Use of alternative receptors different than α-dystroglycan by selected isolates of lymphocytic choriomeningitis virus

Stefan Kunz, a,* Noemí Sevilla, b Jillian M. Rojek, a and Michael B.A. Oldstone a

a Division of Virology, Department of Neuropharmacology, The Scripps Research Institute, La Jolla, CA 92037, USA
b Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid, Cantoblanco, Madrid 2849, Spain

Received 19 March 2004; accepted 13 May 2004
Available online 17 June 2004

Abstract

Long-term infections with viruses permit the generation of variants that evolve specific growth advantages in certain tissues and may show altered disease potentials. The selection of such variants is influenced by the host tissue and often involves virus–receptor interactions. Here we report studies of receptor usage by several lymphocytic choriomeningitis virus (LCMV) isolates that expressed different disease patterns. Consistent with our previous studies, we found that, with one exception, multiple LCMV variants that cause suppression of immune responses bound with high affinity to their cellular receptor α-dystroglycan (α-DG) and were dependent on α-DG for entry and infection. The exception also bound strongly to α-DG but was not dependent on α-DG for entry and infection. In contrast, those variants of LCMV that do not suppress the immune response either displayed low or no binding affinity for α-DG and used alternative receptors in addition to or instead of α-DG for entry and infection. For all α-DG binding variants, α-DG represents the preferred receptor in DG-expressing cells, as soluble α-DG blocked their infection of DG-deficient cells, indicating that binding of α-DG to the viral glycoprotein (GP) at the virion surface interferes with the GP’s interaction with the alternative receptor. Biochemical characterization of the alternative receptor(s) for LCMV indicated that they are either protein(s) or protein-bound entities.

Keywords: Lymphocytic choriomeningitis virus; Receptor; Dystroglycan

Introduction

Lymphocytic choriomeningitis virus (LCMV) is the prototypic member of the arenavirus family (Buchmeier et al., 2001; Oldstone, 2002). Its bisegmented negative-strand genome consists of two single-stranded RNA species, a larger segment encoding the virus polymerase (L) and a small zinc finger motif protein (Z), and a smaller segment encoding the virus nucleoprotein (NP) and glycoprotein (GP) precursor (GPC). GPC is processed into the peripheral GP1 and the transmembrane GP2 by the protease SKI-1/S1P (Beyer et al., 2003; Buchmeier and Oldstone, 1979; Kunz et al., 2003). GP1 is implicated in receptor binding (Borrow and Oldstone, 1992; Parekh and Buchmeier, 1986) and GP2 is structurally similar to the fusion active membrane proximal portions of the GPs of other enveloped viruses (Galagher et al., 2001). Upon receptor binding, arenavirus virions are internalized by uncoated vesicles and released into the cytoplasm by a pH-dependent membrane fusion step (Borrow and Oldstone, 1994; Di Simone and Buchmeier, 1995; Di Simone et al., 1994). Persistent infection with LCMV permits the emergence of variants that have a growth advantage in particular cell types. Closely related variants of the same LCMV strain can have very profound differences, causing unique disease phenotypes that correlate with the tissue from which they were isolated. In mice persistently infected with LCMV ARM53b, brain isolates generally exhibit the disease phenotype of the parental virus, causing an acute infection, which is efficiently cleared by the host’s antiviral CD8+ cytotoxic T lymphocytes (CTLs). In contrast, the majority (>95%) of variants isolated from lymphoid organs cause a persistent infection accompanied by a generalized immunosuppression (Ahmed and Oldstone, 1988; Ahmed et al., 1984; Evans et al., 1994; Sevilla et al., 2004).
Sequence analysis of a large number of immunosuppressive variants consistently revealed an amino acid exchange F260L or F260I (F: wild-type CTL producers, L and I, variants that cause immunosuppression) in GP1. As the selection of variants is influenced by the host cell or tissue, it likely involves virus–receptor interactions.

Several members of the arenavirus family use α-dystroglycan (α-DG) as a cellular receptor, including the Old World arenaviruses LCMV, Lassa fever virus (LFV), and Mobala as well as Clade C New World arenaviruses (Cao et al., 1998; Spiropoulou et al., 2002). α-DG is a ubiquitously expressed, highly versatile cell surface receptor that provides a molecular link between the extracellular matrix (ECM) and the actin-based cytoskeleton and plays a critical role in cell-mediated assembly of basement membranes (Henry and Campbell, 1999; Winder, 2001). Recent studies revealed a striking and consistent correlation between α-DG binding affinity, tissue tropism, and pathological potential of LCMV (Sevilla et al., 2000; Smelt et al., 2001). Immunosuppressive LCMV strains and variants containing an L or I at position 260 of GP1 bind α-DG with high affinity, replicate preferentially in the marginal zone of the white pulp of the spleen, and infect >75% of α-DG+ CD11c+ and DEC 205+ dendritic cells (DCs) (Sevilla et al., 2000). As a consequence of infection, DCs are unable to act as antigen-presenting cells and thus fail to generate an anti-viral T and B cell immune response, resulting in a generalized immunosuppression. In contrast, LCMV strains and variants with lower (2–3 logs less) binding affinity for α-DG localize primarily to the red pulp of the spleen, show only limited infection (<10%) of CD11c+ and DEC 205+ DCs, and generate an effective and efficient antiviral T cell response that clears the acute virus infection. Selection of LCMV variants over time in lymphoid and other organs reveals strong selective pressure for an aliphatic amino acid in position 260 of GP and high α-DG binding affinity (Evans et al., 1994; Sevilla et al., 2000).

Findings reported here revealed that DG (−/−) mouse embryonic stem (ES) cells, which are refractory to infection by most LCMV isolates, can be infected to a certain extent by some LCMV variants (Smelt et al., 2001). The susceptibility of this cell type to some LCMV isolates in the absence of a characterized receptor indicates the existence of alternative receptors. The existence of such alternative receptors for LCMV may be important regarding the disease potential of these viruses as well as shedding light on clade B New World arenaviruses like Machupo, Junin, Guanarito, and Sabia that do not use α-DG as their receptor (Spiropoulou et al., 2002). We investigated the receptor usage of several LCMV isolates with different disease potential. Consistent with previous studies, immunosuppressive LCMV variants bound α-DG with high affinity and critically depended on α-DG for infection. However, one of these variants tested showed strong binding to α-DG but no dependence on α-DG for infection, representing a novel receptor-binding phenotype of LCMV. Concerning non-immunosuppressive LCMV variants, which showed either low or no binding affinity to α-DG, all were able to use alternative receptors in addition or instead of α-DG. We found that in DG-expressing cells, α-DG represents the preferred receptor for all α-DG binding variants and that competitive binding with soluble α-DG interferes with the interaction of the viral GP with the alternative receptor. Biochemical characterization indicates that the alternative receptors for LCMV are either protein(s) or protein-bound entities.

