Susceptibility of B lymphocytes to adenovirus type 5 infection is dependent upon both coxsackie–adenovirus receptor and $\alpha v\beta 5$ integrin expression

Ciarán Richardson,1 Paul Brennan,1 Martin Powell,1 Stuart Prince,1 Yun-Hsiang Chen,3 O. Brad Spiller2 and Martin Rowe1

1Infection and Immunity, Henry Wellcome Research Building, Wales College of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, UK
2Virus Receptor and Immune Evasion Group, Henry Wellcome Research Building, Wales College of Medicine, Cardiff University, Heath Park, Cardiff, UK
3Biomolecular Sciences Building, School of Biology, University of St Andrews, St Andrews, UK

Human lymphocytes are resistant to genetic modification, particularly from recombinant adenoviruses, thus hampering the analysis of gene function using adenoviral vectors. This study engineered an Epstein–Barr virus-transformed B-lymphoblastoid cell line permissive to adenovirus infection and elucidated key roles for both the coxsackie–adenovirus receptor and $\alpha v\beta 5$ integrin in mediating entry of adenoviruses into these cells. The work identified a strategy for engineering B cells to become susceptible to adenovirus infection and showed that such a strategy could be useful for the introduction of genes to alter lymphoblastoid-cell gene expression.

INTRODUCTION

The susceptibility of cells to recombinant adenovirus infection varies considerably and a single defined mechanism of adenovirus infection cannot be assigned to all cell types. Cell-surface receptors such as the coxsackie–adenovirus receptor (CAR) or MHC class I $\alpha 2$ domain (Bergelson et al., 1997; Hong et al., 1997) may initiate adenovirus infection of cells through high-affinity binding with the fibre protein knob domain of the adenovirus (Bergelson et al., 1997; Hong et al., 1997). In addition, interactions between the adenovirus pentons and cell-surface integrins such as $\alpha v\beta 3$ and $\alpha v\beta 5$ have also been shown to facilitate adenovirus infection (Mathias et al., 1994; Wickham et al., 1993). Thus, it has been postulated that CAR and the MHC class I $\alpha 2$ domain mediate adenovirus attachment to the cell and that the integrins promote adenovirus internalization (Bergelson et al., 1997; Hong et al., 1997; Mathias et al., 1994; Wickham et al., 1993). However, this proposed mechanism for adenovirus infection does not appear to be applicable to all serotypes of adenovirus, nor for all cell types. Thus, whilst all subgroup C adenoviruses (which include adenovirus type 5 commonly used for the generation of recombinant vectors) appear to be capable of utilizing CAR, subgroup B adenoviruses use a different fibre receptor (Stevenson et al., 1995). Even when considering only subgroup C adenoviruses, the involvement of receptors and co-receptors appears to vary with cell type. A number of studies have suggested that susceptibility to adenovirus type 5 infection correlates with cell-surface CAR expression and that the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins are dispensable (Buttgereit et al., 2000; Fuxe et al., 2003). In addition, a study by McDonald et al. (1999) has shown that CAR, and not MHC class I, is a receptor for recombinant adenoviruses in cultured human tumour cell lines.

Tumour cells of lymphoid origin have been reported to be quite resistant to adenovirus infection (Cantwell et al., 1996; Prince et al., 1998; Silver & Anderson, 1988). However, a study by Buttgereit et al. (2000) demonstrated effective gene transfer into lymphoma cells, using recombinant adenoviruses combined with the transfection reagent lipofectamine. This study reported that susceptibility to adenovirus infection correlated with cell-surface CAR levels. Human CAR is a 46 kDa member of the immunoglobulin (Ig) CTX subfamily (Chretien et al., 1998) and is composed of two extracellular Ig-like domains, a typical transmembrane domain and a long cytoplasmic domain (Bergelson et al., 1997). The most distal extracellular Ig loop (V region or D1 domain) of CAR allows CAR aggregation by facilitating a homophilic interaction between CAR molecules (Cohen et al., 2001; Honda et al., 2000; van Raaij et al., 2000). CAR localizes to the region of tight junctions in polarized epithelia, suggesting a role in cellular adhesion (Cohen et al., 2001). In terms of recombinant adenovirus infection, CAR functions as a high-affinity receptor for the adenovirus fibre protein (Bergelson et al., 1997). The fibre knob of adenovirus interacts with the D1 domain of CAR (Bewley et al., 2003).
1999) and it has been proposed that CAR is largely responsible for susceptibility to adenovirus infection (Buttgeriet et al., 2000; Fuxe et al., 2003; McDonald et al., 1999).

