbourne, Victoria, Australia) and A. D’Apice (St. Vincent’s Hospital, Melbourne, Victoria, Australia), respectively, as purified protein; P4C2 and P4G9 (hybridoma cell supernatant fluids (SNF), directed against the \( \alpha \) chain (CD49d) of \( \alpha 4\beta 1 \) integrin (Kamata et al., 1995), and P4C10 (purified protein) and QE2.E5 (hybridoma SNF), directed to integrin \( \beta 1 \) chain.
Flow cytometric analysis. Cell surface expression of integrins was detected by indirect immunofluorescent staining of 3 × 10⁵ cells. Confluent RD, HepG2, Caco-2, COS-7 and MA104 cell monolayers were washed twice with PBS and cells were detached by incubation at 37 °C for 5 min in PBS containing 0.1% (w/v) trypsin (Difco) and 0.02% (w/v) EDTA (PBS–trypsin–EDTA). As trypsin treatment can produce proteolysis of the α₂ integrin subunit (Hemler et al., 1987), cells were detached by incubation for 10 min in PBS containing 0.75 mM EDTA (PBS–EDTA) in some experiments. Detached cells were resuspended in DMEM containing 1% (v/v) FCS for 30 min at 37 °C with occasional gentle agitation to allow restitution of surface proteins and then the medium was replaced with PBS containing 1% (v/v) FCS and 0.1% (w/v) Na₂EDTA (PBS–FCS–Az). K562 cells were washed twice in PBS–FCS–Az. Cells of all types were incubated for 45 min on ice with optimal dilutions of MAbs to integrin subunits or isotype-matched control MAbs diluted in PBS–FCS–Az. Optimal MAb dilutions were determined by testing serial dilutions of each MAb on each cell line. For the two-step stain, cells were washed once in PBS–FCS–Az, reacted for 45 min on ice with FITC-conjugated sheep anti-mouse F(ab')₂ fragments (Silenus) diluted 1:50 in PBS–FCS–Az and then washed as before. For the three-step stain, cells were washed twice in PBS–FCS–Az and then reacted as before with biotin-conjugated sheep anti-mouse F(ab')₂ fragments (Silenus) diluted 1:50 in PBS–FCS–Az. After two washes, cells were reacted as before with 3 μl per tube of undiluted phycoerythrin-conjugated streptavidin (Becton Dickinson) and then washed twice. Cells were fixed with 1% (v/v) ultrapure formaldehyde (Polysciences) in PBS before analysis of cellular fluorescence on a FACSort flow cytometer (Becton Dickinson). Viable cell populations were selected by gating dot plots of forward and side scatter and fluorescence intensity histograms of the gated cell populations were constructed. A positive relative linear median fluorescence intensity (RLMFI; median fluorescence intensity with anti-integrin MAb/median fluorescence intensity with control MAb) was defined as ≥1.20 (Wasserman et al., 1994). All anti-integrin MAbs were tested at a range of dilutions and the data obtained at the optimal MAb dilution were used for calculation of the RLMFI value. The optimal MAb dilution was the highest dilution giving the maximum RLMFI value. All MAbs showed dose-dependent binding to the cell lines tested.

The antibody-binding capacity of adherent cell lines was determined by using the Quantum Simply Cellular Kit (Flow Cytometry Standards Corp., San Juan, USA) as suggested by the supplier. The kit contains a mixture of microbeads, consisting of a blank and four populations that express different calibrated binding capacities for mouse IgG MAbs. The beads were treated identically with the test cell lines to derive a calibration curve from which the antibody-binding capacity of each cell line was obtained.