**Results**

**LCMV isolates show either high, low, or no binding affinity to α-dystroglycan**

For studies of receptor usage by different LCMV isolates, we selected the immunosuppressive LCMV variants clone-13 (cl-13), WE54, PBL364, and PBL673, and the non-immunosuppressive LCMV variants ARM53b, CD4-1, CD8-4, and WE2.2, whose origin, disease phenotype, and tropism in the spleen are detailed in Table 1. α-Dystroglycan (α-DG) binding of the variants was quantitatively analyzed in an ELISA-format binding assay. Purified viruses were immobilized in microtiter plates and binding of biotinylated α-DG determined. With immunosuppressive variants, half-maximal binding was observed at 0.5–2 nM α-DG (Fig. 1A). In contrast, α-DG binding to non-immunosuppressive viruses ARM53b, CD4-1, and CD8-4 was not saturable with

<table>
<thead>
<tr>
<th>Table 1 LCMV isolates used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate</td>
</tr>
<tr>
<td>ARM53b</td>
</tr>
<tr>
<td>CD4-1</td>
</tr>
<tr>
<td>CD8-4</td>
</tr>
<tr>
<td>cl-13</td>
</tr>
<tr>
<td>PBL364</td>
</tr>
<tr>
<td>PBL673</td>
</tr>
<tr>
<td>WE54</td>
</tr>
<tr>
<td>WE22</td>
</tr>
</tbody>
</table>

The variants CD4-1 and CD8-4 were isolated from CD4 and CD8 cells from perforin ko mice after 24 days of infection with ARM53b (Sevilla et al., 2000). LCMV cl-13 is a spleen isolate of an adult BALB/WEHI mouse infected persistently since birth with ARM53b (Ahmed et al., 1984). PBL364 and PBL673 were isolated from peripheral blood lymphocytes (PBLs) of perforin ko mice persistently infected with LCMV ARM53b for 7 months (Sevilla et al., 2000). WE2.2 is derived from WE54 and differs from WE54 by only one amino acid exchange, S153F in GP1 (Teng et al., 1996). The phenotype of the immunosuppressive LCMV variants cl-13, PBL364, PBL673, and WE54 is as follows: they induce immunosuppression (IS+), cause persistent infection (P+), and show a tropism for the marginal zone (MZ) and the white pulp (WP) of the spleen. The variants ARM53b, CD4-1, CD8-4, and WE2.2 are non-immunosuppressive (IS−), are cleared by the host immune system (P−), and infect the red pulp (RP) of the spleen. The amino acid in position 260 of GP1 (aa GP1260) is indicated. For the variants WE54 and WE2.2, which both contain an L in position 260 of GP1, the amino acid at position 153 of GP1 is indicated in brackets.
up to 80 nM α-DG and the variant WE2.2 did not show any specific binding to α-dystroglycan (Fig. 1A). As anticipated by their different α-DG binding affinities, 1–4 nM of soluble α-DG was sufficient to achieve 50% inhibition of infection by the immunosuppressive variants but >100 nM was required for 50% neutralization of ARM53b, CD4-1, and CD8-4 (Fig. 1B). As anticipated because LCMV WE2.2 did not bind to α-DG, this variant was not neutralized by soluble α-DG. As a control protein, we used DGFc1, which represents a transcriptional fusion of amino acids 30–181 of α-DG with the Fc moiety of human IgG1 and does not bind to LCMV (Kunz et al., 2001). None of the LCMV variants was significantly neutralized by up to 400 nM DGFc1 (Fig. 1B), demonstrating the specific blocking of infection by soluble α-DG. Together, these data demonstrate that the LCMV isolates fall into three categories of α-DG binding phenotypes: high-affinity binders with an estimated $K_d$ in the low nM range (cl-13, WE54, PBL673, and PBL364), low-affinity binders with $K_d$ >100 nM (ARM53b, CD4-1, and CD8-4), and a nonbinder (WE2.2).

All LCMV isolates contain similar amounts of fully processed GP

The strikingly different binding affinities of immunosuppressive and non-immunosuppressive LCMV variants for α-DG (Fig. 1) are likely due to structural differences in the receptor-binding sites of their glycoproteins (Gs). In addition, different concentrations of GP in the virion envelope or differences in its proteolytic processing may influence the virus–receptor interaction. To address potential differences in GP concentrations in the envelopes of these variants, we quantitatively assessed the ratios of GP to nucleoprotein (NP), which is a component of the ribonucleoprotein (RNP) core of the virion. Purified viruses were immobilized in microtiter plates and the GP/NP ratios in their virions were determined by ELISA using monoclonal antibodies (mAbs) 83.6 anti-LCMVGP2 (Weber and Buchmeier, 1988) and 113 anti-LCMV NP (Buchmeier et al., 1981), which recognize GP2 and NP, respectively, of a wide range of arenaviruses. All variants displayed GP/NP ratios within the same order.
of magnitude (Fig. 2A). To assess potential differences in the proteolytic processing of the GPs of the LCMV variants studied, viral proteins were examined by Western-blot using mAb 83.6, which recognizes the GP precursor (GPC) as well as processed GP2 (Kunz et al., 2003). All LCMV variants contained exclusively fully processed GP in their envelope (Fig. 2B). These data demonstrate that all LCMV isolates contain similar amounts of fully processed GP. The >100-fold different binding affinities of immunosuppressive isolates contain similar amounts of fully processed GP. The non-immunosuppressive LCMV variants for >100-fold different binding affinities of immunosuppressive isolates are therefore likely due to structural differences in the receptor-binding sites of their GPs.

Some LCMV isolates can use other receptors than α-DG for cell entry

To analyze the dependence of infection by the LCMV variants on α-DG, we used DG (−/−) mouse embryonic stem (ES) cells and the corresponding heterozygous DG (+/−) parental line (Henry and Campbell, 1998). In a first set of experiments, DG (−/−) and DG (+/−) ES cells were incubated with virus and infection assessed after 24 and 48 h by detection of LCMV NP. The immunosuppressive variants cl-13 WE54 and PBL673 showed little infection of DG (−/−) ES cells after 24 and 48 h, indicating a strong dependence of infection on α-DG (Fig. 3). In contrast, significant infection of DG (−/−) ES cells at 24 h postinfection was observed with the immunosuppressive variant PBL364. Although ARM53b, CD4-1, and CD8-4 showed <10% infection of DG (−/−) ES cells after 24 h, >50% of cells were infected after 48 h. As previously reported (Smelt et al., 2001), the non-immunosuppressive variant WE2.2 did not depend on α-DG for infection. To exclude the possibility that the differential infection of DG (−/−) and DG (+/−) ES cells was due to factors not related to α-DG deficiency, we reconstituted DG expression in DG (−/−) ES cells. For this purpose, we infected DG (−/−) ES cells with adenoviral (AdV) vectors containing wild-type DG or the α-DG deletion mutant DGH (ΔH30-S485), which is deficient in virus binding (Kunz et al., 2001). Within 48 h after AdV-mediated gene transfer, which resulted in >70–80% of transfected cells expressing heterologous protein, cells were infected with the LCMV variants. Infection was assessed after 24 and 48 h as described above. The susceptibility of DG (−/−) ES cells transfected with wild-type DG to infection with the LCMV variants was similar to the one of DG (+/−) cells (Fig. 3). The lower infection rates observed with the DG-reconstituted DG (−/−) ES cells when compared to DG (+/−) ES are likely because <80% of the cells express DG at the time point of LCMV infection. As expected, transfection of DG (−/−) cells with the nonbinding DG mutant DGH had no significant effect on susceptibility to virus infection. These data indicate that the reduced infection of DG (−/−) ES cells by some LCMV variants is due to lack of α-DG-mediated entry and not caused by a block in a later step of viral replication or gene expression. To assess the role of α-DG on the kinetics of infection, we infected DG (−/−) and DG (+/−) ES cells with a selected subset of LCMV variants (cl-13, WE54, PBL364, ARM53b, and WE2.2) and quantified infection at 12, 24, 36, and 48 h postinfection (Fig. 4). Consistent with the data presented in Fig. 3, the variant WE2.2, which does