This study investigated the susceptibility of B-lymphocyte cell lines to recombinant adenovirus infection and proposes a mechanism by which to enhance this susceptibility. We were particularly interested in Epstein–Barr virus (EBV)-transformed B-lymphoblastoid cell lines (LCLs), which express very high levels of MHC class I at the cell surface (Rowe et al., 1995) but demonstrate a very low rate of infection by recombinant adenovirus vectors (von Seggern et al., 2000). We characterized a role for CAR and \( \nu v / 5 \) integrin in adenoviral entry into lymphocytes and engineered an LCL that contains high levels of both of these proteins. This cell line expressed green fluorescent protein (GFP) from an adenoviral construct and could also be infected with a virus expressing the V protein of simian virus 5 (SV5), which effectively reduced STAT1 protein levels.

**METHODS**

**Cell lines and culture.** DG75 is an EBV-negative Burkitt’s lymphoma (BL) line (Ben-Bassat et al., 1977). Akata is an EBV-positive BL line that displays a latency I pattern of EBV gene expression (Rowe et al., 1999; Takekata & Ono, 1989). IB4 is an EBV-transformed normal lymphoblastoid cell line established by infection of cord blood B lymphocytes with the B95-8 strain of EBV (Sample & Kieff, 1990). Cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM l-glutamine and antibiotics (200 U penicillin ml\(^{-1}\) and 200 g streptomycin ml\(^{-1}\)). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO\(_2\).

**Generation of an anti-CAR monoclonal antibody.** The monoclonal anti-CAR antibody BRAD30 was generated in house. Briefly, recombinant cDNA was engineered that contained the two extracellular Ig regions (V and C2) of CAR followed, in frame, by cDNA encoding the Fc portion of human IgG1. This recombinant cDNA was subcloned into a eukaryotic expression vector containing a hygromycin resistance gene (Yanagawa et al., 2004) and was transfected into CHO cells (ECACC). Stable clones expressing CAR-Fc were generated by propagation in selection medium (RPMI 1640 with 10% fetal calf serum, 2 mM l-glutamine and antibiotics (200 U penicillin ml\(^{-1}\) and 200 g streptomycin ml\(^{-1}\)). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO\(_2\).

**Adenoviral infection of B lymphocytes.** A GFP-expressing recombinant adenovirus (AdGFP) was generated by cloning enhanced GFP (Gormack et al., 1996) under the control of the human cytomegalovirus major immediate-early (hCMV IE) promoter to generate pAL322 (adenovirus transfer vector) and adenovirus was generated by co-transfecting pAL322 and pJM17 (E1-deleted adenovirus type 5 backbone) into 293 cells as described previously (Wilkinson & Akridge, 1992). A recombinant adenovirus expressing the V protein (Didcock et al., 1999) of SV5 (V Adv) was made in a similar fashion. B lymphocytes were washed and resuspended in growth medium at a typical concentration of \( 8 \times 10^5 \) cells ml\(^{-1}\). Aliquots of \( 2 \times 10^5 \) cells in 250 μl were then added to the wells of a 24-well plate. Cells were infected with recombinant adenoviruses at m.o.i. of 15 or 30, as indicated in the text, by the addition of an appropriate amount of recombinant adenovirus to the wells. The plate was then incubated for 2-5 h at 37 °C on a rocking incubator (ST8 drive unit and platform and S.1.600 incubator, speed set at 25; Stuart Scientific). After incubation, 1 ml fresh growth medium was added to each well before placing the plate in a 37 °C incubator for 3 days.

**Generation of glycosylphosphatidylinositol (GPI)-anchored CAR-expressing cells.** Recombinant cDNA encoding the N-terminal V region or D1 domain of CAR fused to the Ser/Thr spacer region and GPI addition signal from human CD55 was created by PCR. Restriction enzyme sites were added at the 5’ end, the CAR–GPI junction and the 3’ end to allow fusion of the cDNA fragments and subcloning into the expression vector listed above for CAR-Fc production. Integrity of the sequence was confirmed by sequencing (ABI), as well as the maintenance of reading frame for the GPI signal. For the generation of DG75 and IB4 stable cell lines expressing GPI-CAR, \( 1 \times 10^6 \) cells were transfected with 8 μg GPI-CAR antibodies were utilized: mouse anti-CAR (zHCAR-BRAD30); mouse anti-\( \nu v / 5 \) (MAB1976Z; Chemicon) and mouse anti-\( \nu v / 5 \) (MAB1961X; Chemicon). Cells were harvested and washed twice in PBS. Approximately \( 2 \times 10^5 \) cells were incubated with the primary antibody, generally at a dilution of 1:100 in PBS containing 10% normal rabbit serum (NRS/PBS), for 30 min at 4 °C. Cells were washed twice with PBS and bound antibodies were detected by incubation of cells for 30 min at 4 °C with a 1:30 dilution of PE-conjugated anti-mouse IgG (R0439; Dako) in NRS/PBS. Cells were given a further two washes in PBS and resuspended in FACS Flow (Becton Dickinson) or in 2% paraformaldehyde in PBS and analysed on a FACScan flow cytometer (Becton Dickinson) with CellQuest Pro software (Becton Dickinson).

