Down-regulation of integrin α1/β1 expression and association with cell rounding in human cytomegalovirus-infected fibroblasts

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Human cytomegalovirus (HCMV) causes a c.p.e. characterized by rounding of the infected cell. Since interactions with the extracellular matrix may be involved in the cell rounding, we have analysed the expression of integrins, which are the main cell surface receptors involved in cell–substrate adhesion and spreading. By FACS analysis, a selective decrease in cell surface expression of α1/β1 integrin was observed in HCMV-infected fibroblasts. This decrease coincides with cell rounding. Immunoprecipitation studies and FACS analysis of permeabilized cells have further demonstrated that total levels of this integrin are decreased in infected cells, suggesting that the reduction in cell surface α1/β1 integrin is not due to a defect in transport to the surface. Furthermore, we have ruled out the possibility that the observed decrease in α1/β1 expression is caused by a cytokine released from the infected cells by showing that the reduction is abolished by inactivating the HCMV with u.v. irradiation, and that conditioned medium from HCMV-infected cells has no effect on expression of α1/β1 integrin in uninfected cells. Concomitant with the reduction in α1/β1 levels, the HCMV-infected fibroblasts show a reduced ability to adhere to laminin and collagen IV. Taken together the data indicate that de novo synthesis of HCMV protein(s) causes a decreased assembly/expression of α1/β1 integrin, coincident with the well characterized morphological alterations of the infected cell.

Introduction

Interaction of cells with extracellular matrix molecules influences many cellular activities including adhesion, migration, differentiation and maintenance of tissue architecture. In part this is mediated through binding of extracellular matrix proteins to members of the integrin family, which are αβ heterodimeric cell surface glycoproteins (Hynes, 1992). Integrins are classified into at least eight subfamilies, designated β1 to β8. Within each subfamily, the β subunit can associate with a number of different α subunits. The specificity and affinity of the integrin is dependent on a particular αβ combination, for example α5/β1 binds only one specific ligand, fibronectin, whereas other integrins such as α3/β1 can interact with several proteins. Many, but not all integrins recognize a peptide sequence, Arg-Gly-Asp (RGD), present in a number of their ligands (Ruoslahti & Pierschbacher, 1986). Integrins also require divalent cations for heterodimer formation as well as for ligand interaction.

Although their primary role is widely recognized as mediating cell–substrate and cell–cell interaction, integrins also act as signalling molecules (Hynes, 1992). The integrins constitute a large family of structurally related proteins, whose widespread distribution and capacity to bind extracellular molecules makes them good candidates for use as virus receptors. Echoviruses utilize the α2/β1 integrin as their cellular receptor (Bergelson et al., 1992) and the αv/β3 and αv/β5 integrins promote adenovirus internalization but not the initial virus attachment (Wickham et al., 1993). There have been fewer reports documenting the effects of virus infection on integrin expression, although α5/β1 integrin expression is enhanced in acute and chronic human immunodeficiency virus infection of CD4+ cells (Weeks et al., 1991). These cells acquire the ability to bind to extracellular glycoproteins fibronectin, collagen IV and laminin and adhesion is accompanied by marked changes in morphology.

Human cytomegalovirus (HCMV), a member of the beta-herpesvirus group, infects the majority of the general population by adulthood. Primary infection is usually subclinical and is followed by asymptomatic lifelong viral persistence in the immunologically competent host. However, reactivation of HCMV is a major cause of death in the immunocompromised, such as...
AIDS patients and individuals undergoing organ transplantation.

A striking phenotypic feature of cells infected with HCMV is the rounded shape that is characteristic of c.p.e. (Albrecht et al., 1989). Reduced adhesiveness is noted concomitant with the rounding. The molecular basis of this phenomenon is unknown but as molecules involved in cell–substrate interactions may be implicated, we have studied the expression of integrins during HCMV infection.