Rotavirus growth curve determination. Confluent RD, HepG2, Caco-2, COS-7 and MA104 cell monolayers in 24-well plates (Nunclon) and K562 cells in exponential phase were washed twice with PBS. K562 cells were suspended in 1 ml aliquots in DMEM at 5 × 10⁵ cells/ml. Cells of all types were incubated with trypsin-activated virus at multiplicities of infection (m.o.i.) of 0.1–10 for 1 h at 37 °C in 5% CO₂/95% air. The inoculum was replaced with DMEM containing 1 μg/ml porcine trypsin and incubation was continued as appropriate. Infection was terminated by freezing at −70 °C and virus was released from cells by two further freeze–thaw cycles. After trypsin activation, titres of harvested virus were determined by indirect immunofluorescent staining of infected cells in MA104 cell monolayers inoculated with serial dilutions of the samples. Virus titres were expressed as the number of fluorescing cell-forming units (f.c.f.u.) per ml (Coulson et al., 1985).

Results

Cell surface expression of integrins

Fig. 1 shows fluorescence intensity histograms determined by flow cytometry for the expression of integrin subunits α₂, α₄, β₁, αX and β₂ on the surface of human non-adherent K562 cells, on human adherent cell lines RD, HepG2 and Caco-2 and on monkey kidney cell lines MA104 and COS-7. All cell lines tested (K562, HepG2, Caco-2, COS-7 and MA104) expressed β₁ integrins. Integrin α₂β₁ was expressed by all adherent cell lines tested (RD, HepG2, COS-7 and MA104) and we have shown previously that Caco-2 cells express α₂β₁ (Coulson et al., 1997). Integrin subunits α₂ and β₂ were also expressed by all adherent cell lines tested. On RD cells, αX and β₂ were detectable by a two-step stain, whereas a three-step stain was required for detection of αX on Caco-2 and MA104 cell lines and for detection of β₂ on HepG2, Caco-2, COS-7 and MA104 cells. This suggests that the latter cell lines express lower levels of αXβ2 than do RD cells. Expression of αX was detected on MA104 cell suspensions produced by using PBS–EDTA but not on those produced with PBS–trypsin–EDTA (Fig. 1b), showing that the extracellular domain of the αX subunit is sensitive to digestion by trypsin. Only RD cells expressed αXβ1 integrin. K562 cells expressed none of the integrins implicated in rotavirus cell entry.

The levels of integrin expression on K562 and RD cells are shown in Table 1. αX and αX integrin subunits were not detected on K562 cells, even with the more sensitive three-step stain and two alternative MAbs. In a three-step stain, the αX subunit was also not detected on K562 cells. In addition, the β₂ subunit was also not detected on these cells with two alternative MAbs in a two-step stain. In contrast, both the MAbs to α4 bound RD cells specifically.

Table 2 provides the levels of integrin expression on HepG2, Caco-2, COS-7 and MA104 cells. Human cell lines HepG2 and Caco-2 showed higher RLMFI values with the anti-αX MAb AK7 than did monkey cell lines COS-7 and MA104. This pattern was also evident in the antibody-binding capacities of these cells with this MAb. All of these cell lines showed at least 13 600 antibody-binding sites per cell for MAb AK7, but HepG2 and Caco-2 cells showed more sites than did COS-7 and MA104 cells. Although it is possible that MAb AK7, being directed to human α2, does not bind as well to...
monkey as to human α2, no evidence for this has been published to our knowledge; thus, comparisons between levels of integrin expression on human and monkey cell lines using this MAb are likely to be reliable.

**Growth of rotaviruses in K562, RD, COS-7 and MA104 cells**

Growth curves of simian rotavirus SA11 and human rotavirus RV-5 in K562 and MA104 cells at a range of m.o.i. are shown in Fig. 2. Irrespective of the m.o.i., the maximum titres of SA11 produced in K562 cells were only 14- to 31-fold higher than the input virus titre (titre of virus associated with cells after incubation of virus with cells for 1 h at 37 °C). In contrast, the maximum titre of SA11 produced in MA104 cells at the low m.o.i. of 0·1 was 925 000-fold higher than the input virus titre. The maximum RV-5 rotavirus titres recorded in K562 cells were only 0·67- to 0·99-fold of the input virus titre, whereas the maximum titre produced in MA104 cells was 440- to 1520-fold higher than the input virus titre. Thus, SA11 showed very limited replication in K562 cells and RV-5 did not
replicate at all. In MA104 cells, SA11 at an m.o.i. of 0–1 grew to a 2000-fold higher titre than did RV-5 at an m.o.i. of 0–2.