**Generation of cell extracts, SDS-PAGE and Western blot analysis.** Cells were counted on a haemocytometer and resuspended in 50 μl PBS per 10^6 cells. An equal volume of 2× gel sample buffer (100 mM Tris/HCl pH 6-8, 20% glycerol, 0.2 M DTT, 4% SDS, 0-02% bromophenol blue) was added and the cells were sonicated using a W385 sonicator (Heat Systems Ultrasomics). Following sonication, samples were heated at 100 °C for 5 min. The solubilized proteins were separated by SDS-PAGE and transferred to PVDF membrane (Amersham) for immunoblotting using an alkaline phosphatase chemiluminescent detection protocol (Rowe & Jones, 2001). Primary antibody incubations were for 80 min at room temperature and primary antibodies were used at the following concentrations: rabbit anti-STAT1 (sc-346; Santa Cruz Biotechnology), 0.2 μg ml\(^{-1}\); rabbit anti-STAT2 (sc-476; Santa Cruz Biotechnology), 0.4 μg ml\(^{-1}\); rabbit anti-actin (A2066; Sigma), 4 μg ml\(^{-1}\), and rabbit anti-CAR (Spiller et al., 2002), 1:500 dilution. Secondary antibody incubations using a 1:10,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (170-6518; Bio-Rad), were for 80 min at room temperature. Specific antibody–protein complexes were detected using CDP-Star (Tropix) chemiluminescence reagent.
expression plasmid by electroporation (Bio-Rad GenePulser II electroporator set at 270 V/950 μF). Following electroporation, cells were reseded in 9.5 ml fresh growth medium and 200 μl fractions were aliquoted into the wells of a 96-well plate. Twenty-four hours post-transfection, cells were treated with hygromycin B to select GPI–CAR transfectants. DG75 and IB4 cells were treated with hygromycin B (Boehringer Mannheim) at concentrations of 400 μg ml⁻¹ and 200 μg ml⁻¹, respectively.

**Cell-surface receptor blocking studies.** Blocking studies were performed with purified azide-free antibodies and the following antibodies were utilized: rabbit anti-CAR (raised in house); mouse anti-xvβ3 (MAB1976Z; Chemicon) and mouse anti-xvβ5 (MAB1961X; Chemicon). Typically, 2.4 x 10⁵ cells per 600 μl growth medium were incubated with purified antibodies at concentrations ranging from 0.03 to 40 μg ml⁻¹ for 40 min at room temperature under sterile conditions. Aliquots of 250 μl of the cell suspension (1 x 10⁵ cells) were subsequently transferred to a 24-well plate and the cells were infected with AdGFP at an m.o.i. of 30. Following recombination, adenovirus infection, 1.25 ml fresh growth medium was added to each well and the 24-well plate was placed in a 37°C incubator. Forty-eight or 72 h post-infection (p.i.), cells were harvested, washed in PBS and fixed in 2% paraformaldehyde in PBS. The percentage of GFP-positive cells was determined by flow cytometry on a FACScalibur flow cytometer with CellQuest Pro software.

**RESULTS**

**CAR expression varies in B-lymphocyte cell lines but all remain refractory to adenovirus infection**

Three model B-lymphocyte cell lines were analysed for CAR cell-surface expression and for susceptibility to adenovirus infection. DG75 is an EBV-negative tumour B-lymphocyte cell line derived from a patient with BL. Akata is an EBV-positive BL cell line exhibiting a latency I pattern of viral gene expression. IB4 is an EBV-immortalized LCL, which exhibits a latency III pattern of viral gene expression. CAR expression was assayed by flow cytometry (Fig. 1a) and by Western blotting (Fig. 1b) using specific antibodies. Flow cytometric analysis showed that the IB4 cell line was CAR negative, the DG75 cell line exhibited intermediate levels of CAR expression on the cell surface and all of the Akata cells were CAR positive (Fig. 1a). This pattern of CAR expression correlated with detection of a 46 kDa band by immunoblot analysis of CAR from total lysates of the same cells (Fig. 1b).