The combination of β1 with one of the α1 to α6 subunits results in integrins that bind collagen, fibronectin or laminin (Hemler et al., 1987) as well as to receptors on other cells (Carter et al., 1990). We have investigated the expression of the α1 to α6 integrin subunits to determine whether HCMV can affect integrin expression and whether this correlates with cell rounding. We found that HCMV selectively causes a large decrease in cell surface expression of α1/β1 integrin in HCMV-infected fibroblasts. This decrease is accompanied by reduced ability of the HCMV-infected cells to adhere to laminin and collagen IV but no differences in adhesion to collagen I, collagen II, or fibronectin were noted. The decrease in α1/β1 integrin expression coincided with cell rounding.

**Methods**

**Cell lines and virus.** Human fetal lung fibroblasts MRC-5 (European Collection of Animal Cell Cultures) were grown in MEM (Gibco) supplemented with 10% fetal calf serum (FCS) and 2 mM-glutamine and used between passage 25 and 35. KP is a primary human dermal fibroblast line (Borysiewicz et al., 1983) used between passage 10 and 20. HCMV strain AD169 was propagated and titred in MRC-5 fibroblasts as described previously (Borysiewicz et al., 1983).

**Antibodies.** Antibodies listed in Table 1 were used at the recommended concentrations. Monoclonal antibodies TS2/7, LM609 and LM142 were provided as ascites, GoH3 was a culture supernatant and the rest of the antibodies were purified.

**Flow cytometry.** Pilot studies indicated that levels of cell surface integrins were unaffected by brief trypsinization. Therefore mock- or HCMV-infected cells were briefly trypsinized with trypsin and EDTA and washed with PBS, 1% FCS and 0.02% azide. They were then fixed in 2% paraformaldehyde in PBS for 15 min at room temperature. After washing with PBS, the cells were incubated with various antibodies (diluted at the working concentration in PBS, 1% FCS and 0.02% azide) for 30 min at 4 °C. Cells were washed to remove unbound antibody and further incubated with fluorescein isothiocyanate (FITC)-conjugated F(ab')2 fragments to mouse IgG or in the case of GoH3 antibody, with FITC-conjugated whole antibody to rat IgG. After 30 min, cells were washed and re-fixed as outlined above. Following further washing, cells were analysed by flow cytometry using a FACScan (Beckton Dickinson).

**Immunoprecipitation.** Mock- or HCMV-infected cells were pre-incubated in methionine-free MEM for 60 min at 37 °C. [35S]Methionine (100 μCi, 50 μCi/mmol; Amersham) was added and cells incubated for 4 h at 37 °C. The cells were washed with ice-cold PBS and lysed on ice in 1% NP40, 10 mM-Tris-HCl pH 7.4, 150 mM-NaCl, 2 mM-EDTA, 2 mM-PMSF and 10 μg/ml leupeptin. The lysates were centrifuged at 13000 g for 20 min at 4 °C. The supernatant was pre-cleared overnight at 4 °C with formalin-fixed Staphylococcus aureus cell suspension (Sigma). Pre-cleared lysates were then incubated with monoclonal antibody TS2/7 or OKT9 for 90 min at 4 °C. This was followed by a further incubation of 30 min with goat anti-mouse Ig. The antigen–antibody complexes were immunoprecipitated with Protein A–Sepharose beads for 45 min. The immunoprecipitates were washed four times and resuspended in Laemmli reducing sample buffer. The immunoprecipitated material was separated by 7.5% SDS-PAGE. After impregnating the gel with Amplify (Amersham), the dried gel was exposed to X-ray film at -70 °C.

**Adhesion assay.** Flat-bottomed tissue culture dishes (96 well) were coated overnight at 4 °C with 100 μl of 10 μg/ml collagen I, collagen II, collagen IV, fibronectin, laminin or BSA. Non-specific sites were blocked by incubation with 2 mg/ml BSA for 2 h at 37 °C. The plates were then ready for use in the adhesion assay.

