Regulation of expression of the reovirus receptor on differentiated HL60 cells

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The reovirus receptor on mammalian cells has not been fully characterized and controversy exists over the nature of this receptor. We report here that the expression of this receptor is dependent on the differentiation status of a human promyelocytic leukaemia cell line (HL60). Phorbol treatment of HL60 cells for 24 h, at a concentration range of 160 nM down to 1 nM, led to differentiation of these cells towards monocytes and a loss of approximately 80% of their ability to bind reovirus in a fluorescence assay. These cells also lost their susceptibility to T1 and T3 reovirus infection. DMSO treatment for 24 h at a concentration of 1-25% (v/v) led to differentiation towards granulocytes. This was accompanied by an increase of approximately 15% in binding of reovirus to these cells. After being infected by T1 or T3 reovirus, the granulocytes produced higher titres of progeny virus than did untreated HL60 cells. Similar differences were noted when virus binding to HL60 cells was assayed using radiolabelled reovirus. These effects were not detected when murine L fibroblasts were treated with DMSO or phorbol. ATCC-derived murine R1.1 cells did not bind reovirus. Competition data indicated that there may be two reovirus receptors on HL60 cells, and that T1 can bind to only one receptor whereas T3 can bind to both receptors. Our data also suggested that the β-adrenergic receptor was unlikely to act as the reovirus receptor on HL60 cells.

Introduction

To initiate an infection, a virus must first attach to its target cell. This process is frequently mediated by a specific viral receptor on the cells, which in turn is an important determinant for viral tropism (White & Littman, 1989). Viral receptors so far identified are normal constituents of the cell surface and usually function as receptors for physiological ligands (hormone, immunoglobulin, growth factor or neurotransmitter). Recent data suggest that the expression of receptors is regulated and correlates with the ability of a virus to generate a productive infection (Kaplan & Racaniello, 1991; Kazazi et al., 1989). The virus mimics the normal ligand structurally or conformationally in order to bind to the receptor. Once the nature of the recognition events between a virus and the host cell receptor is fully understood, they can become targets for the development of antiviral agents (Lentz, 1990).

Reovirus, although not in itself a major human pathogen, has long been used as a laboratory model for viral pathogenesis and for more pathogenic but related veterinary and human virus infections (Tyler & Fields, 1990). Members of the reovirus genus are non-enveloped, 80 nm in diameter, possess a double-protein capsid shell and contain 10 segments of dsRNA (Tyler & Fields, 1990). Three serotypes of reovirus have been identified, but only the T1 and T3 isolates have been studied thoroughly as they can be readily grown in cell culture to high titre. The σ1 protein of reovirus has been shown to mediate binding of the virus to target cells (Lee et al., 1981). This 49K protein occurs as a homotrimer and is distributed icosahedrally at the 12 vertices of the virion (Strong et al., 1991). The σ1 protein also possesses haemagglutinating and neutralizing epitopes (Burstin et al., 1982; Tyler & Fields, 1990).

Information on the nature of the reovirus receptor on mammalian cells is conflicting. Some studies, utilizing anti-idiotype antibodies, have suggested that a T3 reovirus infection is initiated by binding to the β-adrenergic receptor complex (Co et al., 1985b; Donta & Shanley, 1990; Uri Saragovi et al., 1991; Williams et al., 1991). However, the expression of β-adrenergic receptors and the ability to bind reovirus do not correlate on several cell types. Murine L cells and DDT1 smooth muscle cells both bind reovirus well; β-adrenergic receptors are expressed only on the DDT1 cells and not on the L cells (Choi & Lee, 1988; Sawutz et al., 1987). It has been reported, however, that T3 reovirus binds to antagonist (non-functional) domains of the β-adrenergic receptor complex (Donta & Shanley 1990). Additionally,
it is unclear whether T1 and T3 reoviruses utilize the same cell surface receptors. Cross-competition of T1 and T3 has been reported in L cells (Lee et al., 1981) and endothelial cells (Verdin et al., 1989) suggesting that T1 and T3 binding is mediated by the same receptors. However this phenomenon has not been observed by others (Epstein et al., 1984). At this time the nature of the reovirus receptor remains controversial and the field is further complicated by the use of different cell lines and variants of the same cell line by different laboratories. In addition, no information exists on the regulation of expression of the reovirus receptor.