Growth curves of SA11, RRV and human rotaviruses RV-4 and K8 in RD cells are shown in Fig. 3. Depending on the m.o.i. used, the maximum titres of SA11 produced were 132- to 759-fold higher than the input virus titre and those of RRV were 788- to 14 600-fold higher than the input titres. Thus, RD cells are permissive to SA11 and RRV. In contrast, RV-4 titres increased only 1–0- to 1–7-fold over input virus titre and K8 grew to titres 8–1- to 38-fold higher than input virus titres. RD cells are resistant to RV-4 infection but allowed low levels of K8 rotavirus replication.
3. Thus, in COS-7 cells at an m.o.i. of 1

tyintegrin were expected); and AK7 MAbs were comparable; see footnote
‡ (control) in a two-step stain on cells detached with PBS–EDTA;

f a two-step stain;

§ with these MAbs were equivalent. Insufficient quantities of MAb RMAC11 were available to complete the study with this MAb alone.

domain and showed similar RLMFI values at the same concentrations using MA104 cells (see Table 1 and footnote
‡ with a three-step stain.

Values were determined by using various MAbs and staining protocols. Superscript lower-case letters indicate the MAbs and conditions used:
a, 10 µg/ml MAbs RMAC11 (anti-α2) and MOPC21 (control) with both two-step and three-step stains; b, MAbs P4C2 (anti-α4) and ST-3:1 as control (1:2 dilution of hybridoma SNF) with a two-step stain; c, MAbs P4G9 (anti-α4) and RV-5:2 as control (1:2 dilution of hybridoma SNF) with a three-step stain; d, MAbs QE2.E5 (anti-β1) and RV-5:2 (control) with a two-step stain; e, anti-αX MAb 99.1.1.1 and ST-3:1 as control with a two-step stain; f, 80 µg/ml MAbs KB90 (anti-αX) and MOPC21 (control) in a three-step stain; g, 40 µg/ml MAbs KB90 (anti-αX) and MOPC21 (control) in a two-step stain on cells detached with PBS–EDTA; h, 10 µg/ml MAbs MHHM23 (anti-β2) and MOPC21 (control) in a two-step stain; i, 69 µg/ml MAbs MHHM23 (anti-β2) and MOPC21 (control) in a two-step stain.

† Determined from published test and control flow cytometric histograms (Chan et al., 1991; Hemler et al., 1987).

Table 1. Surface expression of integrins on K562 and RD cells
Values are RLMFI given as mean ± range. Positive RLMFI values, indicating that the integrin is expressed, are shown in bold.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>α2</th>
<th>α4</th>
<th>β1</th>
<th>αX</th>
<th>β2</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>0.98 ± 0.15 to 1.04 ± 0.01*</td>
<td>0.92 ± 0.1 to 1.10 ± 0.07</td>
<td>2.74 ± 0.07</td>
<td>0.99 ± 0.03</td>
<td>1.00 ± 0.01*</td>
</tr>
<tr>
<td>RD</td>
<td>1.3†</td>
<td>5.40 ± 0.2</td>
<td>3.79 ± 0.3</td>
<td>&gt; 2.0†</td>
<td>1.61 ± 0.03</td>
</tr>
</tbody>
</table>