Fig. 2. All LCMV isolates contain similar amounts of fully processed GP. (A) Ratios of GP2/NP in LCMV isolates: to determine the GP2/NP ratios in the LCMV variants indicated, three independent preparations were analyzed for each variant. Equal amounts of purified virus were immobilized in triplicate wells of microtiter plates and probed with mAb 83.6 anti-GP2 for the detection of GP. For the detection of NP, virions were briefly permeabilized with 0.1% Triton-X-100 and subsequently incubated with mAb 113 anti-LCMV NP. Primary antibodies were detected with anti-mouse IgG coupled to peroxidase in a color reaction using ABTS substrate. OD (405) was measured using an ELISA reader (n = 3 ± SD). GP2/NP ratios were calculated for each preparation individually: the average OD (405) of the GP2 signal was divided by the average OD (405) of the NP signal. Data shown are mean GP2/NP ratios from three preparations ± SD. (C) Examination of viral GPs by Western-blot: equal amounts of virus were solubilized in SDS-PAGE sample buffer, proteins separated by SDS-PAGE, and transferred to nitrocellulose. Membranes were probed with mouse mAb 83.6 (anti-LCMVG2) and a peroxidase-conjugated anti-mouse IgG using ECL for detection. The positions of mature GP2 and the GP-precursor GPC are indicated.
not depend on α-DG for infection, shows comparable infection kinetics in both cell types. Although the variant PBL364 infects both cell types efficiently, the presence of α-DG facilitates infection. Infection of DG (−/−) ES cells with ARMS3b is significantly delayed when compared to the infection of DG (+/−) ES cells, indicating an important role of α-DG in infection with this variant. As expected based on the strong dependence on α-DG for infection (Fig. 3), the variants cl-13 and WE54 show little infection in DG (−/−) cells, even after prolonged incubation times.

Fig. 3. Some LCMV isolates can use other receptors than α-DG for cell entry. DG (−/−) ES cells were transfected with AdV vectors containing either wild-type DG (DG (−/−) ES cells + AdV DG wt) or DGH (DG (−/−) ES cells + AdV DGH). Untreated DG (−/−) and DG (+/−) ES cells were cultured in parallel. Forty hours after AdV-mediated gene transfer, cells were infected with the indicated LCMV variants at a MOI of 1. Infection levels were assessed after 24 h (black bars) and 48 h (white bars) by immunofluorescence staining with LCMV NP-specific mAb 113. Triplicate samples were counted for each virus and cell line at each time point. Data shown are mean percentages of infected cells ± SD.

Fig. 4. Infection kinetics of DG (−/−) and DG (+/−) ES cells with LCMV variants. DG (−/−) and DG (+/−) ES cells were infected with the LCMV variants cl-13, PBL364, WE54, ARMS3b, and WE2.2 at MOI of 1. The percentages of infected cells were assessed after 0, 12, 24, 36, and 48 h by immunofluorescence staining with LCMV NP-specific mAb 113. Data shown are mean percentages of triplicate samples ± SD.
Binding of LCMVGP to α-DG blocks its interaction with alternative receptors

Though the α-DG-binding LCMV variants PBL364, ARM53b, CD4-1, and CD8-4 can use alternative receptors for cell entry, they infect DG (+/−) ES cells more efficiently than DG (−/−) ES cells (Figs. 3 and 4), suggesting that α-DG facilitates infection. The ability of the glycoproteins (GPs) of these variants to recognize both α-DG and the alternative receptor may allow these variants to use both receptor systems at the same time. Alternatively, binding of the viral GP to α-DG may interfere with its ability to interact with alternative receptor molecules present at the surface of DG-expressing cells. To distinguish between these two possibilities, we tested the ability of soluble α-DG to block the infection of DG (+/−) and DG (−/−) ES cells by the LCMV variants PBL364, ARM53b, CD4-1, and CD8-4, all of which show dual receptor specificity. As a control protein, we used the nonbinding α-DG-Fc fusion protein DGFc1. The results obtained with the neutralization of infection of DG (+/−) ES cells with α-DG and DGFc1 (Fig. 5) were comparable with the results obtained with the neutralization of infection of 3T6 fibroblasts (Fig. 1B). Infection of DG (−/−) ES cells by PBL364, ARM53b, CD4-1, and CD8-4 was significantly blocked by soluble α-DG, but not DGFc1 (Fig. 5). Low nanomolar concentrations of α-DG were sufficient to block infection with the high-affinity binding variant PBL364 while >100 nM α-DG was needed to achieve significant reduction of infection of DG (−/−) ES cells by the low-affinity binders ARM53b, CD4-1, and CD8-4. Consistent with the previous results, soluble α-DG had no effect on the infection of DG (−/−) ES cells with WE2.2 (data not shown). This correlation between the inhibitory concentrations of α-DG and the α-DG binding affinity indicates that binding of soluble α-DG to the GP at the virion surface interferes with the GP’s ability to interact with alternative receptor molecules present on DG (−/−) ES cells.