To examine the potential for regulation of reovirus receptor expression we have studied T1 and T3 reovirus binding to their receptors on the differentiable HL60 line of human promyelocytic leukaemia cells. HL60 cells have been shown to be capable of differentiating to granulocytes or monocytes when treated with DMSO (Collins et al., 1978) or 12-O-tetradecanoyl-phorbol-13-acetate (PMA), a phorbol diester (Rovera et al., 1979), respectively.

This study reports on the regulation of expression of the reovirus receptor in HL60 cells. Receptor expression is markedly decreased when cells are differentiated to macrophages and increased when cells are differentiated to granulocytes. In addition, infectivity of virus correlates well with receptor expression. This is the first report of the regulation of reovirus receptor expression.

Methods

Viruses. Reovirus serotypes T1 (Lang) and T3 (Dearing) were originally obtained from Dr B. Fields, propagated in murine L cells and purified on a CsCl gradient as previously described (Verdin et al., 1989). Virus was radiolabelled by adding, at the virus adsorption stage, 5 mCi of TRAN35S-Label (ICN Biochemicals) to 2.5 x 10^8 L cells, in a 10 ml volume, infected with reovirus at an m.o.i. of 1. The 35S-radiolabelled virus was also purified on a CsCl gradient in the same manner as the non-labelled virus, was stored at 4 °C and was used within 6 weeks of being produced. The specific activity of the labelled virus was 9900 to 11500 c.p.m./µg (5-6 to 6.5 mCi/µg). Virus titres were calculated after performing a plaque assay over L cells as previously described (Verdin et al., 1989).

Cells. The murine L929 connective tissue (fibroblastic) cell line, the R1.1 mouse thymoma cell line and the HL60 cell line were obtained from the ATCC. The L cells were propagated as described elsewhere (Verdin et al., 1989). The R1.1 and HL60 cells were grown as suspension cultures in plastic 75 cm² tissue culture flasks (Bellco (Verdin from the ATCC. The L cells were propagated as described elsewhere (Verdin et al., 1989). The R1.1 and HL60 cells were obtained from the ATCC. R1.1 and HL60 cells were grown as described elsewhere (Verdin et al., 1989). The R1.1 and HL60 cells were grown in RPMI medium containing 10% foetal bovine serum (FBS) and 1% L-glutamine, under a 5% CO₂ atmosphere at 37 °C. The HL60 cells were differentiated by adding DMSO or PMA at the appropriate concentration to 2 x 10⁶ cells/ml in 20 ml cultures and incubating at 37 °C for 24 h.

Antiserum. Separate rabbits were inoculated subcutaneously with 300 µg of purified T1 or T3 reovirus in Freund's incomplete adjuvant. Two further intramuscular injections of the same amount of virus in Freund's incomplete adjuvant were administered at monthly intervals. Two weeks after the last immunization, serum was collected from the rabbits. Immunoglobulins were fractionated from the serum using a Protein A column, eluted from the column using 0.1 M glycine-HCl pH 2.8, and desalted on a Sephadex G-25 column pre-equilibrated with PBS. The Fab fragments of the immunoglobulins were generated by papain cleavage of immunoglobulin fragments. An anti-rabbit fluorescein isothiocyanate (FITC) conjugate (also an Fab fraction) was purchased from Sigma.