Mock- and HCMV-infected fibroblasts were trypsinized and loaded with 100 μCi of sodium [35Cr]chromate (250 μCi/mg; Amersham). After removing the excess chromate by extensive washing, the labelled cells were resuspended at 5×10⁶ cells/ml in RPMI 1640 medium containing 2 mg/ml BSA. A 100 μl sample of the cell suspension was added to each well and the plate centrifuged at 10 g for 5 min to allow even settlement of the cells on the plate surface. After 60 min incubation at 37 °C to allow adhesion, the wells were filled with RPMI 1640 medium and sealed with clingfilm. The plate was inverted and centrifuged at 30 g for 3 min to remove unbound cells. The plate was flicked to remove the medium, fresh medium was added and the plate processed as described above for the second wash. After the final wash, the plate was dried at 37 °C and the adherent cells lysed in 1% Triton-

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Source</th>
<th>Reference</th>
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<tbody>
<tr>
<td>TS2/7</td>
<td>α1/β1</td>
<td>Dr M. Hemler</td>
<td>Hemler et al. (1984)</td>
</tr>
<tr>
<td>P1E6</td>
<td>α2/β1</td>
<td>Chemicon</td>
<td>Wayne et al. (1988)</td>
</tr>
<tr>
<td>P1B5</td>
<td>α3/β1</td>
<td>Chemicon</td>
<td>Wayne et al. (1988)</td>
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<td>HP2/1</td>
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<td>Serotec</td>
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<td>Chemicon</td>
<td>Wayne et al. (1988)</td>
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<td>GoH3</td>
<td>α6/β1</td>
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<td>Sonnenberg et al. (1987)</td>
</tr>
<tr>
<td>LM609</td>
<td>αv/β3</td>
<td>Dr D. Cheres</td>
<td>Cheres &amp; Spiro (1987)</td>
</tr>
<tr>
<td>LM142</td>
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<td>Dr D. Cheres</td>
<td>Cheres &amp; Harper (1987)</td>
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<tr>
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<td>β1</td>
<td>Serotec</td>
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<tr>
<td>W6/32</td>
<td>HLA* A, B, C</td>
<td>Seralab</td>
<td>Barnstable et al. (1978)</td>
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* HLA, human leukocyte antigen.
X100. The number of cells adhering to the different matrix components was measured by determining the radioactivity in aliquots of the lysates by liquid scintillation counting (Betaplate; Wallac).

The experiment was carried out in sextuplicate and the values of background adhesion (measured by using BSA as the coating matrix; typically 1 to 5%) was deducted from the experimental values. Data were expressed as percentage adhesion compared to input cell number.

Results

Time-dependent decrease in cell surface expression of $\alpha 1/\beta 1$ integrin in HCMV-infected embryonic fibroblasts

MRC-5 cells were initially infected with HCMV at 20 p.f.u./cell. These cells displayed the typical morphological changes associated with HCMV infection from 10 h post-infection (p.i.) (Fig. 1); at 10 to 16 h p.i., many of the cells completely round up although they remain adherent and subsequently, partial spreading of the infected cells is observed. We have investigated the expression of integrins known to be involved in cell-substrate adhesion to determine whether cell rounding induced by HCMV is a consequence of disturbance in integrin expression. Uninfected MRC-5 cells were screened using a panel of antibodies listed in Table 1 and were found to express $\alpha 2/\beta 1$, $\alpha 3/\beta 1$, $\alpha 5/\beta 1$, $\alpha 6/\beta 1$ and $\alpha v/\beta 3$ integrins. No $\alpha 4/\beta 1$ expression was noted. In order to determine whether HCMV affects integrin expression, integrin levels were initially determined at 72 h p.i. Expression of most of the integrins tested was only marginally altered in infected cells (10 to 30%). However, the levels of $\alpha 1/\beta 1$ were markedly and consistently depressed (Table 2). There was a five- to 20-fold decrease in levels of $\alpha 1/\beta 1$ in five separate experiments at 72 h p.i. We therefore determined the time-course for the HCMV-induced reduction in $\alpha 1/\beta 1$. By FACS analysis, the level of $\alpha 1/\beta 1$ at the cell surface of HCMV-infected cells was 50% of uninfected-cell levels at 10 h p.i., and 7 to 20% at 16 to 72 h p.i. (Fig. 2). Decreased $\alpha 1/\beta 1$ expression was also found in HCMV-
Decreased expression of α1/β1 integrin in HCMV-infected fibroblasts requires active viral replication