ARM53b, PBL364, CD4-1, and CD8-4 bind to at least partially identical alternative receptors that are distinct from the one(s) used by WE2.2

Next, we addressed the question whether ARM53b, PBL364, CD4-1, CD8-4, and WE2.2 use identical or different receptor molecules to infect DG (−/−) ES cells. To this end, we blocked DG (−/−) and DG (+/−) ES cells with a large excess of UV-inactivated LCMV ARM53b or WE2.2 before infection with the live LCMV variants PBL364, ARM53b, CD4-1, CD8-4, and WE2.2. The blocking virus was produced in BHK21 cells, purified from cell culture supernatant by ultracentrifugation on a renografin gradient, and subjected to UV inactivation as described by Bachmann et al. (1994). Inactivation of the virus was verified by plaque assay and immunofluorescence. To address the possibility that an unknown component may have been co-purified from the BHK21 supernatants that may interfere with the assay, we subjected supernatants of mock-infected BHK21 cells to the same purification and inactivation procedure and used the resulting fractions as a negative control. In DG (−/−) ES cells, previous blocking with UV-inactivated ARM53b, but not the control supernatant, resulted in significant reduction of the subsequent infection with ARM53b, PBL364, CD4-1, and CD8-4, but not WE2.2 (Fig. 6). Preincubation of DG (+/−) ES cells with UV-inactivated ARM53b affected only the subsequent infection with ARM53b, CD4-1, and CD8-4, but not PBL364 and WE2.2. The absence of a significant reduction in case of PBL364 is likely due to its much higher binding
affinity to α-DG when compared to ARM53b (Fig. 1) that allows this variant to successfully compete with the UV-inactivated ARM53b for receptor binding. Blocking of DG (+/−) and DG (−/−) ES cells with an excess of UV-inactivated WE2.2 significantly blocked the subsequent infection with WE2.2, but not ARM53b and its derived variants PBL364, CD4-1, and CD8-4 (Fig. 6). These findings suggest that ARM53b, PBL364, CD4-1, and CD8-4 use at least partially identical receptor molecules to infect DG (−/−) ES cells, which are distinct from the receptor(s) used by WE2.2.

The receptors for LCMV ARM53b and WE2.2 on DG (−/−) ES cells are proteins or protein-bound entities

To analyze the biochemical nature of the receptors for LCMV ARM53b and WE2.2 on DG (−/−) ES cells, we used the FACS-based virus-cell binding assay described by Borrow and Oldstone (1992). DG (−/−) ES cells were incubated with biotinylated virus and bound virus subsequently detected by flow cytometry. The specificity of virus binding was confirmed by a competition assay with unbiotinylated virus (Fig. 7A). In an additional control experiment, biotinylated proteins from fractions of mock-infected BHK21 supernatants were subjected to the same purification procedure as the virus. When tested for binding to DG (−/−) cells in the flow-cytometry-based assay, these biotinylated protein fractions did not show significant binding (data not shown), further confirming the specificity of the binding observed with biotinylated virus. To determine the biochemical nature of the receptor(s) for ARM53b and WE2.2 on DG (−/−) ES cells, we treated the cells with a series of proteases, lipases, or glycosidases, and assessed the effects of enzyme treatment on virus binding by flow cytometry. Treatment with the proteases trypsin and proteinase K resulted in a significant reduction of subsequent virus binding. In contrast, treatment of cells with the phospholipases PLA2, PLC, or PLD did not have a significant effect on virus binding (Fig. 7B). Treatment with PI-PLC, which removes glycosyl-phosphatidyl-inositol (GPI)-anchored moieties from the cell surface, did not reduce virus binding, indicating that the receptor for ARM53b and WE2.2 on DG (−/−) ES cells is not GPI-linked (Fig. 7B). To address the role of N-linked carbohydrates in the binding of ARM53b and WE2.2, DG (−/−) ES cells were treated with N-glycosidase A (PNGaseA), which hydrolyzes all types of N-glycan chains from glycopeptides. O-glycans were removed by O-glycosidase, which hydrolyzes Galβ1-3GalNAc moieties from O-glycans. To test for the potential involvement of specific terminal sugar groups in virus binding, cells were treated with a series of exoglycosidases: α-1,2,3 mannosidase (cleaves specifically Mano-1,2,3R), α-N-acetyl-galactosaminidase (cleaves GalNAcα-1R), α-1,2 fucosidase (cleaves Fuco-1,2R), neuraminidase (cleaves NANAα-2,3,6,8R), and β-N-acetyl-hexosaminidase (cleaves GlcNAcβ1-2,3,4,6R). Although all these enzymes were used at concentrations and under conditions in which they efficiently remove the corresponding sugars from glycopeptides (see Materials and methods), none of the treatments resulted in a significant reduction of the binding of either ARM53b or WE2.2 to DG (−/−) ES cells (Fig. 7B). These findings indicate that the carbohydrate moieties tested are likely not critically involved in virus binding. However, based on the limited range of our reagents, we cannot conclude that carbohydrate structures not tested do not participate in the virus–receptor interaction. Nevertheless, our data indicate that the alternative receptors involved in the infection of DG (−/−) ES cells by
LCMV ARM53b and WE2.2 are most likely either themselves cell surface proteins or biochemical moieties attached to proteins.

Discussion

Here we investigated receptor usage by selected LCMV isolates and make the following points. (1) LCMV immunosuppressive variants show high-affinity binding to α-DG and depend on α-DG for infection. One exception was a variant that showed strong binding to α-DG but was not dependent on α-DG for entry. (2) By contrast, variants of LCMV that do not suppress the immune response either displayed low or no binding affinity for α-DG and used alternative receptors in addition to or instead of α-DG. (3) For all α-DG binding variants, α-DG represents the preferred receptor in DG-expressing cells. (4) Soluble α-DG blocked infection of these variants in DG-deficient cells, indicating that binding of α-DG to the viral glycoprotein (GP) at the virion surface interferes with the GP’s interaction with alternative receptors. (5) Biochemical characterization indicates that the alternative receptors for LCMV are either protein(s) or protein-bound entities.

The use of different receptors by the same virus has been reported for other viruses (reviewed by Baranowski et al., 2001, 2003; Schneider-Schaulies, 2000; Wimmer, 1994) like, for example, human immunodeficiency virus (HIV) type 1 (Michael, 1999), adeno-associated virus (Summerford et al., 1999), sindbis virus (Klimstra et al., 1998), herpes simplex virus (Montgomery et al., 1996), and foot-and-mouth disease virus (Neff et al., 1998). The novelty in our studies with arenaviruses is the finding that virus–receptor interactions of variants of the same virus species during persistent infections allowed the generation of variants that showed specific growth advantages in certain cells, and this process resulted in distinct pathological phenotypes and resultant disease outcomes. The organotypic and cellular selection of LCMV variants in vivo (Ahmed and Oldstone, 1988; Ahmed et al., 1984; Evans et al., 1994; Oldstone et al., 1988; Sevilla et al., 2000) was influenced primarily by virus–receptor interactions.