Fluorescent antibody-binding assay. PBS pH 7.4 containing 1% (w/v) lactic acid, hydrolysate (LAH; Gibco) was used throughout as a washing solution and diluent (PBS-LAH). One-million washed cells were mixed with 6 µg of purified reovirus (0.5 nM virus representing 3 x 10⁹ virus particles per cell) in a 100 µl volume and incubated at 4 °C overnight. In control experiments, no reovirus was added at this stage. In the case of PMA-treated HL60 cells, which had adhered to the plastic flasks, cells were scraped in order to resuspend them. Unbound virus was removed by two washing steps, each followed by gentle pelleting of the cells at 400 g in a bench centrifuge for 5 min. Rabbit anti-reovirus immunoglobulins (Fab) were added at a dilution of 1:100 and incubated at 4 °C for 1 h. The cells were washed twice and anti-rabbit FITC conjugate was added at a dilution of 1:50 and incubated at 4 °C for 1 h. The cells were washed twice more, resuspended in 1 ml volume and 10000 cells were analysed by a flow cytometer. The latter calculates the percentage of cells binding virus by comparing the fluorescence of cells incubated with primary and secondary antibodies and no virus to the fluorescence of cells incubated with virus and both antibodies. The sensitivity of this assay was determined by examining the binding of reovirus to L cells and R1.1 cells. The Fab fragments of immunoglobulins were used in this assay in order to rule out any interference by the expression of Fc receptors on the differentiated HL60 cells.

Radiolabelled virus-binding assay. Radiolabelled T1 or T3 reovirus (6 µg) was added to 10⁶ washed cells in a total volume of 100 µl (0.5 nM virus) and incubated at 4 °C overnight. The cells were washed twice, resuspended in 30 µl PBS-LAH and 3 ml of liquid scintillation cocktail was added. The mixture was counted in an LKB 1215 liquid scintillation counter. These experiments were performed in duplicate and mean results are reported.

β-Adrenergic receptor binding on HL60 cells. The radiolabelled and potent β-adrenergic antagonist (−)-(3H)dihydroalprenolol (3H[DHA]) (100 Ci/mmol; NEN Research Products) was used to follow the expression of the β-adrenergic receptor in differentiated HL60 cells. Another antagonist (−)-(4R,5R)-propranolol hydrochloride (Sigma) was used to compete with the [3H]DHA, and to calculate non-specific binding of the radiolabelled ligand. The binding assay was performed on 0.5 x 10⁶ cells in 100 µl of PBS-LA. The 3H[DHA] was added to a final concentration of 15 nM (180000 c.p.m.) and the cells were incubated at 37 °C for 10 min. The cells were washed twice, resuspended as before and counted in an LKB 1215. In competitive binding assays, the competing ligand was added to the cells which were then incubated at room temperature for 10 min, prior to the addition of [3H]DHA. Non-specifically bound [3H]DHA was calculated by measuring the radioactivity present after blocking with 100 µM-propranolol (Alexander et al., 1975).

Results

Susceptibility of differentiated HL60 cells to T1 and T3 infection

Untreated HL60 cells remained in suspension and were mostly spherical in morphology. T1 and T3 reovirus grew well in these cells (Fig. 1). HL60 cells treated with 1.25%...
DMSO also remained in suspension but possessed subtle morphological differences. Fine projections protruded from these cells. These morphological changes appeared at 18 h post-treatment. T1 and T3 reovirus also grew well in these cells and gave rise to higher titres of progeny virus than those of the untreated HL60 cells (Fig. 1), indicating that the cells remained susceptible to infection. HL60 cells treated with 160 nm-PMA showed more dramatic morphological changes. Most cells were flattened and elongated, after adhering to the plastic surface. These distinct features started to appear at 4 h post-treatment with 10% of the cells being affected. By 18 h post-treatment 80% of the cells were attached and this number rose to 99% at 4 days post-treatment. In these cells, T1 and T3 reovirus grew poorly indicating a very low susceptibility to infection (Fig. 1). T1 reovirus gave rise to a higher titre of progeny virus than T3 virus in all cases using these cells.

Fluorescence binding assay

Control binding of T1 and T3 reovirus to L cells and R1.1 cells was initially assessed. By using our fluorescence assay, binding to L cells was readily demonstrated (Fig. 2a). In the case of T1 reovirus 96% of the cells fluoresced to a higher intensity than the controls not incubated with virus. In the presence of T3 reovirus, the shift represented 93% of the cells (Fig. 2a). When R1.1 cells were used in conjunction with T3 reovirus, only 9% of the cells fluoresced to a higher intensity than control cells (Fig. 2b).