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Uninfected cells</th>
<th>HCMV-infected cells</th>
<th>U.v.-irradiated HCMV-infected cells</th>
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<tbody>
<tr>
<td>HCMV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1/β1</td>
<td>16</td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td>αv/β3</td>
<td>8</td>
<td>9</td>
<td>15</td>
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<td>Conditioned medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1/β1</td>
<td>19</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>αv/β3</td>
<td>10</td>
<td>9</td>
<td>9</td>
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infected adult skin fibroblasts (data not shown). This change was not due to a generalized effect on cell surface proteins since the expression of other integrins (αv) was relatively unchanged (Table 2).

Decreased α1/β1 expression is dependent on multiplicity of HCMV infection

The decrease in α1/β1 expression was dependent on levels of input virus (Fig. 3). As little as 2 to 5 p.f.u./cell was sufficient to induce a change in α1/β1 expression at 72 h p.i. The expression of other integrins was unchanged; αv integrin is shown for comparison in Fig. 3. The reduction in cell surface α1/β1 in HCMV-infected cells required viral gene expression since it was not observed when cells were exposed to u.v.-inactivated HCMV (Table 3). Furthermore, conditioned medium from HCMV-infected fibroblasts, which had been filtered through a Centricon 100 filter (Amicon) to remove viral particles, was unable to reduce α1/β1 expression when added to uninfected cells and incubated for 72 h, suggesting that the effect is not caused by a soluble factor released into the medium by HCMV-infected fibroblasts (Table 3).

Decrease in cell surface α1/β1 integrin level in HCMV-infected fibroblasts occurs at the level of α1 expression

Since the expression of other β1 integrin complexes was relatively unaffected by HCMV infection, we reasoned that the defect in α1/β1 expression might be explained by reduced synthesis of the α1 chain. In order to test this, control or 72 h HCMV-infected MRC-5 cells were metabolically labelled with [35S]methionine and lysates obtained from equivalent cell numbers were immunoprecipitated with the TS2/7 monoclonal antibody, recognizing α1/β1 integrin. The level of α1 subunit (210K) was clearly decreased in HCMV-infected cells compared to uninfected cells (Fig. 4). The expression of other α chains (data not shown) and cell-surface proteins such as the transferrin receptor recognized by antibody OKT9 were unchanged (Fig. 4). Consistent with this observation, we noted that total levels of α1/β1 in

![Figure 3: Decrease in α1/β1 integrin expression is dependent on multiplicity of HCMV infection. MRC-5 fibroblasts were infected with different amounts of HCMV for 72 h. The cells were trypsinized and stained with either monoclonal antibody TS2/7 recognizing α1/β1 integrin (a to f), antibody LM142 recognizing αv integrin (g to l) or an irrelevant antibody (OKT3). The open curves represent background fluorescence obtained with the irrelevant antibody. The filled curves represent staining with TS2/7 or LM142. Cells were either uninfected (a and g) or infected with HCMV at 1 p.f.u./cell (b and h), 2 p.f.u./cell (c and i), 5 p.f.u./cell (d and j), 10 p.f.u./cell (e and k) or 20 p.f.u./cell (f and l).](image-url)
Integrins and HCMV infection

Fig. 4. Immunoprecipitation of the α1/β1 integrin from HCMV-infected fibroblasts. MRC-5 fibroblasts were infected with 20 p.f.u./cell or HCMV for 72 h. The cells were labelled with [35S]methionine and NP40 lysates were then immunoprecipitated with either monoclonal antibody TS2/7 (α1/β1) or OKT9 (transferin receptor; TIR). The immunoprecipitates were separated by 7.5% SDS-PAGE. Lane 1, HCMV-infected cell lysate immunoprecipitated with TS2/7; lane 2, uninfected cell lysate immunoprecipitated with TS2/7; lane 3, HCMV-infected cell lysate immunoprecipitated with OKT9; lane 4, uninfected cell lysate immunoprecipitated with OKT9.