The cellular receptor for Old World arenaviruses like LCMV and Lassa fever virus (LFV) is α-dystroglycan (α-
DG). Studies with LCMV, a BSL2 agent in its natural host, the mouse, allowed us to dissect the role in viral pathogenesis played by the generation of quasi-species of virus and the interaction of such viruses with the α-DG receptor. A consistent correlation between high α-DG binding affinity and the immunosuppressive disease potential of the virus is known (Sevilla et al., 2000; Smelt et al., 2001). However, for some variants of LCMV, it was not clear whether they could use alternative receptors in addition to or instead of α-DG (Smelt et al., 2001). The study here addressed this issue by analysis of receptor binding and usage for several selected LCMV isolates. Examined were the prototypic non-immunosuppressive parental virus LCMV ARM53b, its immunosuppressive variants clone-13 (cl-13), PBL636, and PBL673, and its non-immunosuppressive variants CD4-1, and CD8-4. LCMV ARM53b and cl-13 (Ahmed et al., 1984) differ at the structural level by only two point mutations, K1079Q in the viral polymerase, and F260L in the viral glycoprotein (Salvato et al., 1988, 1991). Reassortants of viral genes mapped the receptor binding and the immunosuppressive phenotype to L or I at amino acid 260 of GP (Dockter et al., 1996; Sevilla et al., 2000; Smelt et al., 2001). The variants PBL634 and PBL673 were isolated from peripheral blood lymphocytes (PBLs) of perforin ko mice persistently infected with LCMV ARM53b for 7 months (Sevilla et al., 2000). The variants CD4-1 and CD8-4 were isolated from CD4 and CD8 cells from perforin ko mice after 24 days of infection with ARM53b. Also analyzed was another pair of closely related LCMV isolates, WE54 and WE2.2, which exhibit strikingly different disease potentials (Sevilla et al., 2001; Teng et al., 1996) and differ from each other by only one amino acid exchange, S153F in GP1 (Teng et al., 1996). In adult immunocompetent mice, WE54 causes a persistent infection accompanied by a generalized immunosuppression, similar to LCMV cl-13, while WE2.2 is not immunosuppressive but easily generates an anti-viral CTL response that clears the virus during an acute infection thereby preventing the establishment of persistent infection (Sevilla et al., 2000; Smelt et al., 2001). In addition, when inoculated into newborn C3H/St mice, WE2.2, but not WE54, causes growth hormone deficiency syndrome, which also mapped to the S153F mutation in GP1 (de la Torre and Oldstone, 1992; Oldstone et al., 1984; Teng et al., 1996).

Using these LCMV viruses, we found four distinct virus–receptor interactions (Table 2). In agreement with previous findings, the variants cl-13, WE54, and PBL673 show high-affinity binding to α-DG and strongly depend on α-DG for infection. Their receptor-binding phenotype is similar to the human pathogenic Old World arenavirus LFV and the Clade C New World arenaviruses Oliveros and Latino (Spiropoulou et al., 2002). The similarity of the receptor-binding phenotypes between these rather distantly related arenaviruses is striking and suggests a strong selective pressure for high-affinity α-DG binding in their natural rodent host species. A novel receptor phenotype was uncovered by study of the variant PBL636. Despite PBL634’s high binding affinity to α-DG, which was comparable to the affinities observed with cl-13, WE54, and PBL673, and correlates with its ability to cause a general immunosuppression and persistent infection (Sevilla et al., 2000), this variant shows negligible dependence on α-DG for infection and efficiently uses an alternative receptor for cell entry. Sequence comparison between ARM53b and PBL634 revealed only three differences in GP1 in addition to the F260L mutation: Y60H, Q73N, and A242E (Sevilla et al., 2002). In addition, a third receptor-binding phenotype is represented by ARM53b and its variants CD4-1 and CD8-4, all of which contain an F at position 260 of GP1. These three viruses bind to α-DG with a >100-fold lower affinity and used an alternative receptor to infect DG-deficient cells. However, the more efficient infection of DG (+/-) ES cells when compared to DG (-/-) ES cells indicated either that α-DG represented the preferred receptor for these variants on DG-positive cells or that α-DG somehow facilitates infection via the alternative receptor. Our data indicate that binding of α-DG to the GPs of ARM53b, PBL634, CD4-1, and CD8-4 blocks their interaction with alternative receptor molecules. The efficiency of blocking by α-DG correlates with the α-DG binding affinity of the virus and may be due to competition for overlapping binding sites or steric hindrance. These findings suggest that on DG-positive cells, α-DG represents the preferred receptor for variants with a dual receptor specificity rather than facilitates infection via alternative receptor(s). The fourth distinct receptor-binding pattern is represented by WE2.2. LCMV WE2.2, in contrast to its parental virus WE54, does not bind to α-DG and shows no dependence on α-DG for cell entry and infection. This suggests that the point mutation S153F (S: WE54, F: WE2.2) in GP1 likely has two effects. First, a total loss of α-DG binding, and second, the ability to recognize a novel receptor molecule expressed on DG (-/-) ES cells. The loss of α-DG binding due to the S153F amino acid exchange likely explains the inability of WE2.2 to cause immunosuppression. In addition, the S153F mutation in GP1 appears to allow WE2.2 to recognize a receptor molecule that is specifically expressed on growth hormone (GH)-producing cells in the anterior pituitary, resulting in a novel disease phenotype. Interestingly, with the exception of their different tropism for GH-producing cells, both WE54 and WE2.2 show a similar

<table>
<thead>
<tr>
<th>Isolate</th>
<th>α-DG binding</th>
<th>α-DG dependence</th>
<th>Receptors used</th>
</tr>
</thead>
<tbody>
<tr>
<td>cl-13</td>
<td>+++</td>
<td>+++</td>
<td>α-DG</td>
</tr>
<tr>
<td>PBL673</td>
<td>+++</td>
<td>+++</td>
<td>α-DG</td>
</tr>
<tr>
<td>WE54</td>
<td>+++</td>
<td>+++</td>
<td>α-DG</td>
</tr>
<tr>
<td>PBL634</td>
<td>+++</td>
<td>–</td>
<td>α-DG + AR1</td>
</tr>
<tr>
<td>ARM53b</td>
<td>+</td>
<td>+</td>
<td>α-DG + AR1</td>
</tr>
<tr>
<td>CD4-1</td>
<td>+</td>
<td>+</td>
<td>α-DG + AR1</td>
</tr>
<tr>
<td>CD8-4</td>
<td>+</td>
<td>+</td>
<td>α-DG + AR1</td>
</tr>
<tr>
<td>WE22</td>
<td>–</td>
<td>–</td>
<td>AR2</td>
</tr>
</tbody>
</table>

AR1 represents the putative alternative receptor(s) for ARM53b, PBL364, CD4-1, and CD8-4, and AR2 the putative receptor molecule(s) utilized by WE2.2.

### Table 2

<table>
<thead>
<tr>
<th>Isolate</th>
<th>α-DG binding</th>
<th>α-DG dependence</th>
<th>Receptors used</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARM53b</td>
<td>+++</td>
<td>++</td>
<td>α-DG + AR1</td>
</tr>
<tr>
<td>PBL634</td>
<td>+++</td>
<td>++</td>
<td>α-DG + AR1</td>
</tr>
<tr>
<td>CD4-1</td>
<td>+</td>
<td>+</td>
<td>α-DG + AR1</td>
</tr>
<tr>
<td>CD8-4</td>
<td>+</td>
<td>+</td>
<td>α-DG + AR1</td>
</tr>
<tr>
<td>WE22</td>
<td>–</td>
<td>–</td>
<td>AR2</td>
</tr>
</tbody>
</table>

AR1 represents the putative alternative receptor(s) for ARM53b, PBL364, CD4-1, and CD8-4, and AR2 the putative receptor molecule(s) utilized by WE2.2.