* Values were determined by using various MAbs and staining protocols. Superscript lower-case letters indicate the MAbs and conditions used: a, 10 µg/ml MAbs RMAC11 (anti-α2) and MOPC21 (control) with both two-step and three-step stains; b, MAbs P4C2 (anti-α4) and ST-3:1 as control (1:2 dilution of hybridoma SNF) with a two-step stain; c, MAbs P4G9 (anti-α4) and RV-5:2 as control (1:2 dilution of hybridoma SNF) with a three-step stain; d, MAbs QE2.E5 (anti-β1) and RV-5:2 (control) with a two-step stain; e, anti-αX MAb 99.1.1.1 and ST-3:1 as control with a two-step stain; f, 80 µg/ml MAbs KB90 (anti-αX) and MOPC21 (control) in a three-step stain; g, 40 µg/ml MAbs KB90 (anti-αX) and MOPC21 (control) in a two-step stain on cells detached with PBS–EDTA; h, 10 µg/ml MAbs MHHM23 (anti-β2) and MOPC21 (control) in a three-step stain; i, 69 µg/ml MAbs MHHM23 (anti-β2) and MOPC21 (control) in a two-step stain.

Table 2. Surface expression of integrins on HepG2, Caco-2, COS-7 and MA104 cells
Values are RLMFI given as mean ± range. Positive RLMFI values, indicating that the integrin is expressed, are shown in bold.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ab-binding capacity for MAb to α2*</th>
<th>Cell surface expression†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α2±</td>
</tr>
<tr>
<td>HepG2</td>
<td>98 200 ± 19 800</td>
<td>21 96 ± 0.10</td>
</tr>
<tr>
<td>Caco-2</td>
<td>19 100 ± 190</td>
<td>15 54 ± 0.58</td>
</tr>
<tr>
<td>COS-7</td>
<td>13 600 ± 0</td>
<td>3.25 ± 0.03</td>
</tr>
<tr>
<td>MA104</td>
<td>17 413 ± 484</td>
<td>4.37 ± 0.12</td>
</tr>
</tbody>
</table>

* Antibody (Ab)-binding capacity (the number of antibody-binding sites on each cell) was determined as described in Methods and is given as mean ± range of two replicates. The binding capacity was determined by using 10 µg/ml MAbs AK7 (anti-α2) and MOPC21 (control) in a two-step stain.

† Values were determined by using various MAbs and staining protocols. Superscript lower-case letters indicate the MAbs and conditions used: a, 10 µg/ml MAbs AK7 (anti-α2) and MOPC21 (control) in a two-step stain, except where indicated; b, MAbs P4G9 (anti-α4) and RV-5:2 as control (1:2 dilution of hybridoma SNF) with a three-step stain; c, 20 µg/ml MAbs P4C10 (anti-β1) and MOPC21 (control) in a two-step stain, unless otherwise indicated (MAbs P4C10 and QE2.E5 gave equivalent RLMFI values, as described in the legend to Fig. 1); d, 40 µg/ml MAbs KB90 (anti-αX) and MOPC21 (control) in a three-step stain on cells detached with PBS–EDTA (the three-step stain was used as low levels of this integrin were expected); e, 35 µg/ml MAbs MHHM23 (anti-β2) and MOPC21 (control) in a three-step stain (the three-step stain was used as low levels of this integrin were expected); f, 20 µg/ml MAbs RMAC11 (anti-αX) and ST-3:3 (control) in a two-step stain (data obtained with RMAC11 and AK7 MAbs were comparable; see footnote ‡); g, MAbs QE2.E5 (anti-β1) and RV-5:2 (control) with a two-step stain.

‡ MAbs AK7 and RMAC11 (which was used for determination of α2 expression on K562 and RD cells) map to overlapping epitopes on the α2 I domain and showed similar RLMFI values at the same concentrations using MA104 cells (see Table 1 and footnote ‡ above). Thus, results obtained with these MAbs were equivalent. Insufficient quantities of MAb RMAC11 were available to complete the study with this MAb alone.

§ The first value was obtained with a two-step stain and the second with a three-step stain.

ND, Not done.