By using the fluorescence binding assay, reovirus binding to untreated HL60 cells was readily demonstrated. A shift of 77% of the cells when T3 was used and one of 75% with T1 reovirus were observed (Fig. 3a). When the HL60 cells were treated with 1.25% DMSO for 24 h, the shift was even greater, representing 96% of the cells with T3 reovirus and 90% with T1 reovirus (Fig. 3b). The PMA-treated (160 nm for 24 h) HL60 cells bound significantly less virus and only 38% (T3) and 46% (T1) of the cells fluoresced to a higher intensity than the controls (Fig. 3c). Cells incubated with antibodies demonstrated only a small degree of fluorescence. No autofluorescence was detected in any of these cells in the
A. A. El-Ghorr and others

Fig. 3. Flow cytometry graphs of the number of cells showing the effect of HL60 differentiation on reovirus binding, against the fluorescence intensity. The solid lines represent the background fluorescence of the cells in the absence of reovirus; the dotted and the bold lines show the fluorescence of the cells in the presence of T3 and T1 reoviruses respectively. The horizontal axes actually represent a logarithmic scale of fluorescence intensity units. (a) Fluorescence intensity of untreated HL60 cells. Both T1 and T3 reoviruses bound efficiently to these cells. (b) Effect of DMSO treatment on the fluorescence pattern of HL60 cells. In this case a higher proportion of the cells fluoresced to a higher intensity than untreated HL60 cells. (c) Pattern of fluorescence observed with PMA-treated HL60 cells. Here, fewer cell fluoresced to a higher intensity than untreated cells.

absence of primary and secondary antibodies (data not shown).

Several repeat experiments, using T1 and T3 reovirus, gave a similar pattern of results. The fluorescence in DMSO-differentiated cells ranged between 106 and 126% of controls and that in PMA-treated cells ranged between 7 and 60% of controls as compared to untreated HL60 cells (100%). When L cells were treated with DMSO and PMA, no morphological changes were observed and no differences in the T1 reovirus binding were detected (data not shown).

A biologically inactive form of the phorbol ester (4xPMA) was also used as a negative control. No differentiation of the HL60 cells was observed with this agent and there was no reduction in the fluorescence intensity of cells (data not shown).

Dose–response curves showed that a 1.25% (v/v) DMSO concentration gave the optimum increase in
fluorescence and concentrations varying between 1 and 160 nM-PMA all markedly decreased cell fluorescence (Fig. 4). A slight peak was observed at 80 nM-PMA, which may be due to experimental variation or to desensitization of the cells to the PMA effect at this dose. Time course studies revealed no difference in the pattern of results between 1, 2, 3, 4 and 5 days treatment with DMSO or PMA (data not shown).

**Binding of radioactive virus**

When binding studies were performed using the radioactive virus, the same pattern of results was obtained as that seen using the fluorescence assay. Control HL60 cells bound 59% of input T1 virus and 45% of input T3 virus. The DMSO-treated HL60 cells bound significantly more virus than untreated cells whereas PMA-treated HL60 cells bound less than 10% of virus added. No major differences were observed between the binding abilities of T1 or T3 reoviruses (Table 1). The R1.1 cells did not bind radioactive virus to any significant extent. The L cells bound virus efficiently as would be expected from a susceptible cell line (Table 1).

**Competitive binding of T1 and T3 reovirus to HL60 cells**

Different concentrations of unlabelled T1 or T3 reovirus were allowed to compete with radiolabelled virus for the receptor sites on HL60 cells (Fig. 5). Specificity of viral binding was demonstrated by inhibition of tracer binding with the addition of unlabelled virus. Unlabelled T1 and T3 reoviruses both individually competed directly with their respective homologous radiolabelled ligand for the receptor complex on HL60 cells. In the heterologous reactions, 10-fold excess unlabelled T1 reovirus inhibited the binding of radiolabelled T3 reovirus by only 40%. Total inhibition, however, was detected when unlabelled T3 virus was reacted with radiolabelled T1 reovirus (Fig. 5).