The similarity of the receptor-binding phenotype is similar to the human pathogenic Old World arenavirus LFV and the Clade C New World arenaviruses Oliveros and Latino (Spiropoulou et al., 2002). The similarity of the receptor-binding phenotypes between these rather distantly related arenaviruses is striking and suggests a strong selective pressure for high-affinity α-DG binding in their natural rodent host species. A novel receptor phenotype was uncovered by study of the variant PBL634. Despite PBL634’s high binding affinity to α-DG.
tissue distribution in susceptible mice, suggesting a similar overall expression pattern of α-DG and the receptor molecule(s) utilized by WE2.2.

The remarkable differences in receptor phenotypes caused by single amino acid changes such as F260L (ARM53b/cl-13) and S153F (WE54/WE2.2) suggest that minimal changes in viral genomes may lead to a shift in receptor usage for virus entry. Single amino acid changes in surface proteins have a major impact on receptor usage, tissue tropism, and disease potential of other viruses, such as measles virus (Hsu et al., 1998), Theiler’s murine encephalitis virus (McCright et al., 1999), polio virus (Colston and Racaniello, 1995), and influenza virus (Skehel and Wiley, 2000). Given the current evidence for a quasi-species population structure of arenaviruses (reviewed by Sevilla et al., 2002), the genetic distance that the virus must bridge to significantly alter its receptor-binding phenotype appears to be rather small.

It is currently not clear how many additional receptor molecules can be used by LCMV isolates. Competition studies with UV-inactivated virus on DG (−/−) cells demonstrated that ARM53b and its variants PBL364, CD4-1, and CD8-4 use at least partially identical alternative receptors. In contrast, the inability of UV-inactivated ARM53b to block infection with WE2.2 and vice versa suggests that these viruses use distinct receptors for infection of DG (−/−) ES cells. To determine the biochemical nature of the cell surface component(s) on DG (−/−) ES cells to which LCMV ARM53b and WE2.2 bind, the effect of selective removal of different host cell membrane components on virus binding was investigated. Treatment of DG (−/−) ES cells with proteases but not phospholipases or glycosidases significantly reduced binding of LCMV ARM53b and WE22, indicating that the cellular receptors for these isolates are either protein(s) or protein-bound entities.

The evolution and selection of viruses have long held the interest of virologists (reviewed by Baranowski et al., 2001, 2003; Woolhouse et al., 2002). Analysis of these events for arenaviruses and especially LCMV in its natural host adds to growing knowledge in this area due to three factors: the ability to manipulate the LCMV genome (reviewed by Lee and de la Torre, 2002), the use of genetics in its natural murine host (reviewed by Rall and Oldstone, 1997), and the accumulated data on the immunobiology and pathogenesis of LCMV infection (reviewed by Oldstone, 2002). In addition, the data derived may have implications for understanding and treatment of the Old World and New World arenaviruses that cause hemorrhagic fevers in humans, an important medical problem.

Materials and methods

Proteins, antibodies, cell cultures, and viruses

For biotinylation, α-DG (purified from rabbit skeletal muscle) was dialyzed against a reaction buffer (0.1 M NaHCO3, 100 mM NaCl, pH 8.3) and exposed to 1 mM NHS-X-biotin (Calbiochem) diluted in cold reaction buffer. The reagent was applied for 20 min on ice with gentle shaking, after which free biotin was quenched by adding glycine, pH 8.0, in a final concentration of 50 mM. Biotinylated α-DG was dialyzed against PBS and biotinylation verified by Western-blot using streptavidin coupled to peroxidase (Pierce) for detection. Protein concentrations were determined by the method of Bradford. The fusion protein DGFc1 represents a transcriptional fusion of amino acids 30–181 of α-DG with the Fc moiety of human IgG1 and was expressed and purified from HEK293T cells as described (Kunz et al., 2001). Monoclonal antibodies (mAb) 113 (anti-LCMV NP) and 83.6 (anti-LCMVGP2) have been described (Buchmeier et al., 1981; Weber and Buchmeier, 1988). HRP-conjugated anti-mouse IgG was from Pierce Chemical Co., Rockford, IL. DG (+/−) and DG (−/−) embryonic stem (ES) cells, and 3T6 mouse fibroblasts cells were maintained as described (Cao et al., 1998). Origin, passage, and characteristics of LCMV ARM53b, cl-13, WE54, and WE2.2 have been described elsewhere (Ahmed et al., 1984; Dutko and Oldstone, 1983; Teng et al., 1996). The variants PBL364 and PBL673 have been isolated from peripheral blood lymphocytes (PBLs) of perforin (−/−) mice, infected at birth with 10^3 pfu ARM53b at 7 months postinfection as described (Sevilla et al., 2000). The variants CD4-1 and CD8-4 were isolated from CD4+ and CD8+ cells of the same 24 days postinfection (Sevilla et al., 2000). Seed stocks of all viruses were prepared by growth in BHK-21 cells. Purified virus stocks were produced and virus titers determined as described (Dutko and Oldstone, 1983).

Detection of LCMVGP and LCMV NP in ELISA

For the determination of GP/NP ratios in LCMV variants, three independent preparations of renograin-purified viruses were analyzed for each variant studied. Purified viruses were coated in triplicate wells in 96-well EIA/RIA high-bond microtiter plates (Corning) for 2 h at 6 °C and nonspecific binding blocked with 1% (wt/vol) BSA/PBS. For the detection of NP, immobilized viruses were fixed with 2% (wt/vol) paraformaldehyde in PBS, washed three times in PBS, and treated with 0.1% (wt/vol) Triton X-100 for 10 min at room temperature. mAbs 83.6 (anti-LCMVGP2) and 113 (anti-LCMV NP) were applied in 1: 100 dilution for 2 h at 6 °C and detected with peroxidase-conjugated anti-mouse IgG (1: 1000) in a color reaction using 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate. OD (405) was measured with an ELISA reader. For the determination of specific binding, background binding to BSA was subtracted. GP2/NP ratios were calculated for each preparation individually: the average OD (405) of the GP2 signal was divided by the average OD (405) of the NP signal.
Immunoblotting

Purified viruses were lysed in preheated (95 °C) SDS-PAGE sample buffer: 2% (wt/vol) SDS, 50 mM Tris–HCl, pH 6.8, 100 mM DTT. Proteins were separated by gel electrophoresis and transferred to nitrocellulose. After blocking in 5% (wt/vol) skim milk powder in PBS, membranes were incubated with the primary antibody mAb 83.6 anti-LCMVGFP2 in a dilution of 1:1000 in 2% (wt/vol) skim milk powder, PBS for 12 h at 6 °C. After several washes in PBS, 0.1% (wt/vol) Tween 20 (PBST), the secondary antibody, goat anti-mouse IgG coupled to peroxidase was applied 1: 5000 in PBST for 1 h at room temperature. Blots were developed by enhanced chemiluminescence (ECL) using Super Signal West Pico ECL Substrate (Pierce) and signals were recorded on autoradiographic film (Kodak, Rochester, N.Y.).