Growth curves of SA11, RV-5, Wa and ST-3 rotaviruses in COS-7 cells are shown in Fig. 4. All rotavirus strains tested replicated in these cells, with maximum titres being 800-fold above input virus titre for SA11, 92-fold above input for RV-5, 15-fold above input for Wa and 2.3-fold above input for ST-3. Thus, in COS-7 cells at an m.o.i. of 1:0, RV-5 grew to a higher titre than did Wa and ST-3 replicated to a low titre only. In MA104 cells, Wa rotavirus grew to a maximum titre 1760-fold above the input virus titre and ST-3 grew to a maximum titre 34-fold above the input titre. All rotavirus strains tested grew to significantly lower titre in COS-7 cells than in MA104 cells.
Comparison of cellular expression of integrins with cellular susceptibility to infection with monkey and human rotaviruses

As shown in Table 3, the cell line that did not express any integrin implicated in rotavirus cell entry (K562) was not susceptible to RV-5 rotavirus infection and showed the lowest titre of SA11 at 20 h post-infection (p.i.) (m.o.i. = 0·1) of any cell line tested. On RD cells, expression of α2 integrin is just detectable by flow cytometry (Chan et al., 1991; Hemler et al., 1987), giving an RLMFI of 1–3, and we showed that these cells do express α4β1 and αXβ2. SA11 replicated to a 1·2-fold higher titre in RD cells than in K562 cells and human strains replicated to a low level. Higher levels of α2 integrin were expressed on Caco-2, COS-7 and MA104 cells, all of which supported both SA11 and human rotavirus infection at moderate to high levels. Expression of no, low or high levels of α2 integrin correlated with no, low and moderate levels of human rotavirus replication. The absence of αXβ2 also correlated with the inability of RV-5 to grow in K562 cells and with the low level of SA11 replication in these cells. Overall, all cells expressing αXβ2 were susceptible to rotavirus infection to varying degrees.

The correlation between the α2 integrin RLMFI value and the titre of SA11 produced at 20 h p.i. after infection at an m.o.i. of 0·1 was analysed for K562, RD, COS-7, MA104 and Caco-2 cells (Fig. 5). There was a highly significant correlation...
Fig. 4. Growth of rotavirus strains SA11, RV-5, Wa and ST-3 in COS-7 and MA104 cells. M.o.i. = 1. Bars represent 95% CI.

Table 3. Comparison of cellular integrin expression with susceptibility to rotavirus infection

<table>
<thead>
<tr>
<th>Cellular integrin expression (RLMFI)</th>
<th>Rotavirus growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>α2</td>
</tr>
<tr>
<td>K562</td>
<td>1.0</td>
</tr>
<tr>
<td>RD</td>
<td>1.3±</td>
</tr>
<tr>
<td>COS-7</td>
<td>3.3</td>
</tr>
<tr>
<td>MA104</td>
<td>4.4</td>
</tr>
<tr>
<td>Caco-2</td>
<td>15±</td>
</tr>
</tbody>
</table>

* Mean titre ± SD (10−9 × f.c.f.u./ml) of virus produced at 20 h p.i. at an m.o.i. of 0.1.
† Defined as the given range of the virus replication index (VRI), which is the ratio of the maximum virus titre produced to the input virus titre (titre of virus associated with cells at 1 h p.i.), scored as follows: —, VRI < 1; ±, 1 ≤ VRI < 10; +, 10 ≤ VRI < 100; 2+, 100 ≤ VRI < 1000; 3+, 1000 ≤ VRI < 10000.
‡ Determined from published test and control flow cytometric histograms (Chan et al., 1991; Hemler et al., 1987).
§ Data taken from Kirkwood et al. (1998).
ND, Not done.