Susceptibility to adenovirus infection was monitored by infecting cells with AdGFP at an m.o.i. of 30 and analysing the percentage of GFP-positive cells at 72 h p.i. by flow cytometry (Fig. 1c). Despite the range of CAR expression levels amongst the cell lines, none of the cell lines was particularly susceptible to adenovirus infection. Indeed, less than 5% of the cells in these cultures expressed GFP (Fig. 1c). In contrast to these low percentages, certain fibroblast cell lines can give greater than 90% GFP-positive cells when infected with similar m.o.i. of AdGFP (data not shown). These experiments showed that B lymphocytes are refractory to adenovirus infection.

**Increased CAR expression does not correlate with increased infection of B lymphocytes**

For mouse T lymphocytes and human dendritic cells, the efficiency of infection by adenovirus has been increased by the engineered expression of CAR (Hurez et al., 2002; Stockwin et al., 2002). To investigate this issue in B lymphocytes, stable lines of IB4 LCL and DG75 cells were generated with increased CAR expression. Initial experiments were performed with a plasmid vector encoding full-length CAR. However, expression levels of CAR invariably remained low or undetectable and therefore it was not possible to interpret the observed failure to obtain enhanced adenovirus infectivity (data not shown). Subsequently, we chose to use GPI–CAR, a truncated form of human CAR with the transmembrane and cytoplasmic domains replaced with a GPI anchor. GPI–CAR has a number of advantages compared with full-length human CAR. Importantly, deletion of the cytoplasmic tail of CAR limits the signaling potential of the protein and inhibits its degradation. The transmembrane and cytoplasmic domains are not required for adenovirus infection (Leon et al., 1998; Wang & Bergelson, 1999) and this was validated for our constructs prior to introduction of this plasmid into B lymphocytes (data not shown). IB4 and DG75 cells were transfected by electroporation with 8 μg GPI–CAR expression plasmid. Twenty-four hours post-transfection, cells were treated with hygromycin B to select GPI–CAR transfectants. Hygromycin B-resistant lines that grew out were analysed for CAR cell-surface expression and susceptibility to adenovirus infection.

Clones were generated with a wide range of CAR expression levels (Fig. 2). Data from one representative clone of DG75 and two clones of IB4 are shown in Fig. 2(a). In each case, a large increase in CAR expression was detected (compare Figs 1a and 2a). Twelve hygromycin B-resistant DG75 GPI–CAR lines were generated. Fig. 2(b) shows the level of adenovirus infection of these cell lines as a function of the level of CAR expression. Susceptibility to adenovirus infection was monitored by infecting cells with AdGFP at an m.o.i. of 30 and analysing the percentage of GFP-positive cells at 72 h p.i. by flow cytometry. The graph revealed no correlation between the amount of CAR expressed at the cell surface and susceptibility to AdGFP infection. Infection rates were generally less than 8% for all clones tested, although one clone did exhibit an infection rate of approximately 15%. However, these infection rates fall short of the rates observed in T lymphocytes and dendritic cells expressing high levels of CAR (Hurez et al., 2002; Stockwin et al., 2002).

A similar analysis was performed on the hygromycin B-resistant IB4 GPI–CAR clones and again the observed increase in CAR expression was found not to correlate with adenovirus infection. For example, clone 3 (IB4 GPI–CAR 3) exhibited high levels of CAR expression but remained refractory to adenovirus infection (compare Fig. 2a and c). However, one IB4 clone (IB4 GPI–CAR 8) was unusual in
that it exhibited a notably elevated susceptibility to adenovirus infection. Infection of the IB4 GPI–CAR 8 cell line with AdGFP at an m.o.i. of 30 resulted in 55% of the cells staining GFP positive at 72 h p.i. (Fig. 2c). This was in contrast to the IB4 GPI–CAR 3 cell line in which approximately 4% of the cells stained GFP positive at 72 h p.i. (Fig. 2c). In seven
different experiments, infection of IB4 GPI–CAR 8 cells with AdGFP at an m.o.i. of 30 resulted in 45.5 ± 3.7\% (mean ± SEM, \(n=7\)) of cells expressing GFP (range 29–56\%). However, it was clear that high-level CAR expression on the B lymphocytes tested did not generally translate into an increased susceptibility to adenovirus infection.