Table 4. Total levels of α1/β1 expression in HCMV-infected fibroblasts

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Cell surface expression*</th>
<th>Cell surface and intracellular expression*</th>
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<tr>
<td></td>
<td>Uninfected</td>
<td>HCMV-infected</td>
</tr>
<tr>
<td>α1/β1</td>
<td>20</td>
<td>5.8</td>
</tr>
<tr>
<td>α2/β1</td>
<td>27</td>
<td>26</td>
</tr>
</tbody>
</table>

* Mean fluorescence intensity.

permeabilized cells were decreased whereas that of α2/β1 was not (Table 4).

Decreased adhesion of HCMV-infected MRC-5 cells to extracellular matrices, collagen IV and laminin

Since the α1/β1 integrin is the receptor for collagen and/or laminin, we tested whether the decreased integrin levels in HCMV-infected fibroblasts altered interaction with extracellular matrix proteins. Cells (32P-labelled) were allowed to adhere to plastic coated with different extracellular matrix proteins. Uninfected MRC-5 cells adhered to collagen I, II and IV, fibronectin and laminin but not to BSA (Fig. 5). After HCMV infection for 72 h, the adherence of the cells to collagen I and II and fibronectin was unaltered (Fig. 5). In contrast, adherence to collagen IV and laminin was decreased after HCMV infection. This decrease occurred as early as 24 h p.i. (data not shown). Therefore, reduced expression of the α1/β1 integrin at the cell surface correlated with reduced adhesion to laminin and collagen IV. This double specificity has been also reported in other cellular systems (Clyman et al., 1990).

Discussion

During cases of HCMV disease, the viral particles can be detected in many tissues yet the sites of persistence in healthy individuals remain poorly defined. Although there is some evidence that HCMV persists in endothelia, epithelia and monocytes (Ho, 1991), in vitro HCMV will only readily produce a productive infection in human fibroblasts.

HCMV causes cell rounding 10 to 24 h p.i. This is not due to cell death as the cells recover and partially re-spread from 48 to 72 h p.i. Since integrins are the main receptors for interaction with the extracellular matrix, we studied their expression in HCMV-infected fibroblasts. We report that HCMV-induced changes in cell morphology coincided with decreased expression of the α1/β1 integrin.

The decrease in α1/β1 at the cell surface could be due
to a decrease in either \( \alpha_1 \) or \( \beta_1 \) biosynthesis. Normally the \( \beta_1 \) chain is synthesized in excess compared with \( \alpha_1 \), and the two associate intracellularly before transport to the cell surface. Dimer expression, therefore, appears to be regulated by the rate limiting synthesis of the \( \alpha \) subunit (Heino et al., 1989). Briesewitz et al. (1993) have shown this to be specifically the case for the \( \alpha_1/\beta_1 \) integrin. In situations where \( \alpha_1/\beta_1 \) expression is increased, such as treatment with retinoic acid (Rossino et al., 1991) or cytokines (Santala & Heino, 1991; Defilippi et al., 1992), synthesis of the \( \alpha_1 \) subunit is increased whilst \( \beta_1 \) synthesis is unchanged, although occasionally regulation of expression of both \( \alpha \) and \( \beta \) units has been described (Wahl et al., 1993). The down regulation of \( \alpha_1/\beta_1 \) expression in HCMV-infected fibroblasts described in this study is probably due to the reduction of \( \alpha_1 \) subunit synthesis, based on immunoprecipitation and FACS analysis of integrin expression in permeabilized cells, together with the finding that other integrins of the \( \beta_1 \) family were unaffected.