Fig. 5. Correlation of growth of SA11 rotavirus with cellular α2 integrin expression. The data for SA11 growth (expressed as the mean titre ± SD of virus produced at 20 h.p.i. from cells infected at an m.o.i. of 0.1) and α2 integrin expression (expressed as the log_{10} of the RLMFI) taken from Tables 1–3 for K562, RD, COS-7, MA104 and Caco-2 cells are represented as open squares. The line shows the best fit determined by non-linear regression analysis (r² = 1.00) and corresponds to a sigmoidal dose-response curve with variable slope.

between these parameters by the two-tailed Pearson test (P = 0.005) and the data were best fitted by a sigmoidal dose-response curve with variable slope (r² = 1.00). This suggests that SA11 may be binding allosterically to α2 integrin as a...
result of the interaction of multimeric virus-binding sites with increasing numbers of receptors. The titres of SA11 produced after one cycle of virus replication correlated quantitatively with the level of cellular expression of α2 integrin, and cellular levels of α2 integrin expression may therefore be important in determining SA11 rotavirus yields.

**Discussion**

If integrins play an important role in rotavirus entry into cells, their cellular expression should be necessary for the cells to be fully permissive to rotavirus infection. In addition, levels of cellular integrin expression might be expected to correlate with levels of rotavirus replication. In this study, we have shown that cell lines that allow any replication of human rotaviruses express α2β1 integrin and (when tested) αXβ2 integrin, whereas the K562 cell line, which was totally non-permissive to human rotavirus RV-5 infection, did not express these integrins or the other integrin implicated as a rotavirus receptor, α4β1. The level of human rotavirus replication, within broad ranges of virus replication index (VRI), correlated positively with the level of expression of α2 integrin subunit on human cell lines. Thus, cellular expression of α2β1, and probably αXβ2, is consistent with these integrins being important for human rotavirus cell entry. Titres of simian rotavirus SA11 produced 20 h after infection at an m.o.i. of 0-1 correlated quantitatively with levels of expression of the α2 integrin, expressed as an RLMFI. This correlation of α2β1 expression with SA11 yield provides further support for the role for this integrin as a rotavirus receptor. A similar correlation between SA11 rotavirus yields and the α2 integrin RLMFI was observed for K562 cells transfected with α2 integrin and/or expressing additional α2 integrin due to phorbol ester treatment (Hewish et al., 2000).

In contrast to human rotaviruses, SA11 was able to replicate to a low level in K562 cells, which did not express any integrin implicated in rotavirus cell entry. This suggests that another receptor(s), in addition to integrins, is used by SA11 in K562 cells, but that α2β1 and possibly αXβ2 are required for efficient SA11 replication in cell culture. As SA11, but not human rotaviruses, can use sialic acid for cell attachment (Fukudome et al., 1989), this is suggested to be the most likely candidate receptor for SA11 on K562 cells.

Our results are in full agreement with previous reports of the cell surface expression of these integrins. Of the β1 integrins, K562 cells have been shown to express only α5β1 (Hemler et al., 1987) and were also found to lack β2 integrins, including αXβ2 (Uciechowski & Schmidt, 1989). On RD cells, 32% of the β1 expressed was as α4β1, whereas only 3% was as α2β1 (Hemler et al., 1987). On HepG2 cells, 53% of the β1 expressed was as α2β1 but no α4β1 was detected, so RD cells expressed much less α2β1 than did HepG2 cells (Hemler et al., 1987). Caco-2 and MA104 cells have been shown to express α2β1 (Basson et al., 1992; Coulson et al., 1997) and β2 (Coulson et al., 1997). The expression of β2 integrins on non-lymphoid cells is controversial but, in humans, β2 integrins have been detected on rectal epithelial cells (Hussain et al., 1995) and αXβ2 has been found on isolated enterocytes (Martin-Villa et al., 1997). Our studies showed that αXβ2 detection in flow cytometric studies can depend on the use of a protease-free cell dissociation buffer, as has been reported previously for α4β1 detection (Hemler et al., 1987).