Adenovirus infection of IB4 GPI–CAR 8 cells is dependent on CAR

Given the lack of correlation between CAR expression and adenovirus infection, it was important to determine whether this adenoviral entry into IB4 GPI–CAR 8 cells was
dependent upon CAR. We performed antibody-blocking experiments in which IB4 GPI–CAR 8 cells were pre-incubated with purified anti-CAR antibody. A control population and the pre-incubated population were subsequently infected with AdGFP at an m.o.i. of 30 and GFP expression was determined at 72 h p.i. by flow cytometry. In the experiment illustrated in Fig. 3(a), pre-incubation of cells with anti-CAR antibody decreased the percentage of GFP-positive cells from 53 to 3%. Similar results were obtained in two repeat experiments, and blocking of CAR resulted in a reduction in the receptiveness of the cell line to adenovirus infection of 92 ± 3 % (mean ± SEM, n = 3). Furthermore, titration of the purified CAR antibody revealed that this degree of resistance to adenovirus infection was observed using the antibody at concentrations ranging from 0.03 to 20 µg ml⁻¹ for 40 min at room temperature. A control and the pre-incubated populations were infected with AdGFP at an m.o.i. of 30 and the percentage of GFP-positive cells was determined at 72 h p.i. by flow cytometry. In the graph, the concentration of CAR antibody (µg ml⁻¹) is plotted against the percentage inhibition of GFP expression.

IB4 GPI–CAR 8 cells express high levels of αvβ3 and αvβ5 integrin

Despite the fact that adenovirus infection was found to be CAR dependent, our previous results showed that CAR expression alone was not sufficient for infection and therefore adenovirus most likely requires an additional co-factor to mediate infection of B lymphocytes. To investigate this question, we examined expression of the secondary internalization receptors, αvβ3 and αvβ5 integrins. Integrin staining of αvβ3 and αvβ5 was performed on the DG75, Akata, IB4, IB4 GPI–CAR 3 and IB4 GPI–CAR 8 cell lines (Fig. 4) using monoclonal anti-αvβ3 and anti-αvβ5 antibodies and flow cytometry. Both the DG75 (EBV-negative BL) and Akata (EBV latency I BL) cell lines exhibited negligible αvβ3 and αvβ5 integrin staining. The parental IB4 (EBV-immortalized LCL) cell line and the two IB4 GPI–CAR clones showed strong positive staining for αvβ3. Significantly, the IB4 GPI–CAR 8 cell line exhibited strong αvβ5 cell-surface expression compared with the IB4 parental and IB4 GPI–CAR 3 cell lines, which showed weak αvβ5 staining. There is no reason to suppose that the elevated expression of αvβ5 on the IB4 GPI–CAR 8 clone was in any way induced by expression of GPI–CAR itself. Nevertheless, the rare fortuitous expression of αvβ5 on the IB4 GPI–CAR 8 cell line implied that this integrin could be additionally required for adenovirus infection of B lymphocytes.

Blocking the αvβ5 receptor on IB4 GPI–CAR 8 cells reduces their susceptibility to adenovirus infection

To establish the importance of integrins in mediating adenovirus infection of the IB4 GPI–CAR 8 cell line, blocking studies were performed in which purified anti-αvβ3 and anti-αvβ5 antibodies were used to block the integrins on the cells. A control population and the pre-incubated populations were subsequently infected with AdGFP at an m.o.i. of 30 and GFP expression was determined at 72 h p.i.
by flow cytometry. The result of a representative blocking experiment from three separate experiments is illustrated in Fig. 5. Anti-αvβ3 antibody did not diminish the susceptibility of the IB4 GPI–CAR 8 cell line to adenovirus infection. In the experiment illustrated, the percentage of GFP-positive cells remained at 29%. In contrast, anti-αvβ5 antibody decreased the percentage of GFP-positive cells from 29 to 19% (i.e., a 34% reduction in infectivity). Over three separate experiments, blocking of the αvβ5 receptor resulted in a reduction in the receptiveness of the IB4 GPI–CAR 8 cell line to adenovirus infection of 31.6 ± 2.9% (mean ± SEM, n = 3). The blocking studies may underestimate the role of the αvβ5 receptor in mediating adenovirus infection, as it was difficult to determine how efficiently the antibody blocked the receptor. Indeed, in one experiment, pre-incubation of the IB4 GPI–CAR 8 cell line with purified mouse anti-αvβ5 integrin antibody from a different source (sc-13588; Santa Cruz Biotechnology) resulted in a 55% decrease in the adenovirus infection rate of the IB4 GPI–CAR 8 cell line (data