**β-Adrenergic receptor binding**

Untreated HL60 cells bound only a small proportion of the input [3H]DHA. However this binding was specific and could be blocked with non-labelled ligand (Fig. 6). The use of 15 nm-propranolol blocked 45% of the specific binding of 15 nm-[3H]DHA, and binding was completely inhibited by the addition of 100 μM-propranolol. The expression of the β-adrenergic receptor complex by differentiated HL60 cells followed a pattern opposite to that of the expression of the T1 and T3 reovirus receptors (Fig. 7). The untreated and DMSO-treated HL60 cells

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**Table 1. The percentage of input radiolabelled T1 and T3 reovirus bound to different cell lines**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Bound virus (%)</th>
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<tbody>
<tr>
<td></td>
<td>T1</td>
</tr>
<tr>
<td>HL60</td>
<td>59</td>
</tr>
<tr>
<td>HL60 + 1.25% DMSO</td>
<td>74</td>
</tr>
<tr>
<td>HL60 + 160 nM-PMA</td>
<td>8</td>
</tr>
<tr>
<td>R1.1</td>
<td>1</td>
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<tr>
<td>L</td>
<td>94</td>
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* Mean results of duplicate assays are reported.
Fig. 7. The binding of radiolabelled ligand to differentiated (1.25% DMSO and 160 nm-PMA) and undifferentiated HL60 cells. A total of 15 nM [3H]DHA and 0.5 nm of each reovirus were used on 0.5 × 10^6 cells in each assay. The binding observed using undifferentiated HL60 cells was taken as 100% for comparison purposes. The means of duplicate results are reported and the range is represented. The pattern observed for the expression of the T1 and T3 reovirus receptors diverged broadly from that for the expression of the β-adrenergic receptor.

bound similar quantities of β-adrenergic ligand while specific binding to PMA-treated cells increased by approximately twofold, whereas T1 and T3 reovirus binding increased in DMSO-treated cells and markedly decreased in PMA-treated cells (Fig. 7).

**Discussion**

Controversy exists regarding the nature of the reovirus receptor. Although binding parameters in a variety of cells are quite similar (Choi & Lee, 1988; Choi et al., 1990; Co et al., 1985b; Epstein et al., 1984; Maratos-Flier et al., 1983; Paul & Lee, 1987; Verdin et al., 1989), the biochemical characteristics of the reported receptors are inconsistent (Choi et al., 1990; Co et al., 1985b; Verdin et al., 1989). Some reports have shown that T1 and T3 reovirus bind to sialylated glycoproteins on the surface of L cells (Choi et al., 1990; Pacitti & Gentsch, 1987). On human erythrocytes, the sialoglycoprotein glycophorin has been identified as the reovirus receptor (Paul & Lee, 1987). Yet another report detected a 54K protein on rat endothelial cells that acted as a receptor for both the T1 and T3 serotypes (Verdin et al., 1989). The differences reported in the literature reflect this controversy and point to the possibility that reoviruses utilize multiple receptors which differ on different cell lines.

In addition, although receptors are thought to be differentially expressed in various organs and thus account for differences in viral tropism in vivo, no information is available regarding the regulation of reovirus receptor expression. HL60 cells are a promyelocytic cell line which is susceptible to terminal differentiation when treated with either DMSO or phorbol ester. We have demonstrated that these cells possess specific receptors for T1 and T3 reovirus and that differentiation of HL60 cells leads to profound alterations in reovirus binding and by inference in reovirus receptor expression.

Reovirus binding was demonstrated using both a fluorescence binding assay and radioactively labelled virus. Binding to HL60 cells using either assay was less than that seen in L cells. This was probably a reflection of a larger number of reovirus receptors on the L cells which also represent a homogeneous cell population. Both assays showed that HL60 differentiation was accompanied by increased expression of the reovirus receptor by the DMSO-induced differentiation to granulocytes, and much reduced expression by the PMA-induced differentiation to monocytes. This differential expression was paralleled by the increased susceptibility of DMSO-treated cells to infection by T1 and T3 reovirus and the inability of PMA-treated cells to be infected by either virus. Thus viral tropism was strongly linked to the presence and concentration of receptors on the target cell for both T1 and T3 reovirus. This receptor regulation effect was not due to a physicochemical interaction of the PMA or DMSO with the virus or receptor since treating L cells with these agents did not alter the reovirus binding to L cells and inactive phorbol had no effect.