A number of mechanisms could explain the effect of HCMV on \( \alpha_1/\beta_1 \) expression. Firstly, the decreased \( \alpha_1/\beta_1 \) expression may be mediated by soluble factors either present in the virus inoculum or released into the medium during the course of HCMV infection, since integrin expression can be modulated by cytokines. Interleukin 1\( \beta \) (IL-1\( \beta \)) and tumour necrosis factor \( \alpha \) (TNF-\( \alpha \)) increase \( \alpha_1/\beta_1 \) expression in human skin fibroblasts and osteosarcoma cells by increasing the expression of the \( \alpha_1 \) subunit without affecting the \( \beta_1 \) chain (Santala & Heino, 1991), whereas in human umbilical vein endothelial cells (HUVECS) these cytokines decrease \( \alpha_6/\beta_1 \) expression by reducing the expression of \( \alpha_6 \) subunit (Defilippi et al., 1992). Furthermore TNF-\( \alpha \), but not IL-1\( \beta \), induces expression of \( \alpha_1/\beta_1 \) in HUVECS, which do not normally express this integrin. In addition, integrins can be modulated by other cytokines such as transforming growth factor \( \beta \) (Wahl et al., 1993) and morphogens such as retinoic acid (Rossino et al., 1991). In this study however, the decrease in \( \alpha_1/\beta_1 \) expression does not appear to be cytokine-mediated. Firstly, we prepared conditioned medium from HCMV-infected fibroblasts from which the virus particles had been removed by filtration. Addition of this medium to uninfected cells did not significantly affect \( \alpha_1/\beta_1 \) expression, indicating that soluble factors/epitopes produced by the virus-infected cells were not responsible. Secondly, a virus inoculum from which virus particles had been removed or inactivated by u.v. irradiation was ineffective in reducing \( \alpha_1/\beta_1 \) levels, suggesting that cytokines potentially present in the virus inoculum did not induce this effect.

A second possible explanation to be considered is that HCMV binding to its receptor, or release of viral proteins present in the virus particle causes altered integrin expression. Since u.v.-inactivated virus preparations are ineffective in modulating \( \alpha_1/\beta_1 \) expression, it would seem that \textit{de novo} virus protein synthesis is required. It remains possible, although unlikely, that u.v. inactivation damages an input virion protein involved in inhibition of integrin expression.

A third possibility, and the one that we favour, is that viral gene expression results in the production of proteins that, either directly or indirectly, disrupt \( \alpha_1/\beta_1 \) expression. In particular the IRS1 gene (Jones & Muzithras, 1992a) has been shown to have a role in HCMV-induced cell rounding; HCMV mutants lacking this gene are unable to induce cell rounding (Jones & Muzithras, 1992b). The IRS1 gene has a transcriptional trans-activating activity, which may switch off \( \alpha_1 \) chain synthesis, either by a direct or indirect means.

Integrin \( \alpha_1/\beta_1 \) was first described as the VLA-1 antigen in activated lymphocytes (Hemler et al., 1984). Subsequently it has been shown to be present in many cell types where it functions as a collagen and laminin receptor (Forsberg et al., 1990; Gullberg et al., 1990; Hall et al., 1990; Ignatius & Reichardt, 1988; Kramer & Marks, 1989). We observed a reduction in collagen IV and laminin adhesion in HCMV-infected fibroblasts showing that in fibroblasts, \( \alpha_1/\beta_1 \) acts as a dual receptor. The reduction was only partial because other integrins that bind to collagen IV and laminin are present on fibroblasts and probably compensate for the reduced \( \alpha_1/\beta_1 \) levels. Interestingly, endothelial cells infected with herpes simplex virus type 1 also showed a reduction in adhesion to laminin and collagen IV (Visser et al., 1989), although the integrins involved were not identified.

Whilst the relevance of the findings reported here are presently unclear, integrin receptors are known to play an important role in cell signalling processes, including calcium mobilization, protein phosphorylation, cytoskeletal interaction and alteration in cytoplasmic pH (Hynes, 1992). These events in turn may play a role in induction of gene expression or differentiation of specific cell types, which has been observed in response to engagement of integrins by their ligands or antibodies. So far, induction of the protease gene in synovial fibroblasts (Werb et al., 1989), inhibition of terminal keratinocyte differentiation (Adams & Watt, 1989) and modulation of myogenesis (Menko & Boettiger, 1987) have been documented. Whether any or all of the signalling events mediated by integrins play an important role in cell rounding and the biology of HCMV infection remains to be determined.

We gratefully thank Drs M. E. Hemler (U.S.A.), D. A. Cheresh (U.S.A.) and A. Sonnenberg (The Netherlands) for their generosity in providing antibodies. This work was supported by the Wellcome Trust and University of Wales College of Medicine Research Initiative Fund.
References