**Fig. 4.** Flow cytometry analysis of αvβ3 and αvβ5 integrin cell-surface expression on DG75, Akata, IB4, IB4 GPI–CAR 3 and IB4 GPI–CAR 8 cell lines. Cell lines were stained with mouse anti-αvβ3 integrin antibody or mouse anti-αvβ5 integrin antibody at a concentration of 10 μg ml⁻¹. Bound antibody was labelled using a 1:30 dilution of PE-conjugated anti-mouse IgG and integrin expression was analysed by flow cytometry. As a negative control, cells were stained in an identical manner using mouse anti-BZLF1 antibody as an isotype control primary antibody. To assist in comparing cell lines, the CAR expression and AdGFP infectivity profiles of the cell lines are summarized to the left of the histograms.
Combined blocking of CAR and \(\alpha v/\beta 5\) results in more efficient inhibition of adenovirus infection than that achieved by blocking CAR and \(\alpha v/\beta 5\) separately

Adenoviral entry into IB4 GPI–CAR 8 cells thus depends on both CAR and \(\alpha v/\beta 5\) expression. To demonstrate further the importance of both of these receptors in mediating adenovirus infection of the IB4 GPI–CAR 8 cell line, combined blocking studies were performed in which both CAR and the \(\alpha v/\beta 5\) receptor were targeted for inhibition. A titration was performed to identify a concentration of purified anti-CAR antibody that resulted in partial inhibition of adenovirus infection (see Fig. 3b). A concentration of 0·67 \(\mu\)g anti-CAR antibody ml\(^{-1}\) was found to result in a 65\% decrease in the receptiveness of the IB4 GPI–CAR 8 cell line to adenovirus infection. This concentration of anti-CAR antibody was used in the combined blocking studies. IB4 GPI–CAR 8 cells were pre-incubated with purified anti-CAR antibody, purified anti-\(\alpha v/\beta 5\) antibody, or a combination of both anti-CAR and anti-\(\alpha v/\beta 5\) purified antibodies. A control population and the pre-incubated populations were infected with AdGFP at an m.o.i. of 30. GFP expression was determined at 72 h p.i. by flow cytometry (Fig. 6). At the dilutions of antibody chosen, individual blocking of CAR and the \(\alpha v/\beta 5\) receptor resulted in a decrease in the percentage GFP-positive cells from 44 to 15 and 26\%, respectively. Notably, the greatest inhibition of adenovirus infection was observed when both CAR and \(\alpha v/\beta 5\) were targeted. The combined blocking of both receptors resulted in a decrease in the percentage of GFP-positive cells from 44 to 7\%. These results strongly suggest that both CAR and \(\alpha v/\beta 5\) function together to mediate adenovirus infection of the IB4 GPI–CAR 8 cell line.

**IB4 GPI–CAR 8 cells can be used to investigate the function of proteins delivered by a recombinant adenovirus in an LCL**

The V protein of SV5 has been shown specifically to target STAT1 for proteasomal degradation in fibroblast cell lines (Didcock et al., 1999). It is postulated that this specific degradation of STAT1 is a mechanism by which SV5 counteracts the anti-viral effects of interferons. A recombinant adenovirus expressing the V protein of SV5 was used to infect the IB4 GPI–CAR 8 cell line at an m.o.i. of 15. The IB4 GPI–CAR 8 cell line was also mock infected and infected with AdGFP at an m.o.i. of 15 as a control for adenovirus infection. Seventy-two hours p.i., samples were harvested and total lysates were prepared. The lysates were separated by SDS-PAGE and analysed by Western blotting using antibodies specific to STAT1, STAT2 or actin (Fig. 7). The infection efficiency of the experiment was monitored by flow cytometry analysis of IB4 GPI–CAR 8 cells infected with AdGFP at an m.o.i. of 15: approximately 33\% of the cells exhibited GFP fluorescence at 72 h p.i.