On undifferentiated HL60 cells, the T3 serotype receptor appeared to be distinct from the T1 reovirus receptor. Significant inhibition of virus binding was detected when varying amounts of the homologous serotype were used to compete for the receptor sites. Unlabelled T3 also competed well for the T1 reovirus receptor but unlabelled T1 could not compete efficiently for the T3 reovirus receptor. This finding for HL60 cells agrees with recently published results which were obtained using L cells (Ambler & Mackay, 1991) and endothelial cells (Verdin et al., 1989). The combined data suggest that two different reovirus receptors may exist on these cell lines, a receptor to which only T3 binds and another to which T1 and T3 bind equally well. Alternatively, there may be a single receptor for which serotype 3 has the higher affinity, although T1 virus yield was slightly higher in these cells. Further study is required to establish whether differences in binding between T1 (Lang) and T3 (Dearing) reovirus are simply strain-specific or would be representative of all T1 strains compared to all T3 strains.

The presence of reovirus receptors on R1.1 cells has been debated. The R1.1 cells that we used were obtained from the ATCC and were found to bear very few, if any, reovirus receptors. These cells could not be infected by T1 or T3 reovirus (data not shown; Choi & Lee, 1988; Williams et al., 1991). There have been reports, however,
that R1.1 cells do possess T3 reovirus receptors (Co et al., 1985b; Epstein et al., 1984), but even these latter cells could not be infected by reovirus. Our findings confirm the data of Choi & Lee (1988) that the R1.1 cells from the ATCC do not express reovirus receptors and suggest that two distinct subpopulations of R1.1 cells may exist.

Some studies have suggested that the β-adrenergic receptor on some cell lines acts as the reovirus receptor (Co et al., 1985b; Donta & Shanley, 1990). Some of these reports have been based on one anti-idiotype antibody shown to be the anti-receptor by competitive inhibition of virus binding (Kauffman et al., 1983; Noseworthy et al., 1983). This anti-idiotype antibody was used to isolate a 67K glycoprotein on R1.1 cells (the subpopulation capable of binding reovirus) with structural and biochemical similarities to the β-adrenergic receptor (Co et al., 1985a). When we evaluated β-adrenergic binding with differentiation of HL60 cells, we found that the expression of the β-adrenergic receptor complex was regulated in a discordant manner indicating that this receptor complex was unlikely to mediate reovirus binding in these cells.

Other viral receptors have been shown to be regulated by various factors. Mitogenic activation of B and T lymphocytes leads to an upregulation of the expression of cellular receptors for herpes simplex virus I (Bouayyad & Menezes, 1990). On the other hand, CD4+ monocytes derived from the blood of normal human volunteers could be infected with human immunodeficiency virus (HIV) whereas plastic-adherent macrophages, from the same source, were down-regulated for CD4 and could not be infected by HIV (Kazazi et al., 1989). The poliovirus receptor has been shown to be expressed in a variety of forms by different cell types, due to alternative splicing (Koike et al., 1990). This may in turn regulate the permissivity of the cells for poliovirus infection. HeLa cells resistant to poliovirus infection had their susceptibility restored following treatment with 5-azacytidine, a cytidine analogue and a potent nucleic acid demethylating agent (Kaplan & Racaniello, 1991). The resistance of these cells appeared to be due to the down-regulation of the poliovirus receptor RNA, and methylation seemed to play a role in regulating the expression of the receptor gene.

Unlike previous reports that demonstrated one-way regulation of viral receptor expression of other viruses, the reovirus receptor can be both upregulated (by DMSO treatment) and down-regulated (by PMA treatment) in the same cell line. This phenomenon may prove to be a useful tool for studying the expression of viral receptors and for removing some of the controversy surrounding the reovirus receptor and its identification (on HL60 cells at least) using techniques such as subtractive hybridization.

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References


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