Replication of rotaviruses in K562 and RD cells has not been studied previously, although growth studies with some human and monkey rotavirus strains in MA104. HepG2 and Caco-2 cells have been reported (Estes et al., 1979; Kitamoto et al., 1991; Ramig & Galle, 1990). Our SA11 growth curves in MA104 cells are similar to those reported previously (Estes et al., 1979; Kitamoto et al., 1991; Ramig & Galle, 1990). Growth curves of SA11 in CV-1 cells (Estes et al., 1979) were similar to our results in COS-7 cells, which are a derivative of the CV-1 line (Gluzman, 1981). Levels of rotavirus growth in HepG2, Caco-2 and MA104 cells were shown to be determined by the origin of gene 4, encoding VP4 (Kirkwood et al., 1998; Ludert et al., 1996; Ramig & Galle, 1990). Growth curves for RV-5, RV-4, K8 and ST-3 have not been reported previously.

Although there was an overall correlation between cellular expression of α2β1 (and possibly αXβ2) and rotavirus replication, quantitative differences were discernible in the replication of different rotavirus strains in given cell lines. In particular, RD cells showed unusual relative growth curves for RRV, SA11 and human rotaviruses. SA11 replicated to a 100-fold lower titre in RD cells than did RRV, whereas these two strains have been reported to replicate to similar titres in MA104, Caco-2 and HepG2 cells (Kitamoto et al., 1991). The difference between the maximum titres of human rotaviruses and of RRV in RD cells (1000-fold) was greater than that observed in MA104 cells (100-fold; Kitamoto et al., 1991). Particularly for the human strains, which do not appear to use sialic acid, it is likely that additional cellular receptors exist. One candidate receptor may be β-D-galactose, as infection of cells with Wa and SA11 is blocked by the *Ricinus communis* agglutinin I (Jolly et al., 1999; Superti & Donelli, 1995).

Only RD cells expressed α4β1, so this integrin cannot be a necessary prerequisite for rotavirus infection. As α4 integrin expression is restricted mainly to cells of the immune system, it is likely that rotavirus may interact via α4 with these cells and modulate their function. Rotavirus infection in children results in a specific circulating memory T cell response that is mainly CD4+ and α4β7+. In the murine model, memory B cells providing the secretory IgA response and protective humoral immunity also express α4β7 (Franco & Greenberg, 1999). It will be interesting to examine whether rotavirus can bind to or infect these α4β7+ B and T cells via α4 integrin.

In Caco-2 cells, but not in RD or MA104 cells, the αX integrin subunit was more readily detected than was the β2 subunit. This pattern of apparently greater expression of αX than β2 has been observed previously for mononuclear cells in
the intestine, which showed greater expression of each of the β2 integrin partners (αL, αM and αX) than of β2 (Bernstein et al., 1996). In a study in which β2 expression was detected by using the same MAb used in our study (MHM23), small bowel lamina propria T cells also showed greater expression of αL than β2 (Smart et al., 1991). It has been suggested that this may be due to conformational changes in the integrin heterodimer that result in β2 epitope masking (Smart et al., 1991) or that another β partner for αL, αM and αX may exist (Bernstein et al., 1996).

The role of αXβ2 integrin in rotavirus cell entry is not yet determined. Although expression of this integrin is generally considered to be restricted to immune cells, we have detected αXβ2 on rotavirus-permissive cell lines and others have found αXβ2 on enterocytes, so it is possible that αXβ2 plays a role in rotavirus entry into permissive cells in vivo and in vitro. As αXβ2 and α4β1 on neutrophils are important in adhesion of these cells at sites of inflammation and αXβ2 is important for monocyte/macrophage and dendritic cell function and for homing of intraepithelial cells to the small intestine (Shibahara et al., 2000), rotavirus interaction with αXβ2 and/or α4β1 may affect these immune responses. Transfection of cells with integrins should provide a useful model for studying the requirements of rotavirus usage of integrins and other molecules during cell attachment and entry. Studies on rotavirus binding to and infection of αXβ2-transfected K562 cells are in progress.

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