The blots shown in Fig. 7 revealed that IB4 GPI–CAR 8 cells treated with recombinant adenovirus expressing the

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*Fig. 5. Adenoviral entry into IB4 GPI–CAR 8 cells is regulated by \(\alpha v/\beta 5\). IB4 GPI–CAR 8 cells were pre-incubated with either purified mouse anti-\(\alpha v/\beta 3\) integrin antibody or purified mouse anti-\(\alpha v/\beta 5\) integrin antibody at a concentration of 20 \(\mu\)g ml\(^{-1}\) for 40 min at room temperature. A control and the pre-incubated population were infected with AdGFP at an m.o.i. of 30. The percentage of GFP-positive cells was determined at 72 h p.i. by flow cytometry and is indicated on each histogram.*
V protein of SV5 had reduced levels of STAT1. Mock-infected cells or cells treated with AdGFP did not exhibit a notable reduction in the level of STAT1 compared with uninfected cells. The specific targeting of STAT1 for degradation by the V protein was confirmed by the STAT2 blot, which revealed similar levels of STAT2 in all lanes. The actin blot confirmed equal loading of the lanes. This STAT1 degradation phenomenon was apparent even though only an estimated 33% of the cells were infected with recombinant adenovirus. These data suggest that the V protein of SV5 is capable of targeting STAT1 for degradation in an EBV-immortalized LCL, which is consistent with previous observations demonstrating specific STAT1 degradation by the V protein of SV5 in fibroblast cell lines (Didcock et al., 1999). More importantly, the results demonstrate the feasibility of using the IB4 GPI–CAR 8 cell line as a target for functional studies of proteins introduced using recombinant adenovirus vectors.

**DISCUSSION**

Investigating the function of proteins of interest in EBV-infected B lymphocytes *in vitro* will improve our understanding of how EBV influences malignancy *in vivo*.

**Fig. 6.** Investigating the relationship between CAR and αvβ5 in mediating adenovirus infection of the IB4 GPI–CAR 8 cell line. IB4 GPI–CAR 8 cells were pre-incubated with one of the following: purified rabbit anti-CAR antibody at a concentration of 0.67 µg ml⁻¹; purified mouse anti-αvβ5 integrin antibody at a concentration of 20 µg ml⁻¹; or a combination of both purified CAR and purified αvβ5 antibodies at concentrations of 0.67 µg ml⁻¹ and 20 µg ml⁻¹, respectively. Cells were pre-incubated for 40 min at room temperature. A control population and the pre-incubated populations of cells were infected with AdGFP at an m.o.i. of 30. The percentage of GFP-positive cells was determined at 72 h p.i. by flow cytometry and is indicated on each histogram.

However, EBV-infected B lymphocytes, in particular EBV-immortalized LCLs, are resistant to most gene transfer approaches, which has severely retarded protein functional studies in these cells. For example, transfection of EBV-immortalized LCLs with plasmid DNA generally results in transfection efficiencies of less than 5% (White et al., 2002).

In a number of studies, the susceptibility of human cells to adenovirus infection has been shown to correlate with cell-surface CAR expression (Fuxe et al., 2003; McDonald et al., 1999; Stockwin et al., 2002). The present study clearly shows that this is not the case for human B-lymphocyte lines. There was no correlation between CAR cell-surface expression and susceptibility to adenovirus infection in three model B-lymphocyte cell lines each exhibiting a different EBV status, despite the generation of cell lines overexpressing GPI–CAR. Interestingly, EBV-immortalized LCLs have been shown to express very high levels of MHC class I on the cell surface (Rowe et al., 1995), which has also been proposed to mediate adenovirus infection of target cells (Hong et al., 1997). Nevertheless, the IB4 LCL was not particularly susceptible to adenovirus infection (Fig. 1). The IB4 GPI–CAR clone 8 cell line proved to be distinct from the other IB4 GPI–CAR clones generated in that it displayed a notably enhanced susceptibility to adenovirus infection (Fig. 2). This increased level of adenovirus infection could be effectively blocked by an antibody to CAR. Together these data clearly show that CAR is required but not sufficient for adenovirus infection of B lymphocytes.