Binding of biotinylated α-DG and to LCMV variants

α-DG purified from rabbit skeletal muscle was biotinylated with the reagent NHS-X-biotin as described above. To test for biological activity of biotinylated α-DG, 10^5 pfu of LCMV ARM and cl-13 was incubated with 0, 1, 2, 5, 10, and 20 nM of either biotinylated or unmodified α-DG for 2 h on ice. The inoculum was then added to 10^5 CHO-K1 cells cultured in a M24 plate (MOI = 1) for 45 min. Cells were washed and incubated for 16 h. LCMV infection was assessed by immunofluorescence staining for LCMV NP as described in Cao et al. (1998). Cells (100) were counted and NP-positive cells scored (n = 3 ± SD). As the neutralization of LCMV cl-13 infection of biotinylated α-DG was found to be not significantly different from unmodified α-DG, the virus-binding properties of α-DG are apparently not affected by biotinylation. To test binding to biotinylated α-DG, LCMV variants were immobilized in microtiter plates and incubated with the indicated concentrations of biotinylated α-DG. Bound biotinylated α-DG was detected with peroxidase-conjugated streptavidin in a color reaction using ABTS substrate. OD (405) was recorded in an ELISA reader. For the determination of specific binding, background binding to BSA was subtracted.

Infection of embryonic stem (ES) cells

DG (−/−) and DG (+/−) ES cells were plated in gelatin-pretreated 96-well plates in a density of 10^4 cells/well. AdV-mediated gene transfer of wild-type DG and the DG deletion mutant DGH was performed as described in Cao et al. (1998). After 48 h, LCMV was added to AdV-transfected DG (−/−) ES cells as well as untreated control DG (−/−) and DG (+/−) cells at a MOI of 1 and incubated for 1 h at 37 °C. The viral particles were removed, cells washed twice with DMEM, and fresh medium added. After the time points indicated, infected cells were quantified by immunofluorescence staining for LCMV NP as described (Smelt et al., 2001).

Neutralization of LCMV infection with α-DG

Neutralization of LCMV infection with α-DG was performed as described in Cao et al. (1998). 3T6 mouse fibroblasts, DG (−/−), or DG (+/−) ES cells were cultured in M96 tissue culture plates (2 × 10^4 cells/well). The LCMV variants (MOI = 1 for the infection of 3T6 cells and MOI = 3 for the infection of DG (−/−) and DG (+/−) ES cells) were incubated with the indicated concentrations of α-DG for 1 h on ice. As a control, the nonbinding α-DG-Fc fusion protein DGC1 (Kunz et al., 2001) was used. The inoculum was added to the target cells for 45 min at 37 °C, 5% (vol/vol) CO₂. Cells were washed and incubated for 16 h. LCMV infection was assessed by immunofluorescence staining for LCMV NP. For infection of 3T6 and DG (+/−) ES cells, 100 cells were counted and LCMV NP-positive cells scored. In case of infected DG (−/−) ES cells, 300 cells were counted.

Blocking of infection of cells with LCMV with UV-inactivated virus

LCMV ARM53b and WE2.2 were produced in BHK cells, purified by renografin gradient (Dutko and Oldstone, 1983), tittered on Vero cells, and UV-inactivated as described (Bachmann et al., 1994) using 10 min of UV irradiation for inactivation. Inactivation was verified by the absence of LCMV NP expression in Vero cells 48 and 72 h after infection with UV-inactivated preparations (MOI = 0, 1, 10, 100) by immunofluorescence staining as well as plaque assay on Vero cells. As a control, we used supernatant of mock-infected BHK21 cells that was subjected to the same purification and inactivation procedures as used for the virus. For blocking, DG (−/−) and DG (+/−) ESC, cultured in gelatin-coated 96-well plates (2 × 10^5 cells/well), were incubated with 100 μl/well UV-inactivated LCMV (10^5 pfu/ml in 50% OPTIMEM) or the mock-infected supernatant control in 50% OPTIMEM for 2 h on ice. Live LCMV (2 × 10^3 pfu; MOI = 1) was mixed with 10^5 pfu/ml UV-inactivated LCMV or mock-infected supernatant control in 50% OPTIMEM in a total volume of 100 μl and the inoculum added to the cells. After 45 min of incubation at 37 °C, 5% CO₂, supernatants were removed, cells washed four times with medium (w/o ESGRO) and incubated for 16 h. For immunofluorescence staining, the medium was removed and cells fixed in 2% formaldehyde/PBS for 10 min at room temperature, wells were washed three times with PBS, cells blocked with 1% (vol/vol) FBS, PBS, and permeabilized with 1% (vol/vol) FBS, 0.1% (wt/vol) saponin, PBS for 15 min at room temperature. MAb 1-1-3 was diluted 1:100 in PBS/1% FBS/0.1% saponin and incubated for 45 min at room temperature. Secondary antibody (goat anti-mouse IgG-FITC, 1: 40 in 1% (vol/vol) FBS, 0.1% (wt/vol) saponin) PBS was applied for 45 min in the dark. For quantitative assessment of infection in DG (−/−) ES cells, triplicates of 500 cells were counted and LCMVNVP-positive
cells scored. In case of DG (+/−) cells, triplicates of 100 cells were counted.

**Binding of biotinylated virus to ES cells**

For biotinylation of LCMV ARM53b and WE2.2, we used a modified version of the protocol described by Borrow and Oldstone (1992). Briefly, purified virus was dialyzed against reaction buffer (0.1 M NaHCO₃, 100 mM NaCl, pH 8.0) and biotinylation was accomplished by addition of 1 mM NHS-X-biotin (final concentration) from a 100 mM stock solution in water-free DMSO. After 20 min on ice under gentle shaking, another 1 mM NHS-X-biotin (final concentration) was added and the reaction let go for another 20 min. After a total reaction time of 40 min, the reaction was quenched by adding cold 50 mM glycine (final concentration) pH 8.0 for 10 min. Virus was dialyzed against PBS and infectivity checked by plaque assay on Vero cells. Only biotinylated virus retaining >30% of infectivity was used in experiments. The extent of biotinylation of the virus is tested by ELISA using POD-conjugated streptavidin for detection.