The present study identified αvβ5 as a co-factor that works in combination with CAR to mediate adenovirus infection...
of the IB4 GPI–CAR 8 cell line. The elevated \(\alpha v/\beta 5\) expression observed on the IB4 GPI–CAR 8 cell line (Fig. 4) most likely arose as a result of the hygromycin B selection and cloning process, which resulted in the fortuitous isolation of both CAR- and \(\alpha v/\beta 5\)-positive cells. The importance of \(\alpha v/\beta 5\) is highlighted by the fact that the adenovirus-resistant DG75, Akata, IB4 and IB4 GPI–CAR 3 cell lines were virtually \(\alpha v/\beta 5\) negative (Fig. 4). Whilst numerous studies have implicated CAR and integrins in adenovirus binding and entry, the precise molecular requirement appears to depend on the cell type and the adenovirus serotype. To our knowledge, this study is the first to demonstrate the importance of both a primary (CAR) and a secondary (\(\alpha v/\beta 5\)) receptor in mediating adenovirus type 5 infection of a B-lymphocyte cell line. Two previous studies have reported that the susceptibility of tumour cells to adenovirus infection correlates well with cell-surface CAR expression and that this susceptibility appears to be independent of \(\alpha v/\beta 3\) and \(\alpha v/\beta 5\) integrin expression (Buttgereit et al., 2000; Fuxe et al., 2003). However, our data are more in line with another study showing that the levels of \(\alpha v/\beta 5\) may predict the susceptibility of human lung cancer cells to adenovirus-mediated gene transfer independently of \(\alpha v/\beta 3\) levels (Takayama et al., 1998).

It is pertinent to note that the results obtained in this study may not be applicable for all adenoviral serotypes. For example, adenovirus type 37 has been reported to use sialic acid instead of CAR for virus attachment (Arnberg et al., 2000). In addition, the adenovirus types 11 and 35 differ from adenovirus type 5 in showing high binding efficiencies to some committed haematopoietic cell lines (Segerman et al., 2000). However, this increased binding efficiency does not necessarily translate into increased infectivity. Most notably, the B-cell line used in the study by Segerman et al. (2000), which was also examined in our study, remained refractory to infection by adenovirus types 11 and 35.

Whilst in the present study we chose to modify the target cell to increase the efficiency of adenovirus transduction, an alternative approach involves modification of the fibre protein of the adenovirus to alter its tropism. This is potentially an attractive approach for gene therapy, where retargeting the virus to a different tissue may be especially advantageous (reviewed by Wickham, 2003), and in cases where experimental modification of the target cell is not an option. Interestingly, a study by von Seggern et al. (2000) demonstrated that the susceptibility of EBV-immortalized LCLs to adenovirus infection could be increased by replacing the fibre protein knob of an Ad5–\(\beta 1\)gal recombinant adenovirus with the fibre protein from adenovirus type 3. In that study, the efficiency of infection varied considerably between different LCLs and inspection of the data suggests that this variability may correlate with expression of \(\alpha v/\beta 5\). However, while modification of the fibre protein clearly increased the susceptibility of LCL to adenovirus infection, it is notable that in the order of 50,000 virus particles per cell were required to achieve expression of the adenovirus-transduced gene in 20–50 % of the cells. In our experiments with the CAR-expressing IB4 LCL, a similar percentage of cells was transduced with conventional adenovirus type 5 recombinant vector at an m.o.i. of 30.

The engineering of a B-cell line that is susceptible to adenovirus 5 infection provides a valuable tool for EBV research. However, the molecular characterization of the adenoviral entry provides a strategy for the generation of other cell lines to augment or enhance adenoviral entry. We have shown that the V protein of SV5 can reduce STAT1 protein levels, demonstrating that adenovirus can be used for protein knockout studies in these cells. Thus, the increased susceptibility of this cell line to adenovirus infection provides an opportunity by which the function of proteins of interest may be investigated in an LCL, using recombinant adenoviruses as the gene delivery vehicles. Historically, the resistance of LCLs to traditional transfection approaches (White et al., 2002) has limited protein functional studies in these cells. Notably, this effect was observed by infecting the IB4 GPI–CAR 8 cell line with adenovirus expressing SV5 V protein at an m.o.i. of 15. Such low m.o.i. of adenovirus are unlikely to exert any toxic effects on the target-cell population and thus the reliability of such functional studies is enhanced.

In summary, this study has demonstrated the feasibility of using recombinant adenoviruses as gene delivery vehicles into human B lymphocytes in vitro. It showed that both CAR and \(\alpha v/\beta 5\) are involved in mediating adenovirus infection of IB4 lymphoblastoid cells. The identification of a mechanism for adenovirus infection of B lymphocytes provides an opportunity by which B lymphocytes may be modified to increase their susceptibility to recombinant adenovirus infection. In addition, cell-surface expression levels of CAR and \(\alpha v/\beta 5\) may be used to predict the susceptibility of malignant B lymphocytes to adenovirus infection and perhaps the likely efficiency of adenovirus-mediated gene therapy directed against B lymphocytes.

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