**Binding assay (modified from Borrow and Oldstone, 1992)**

DG (+/−) ES cells were cultured in gelatin-coated M6 tissue culture plates (2 × 10⁵ cells/well) for 48 h, washed three times with PBS, and detached using enzyme-free dissociation solution (Sigma) for 5–10 min at 37 °C. Cells were removed using a cell scraper, dissociated by titration (five times) with a blue tip, and centrifuged for 5 min at 1200 rpm. The pellet was resuspended in 1% (vol/vol) FBS/0.1% (wt/vol) sodium azide/PBS (FACS-buffer) and blocked for 15 min on ice. Cells (5 × 10⁵) were transferred to each well of M96 plates, centrifuged for 5 min at 1200 rpm, and resuspended in 50 µl FACS buffer containing biotinylated LCMV ARM53b (10⁸ pfu/ml) or LCMV WE2.2 (10⁷ pfu/ml). Incubation was for 2 h on ice under shaking. After two rapid wash-steps in FACS-buffer at 4 °C, cells were re-suspended in 4% PFA and fixed for 10 min on ice. After three washes in FACS buffer, biotinylated virus was detected by adding FITC-conjugated streptavidin (1:100 in FACS-buffer) for 45 min in the dark. After three wash-steps in FACS buffer, cells were fixed with 4% (wt/vol) paraformaldehyde, PBS for 10 min at room temperature, washed three times with PBS, and analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose CA) using Cell Quest software. Specificity of binding was demonstrated in two ways: (1) by the ability of unlabelled virus (but not proteins from fractions of mock-infected BHK21 supernatants that were subjected to the same purification procedure as the virus) to block binding of biotinylated virus to DG (+/−) ES cells. For competition experiments, cells were preincubated with 0, 0.1, 0.5, 1, and 5 × 10⁸ pfu/ml of unlabelled virus for 1 h on ice. Biotinylated LCMV ARM53b (5 × 10⁷ pfu/ml) or LCMV WE2.2 (10⁷ pfu/ml) was added in the presence of the same concentrations of unlabelled virus. For the control experiments, we used the corresponding dilutions of purified supernatants of mock-infected BHK cells for the competition assay. (2) We biotinylated proteins from fractions of mock-infected BHK21 supernatants that were subjected to the same purification procedure as the virus using the protocol described above. When tested for binding to DG (+/−) cells in the flow-cytometry-based assay, these biotinylated protein fractions did not show significant binding, indicating that the specific binding observed with biotinylated virus was due to binding of the virus and not a copurified protein component present in the viral preparation.

**Enzyme treatment of DG (+/−) ES cells**

Single-cell suspensions of DG (+/−) ES cells were prepared as described above. For enzyme treatment, 10⁵ cells were transferred to M96 plates (triplicates) and washed twice in HBSS. Enzyme treatments of cells in suspension were carried out as described by Borrow and Oldstone (1992).

**Proteases**

Cells were resuspended in 100 µl HBSS, pH 7.5, containing 10 µg/ml proteinase K from T. album (Roche) or 10 µg/ml trypsin from bovine pancreas (Sigma). After incubation for 1 h at 37 °C, 10% (vol/vol) FBS was added to quench proteases. Cells were washed twice in FACS-buffer and subjected to the binding assay with biotinylated virus.

**Phospholipases**

Cells were resuspended in 100 µl HBSS, pH 8.0, containing 10 U/ml Phospholipase A₂ (PLA₂) from Streptomyces violaceoruber (Sigma), 100 µl HBSS, pH 7.3 containing 10 U/ml phospholipase C (PLC) from Clostridium perfringens (Sigma), 100 µl HBSS, pH 6.5, containing 100 U/ml phospholipase D (PLD) from Streptomyces species, 100 µl HBSS, pH 8.0, containing 100 U/ml PI-specific phospholipase C (PI-PLC) from Bacillus cereus.

**Endoglycosidases**

Cells were resuspended in 100 µl of 1% (wt/vol) BSA, HBSS, pH 7.5, containing 1 mU/ml N-glycosidase A from sweet almonds (PNGaseA, Roche), 1% (wt/vol) BSA, HBSS, pH 7.5, containing 10 mU/ml O-glycosidase from Diploloccus pneumoniae (Roche).

**Exoglycosidases**

Cells were resuspended in 100 µl of 1% (wt/vol) BSA, 50 mM HEPES, 5 mM CaCl₂, pH 6.5, containing 10 U/ml α-1,2 mannosidase from Xanthomonas manihotis (New England Biolabs, NEB), 1% (wt/vol) BSA, HBSS, pH 7.5, containing 10 U/ml α-N-acetyl-galactosaminase from E. coli (NEB), 1% (wt/vol) BSA, HBSS, pH 7.5, containing 10 U/ml α-1,2 fucosidase from X. manihotis (NEB), 1% (wt/vol) BSA, HBSS, pH 6.5, containing 100 U/ml neur-
aminidase from \textit{C. perfringens} (NEB), and 1% (wt/vol) BSA, HBSS, pH 6.5, containing 100 U/ml \textit{b}-N-acetylhexosaminidase from \textit{Streptomyces plicatus} (NEB). In control samples, cells were incubated with buffer only. Enzyme treatment was for 1 h at 37 °C if not indicated otherwise. Cells were then washed twice in FACS buffer and cell viability checked by staining with 7-amino-actinomycin (7-AAD) (BD Biosciences). Briefly, 10 μl of cell suspension was removed from each sample and 1 μl of 1: 10 dilution of 7-AAD stock added to sample. After incubation for 10 min at room temperature, cells were washed twice in FACS buffer and examined by fluorescence microscopy using a Zeiss Axiovert S100 microscope (Carl Zeiss Inc. Thornwood, NY) fitted with a 20× objective, an AxioCam digital camera, and an automated stage. Triplicates of 100 cells in phase were counted and cells showing red fluorescence scored as dead or dying. The percentage of viable cells in phase was calculated accordingly. Cell viability after treatment with enzyme/without enzyme was as follows: PLA2: 81% (+9)/88 (+11), PLC: 76% (+12)/86 (+9), PLD: 64% (+13)/80 (+12), PI-PLC: 80% (+12)/92 (+9), PNGaseA: 71% (+9)/69 (+11), O-glycanase 92% (+10)/96 (+8), \alpha-1,2 mannosidase: 65% (+7)/71 (+11), \alpha-N-acetyl-galactosaminase: 89% (+9)/85 (+10), \alpha-1,2 fucosidase: 91% (+8)/88 (+12), neuraminidase: 61% (+9)/62 (+9), and \beta-N-acetyl-hexosaminidase: 71% (+6)/62 (+9); proteinase K: 72 (+11); Trypsin: 79 (+6). For subsequent binding to biotinylated virus, 5 × 10^5 live cells were transferred to each well of M96 plates, centrifuged for 5 min at 1200 rpm, and incubated with either biotinylated LCMV ARM53b (10^8 pfu/ml) or biotinylated LCMV WE2.2 (10^7 pfu/ml) in FACS buffer, and incubated for 2 h on ice under shaking. After two rapid wash-steps in FACS-buffer at 4 °C, cells were re-suspended in 4% PFA and fixed for 10 min on ice, and bound virus detected by flow cytometry as described above.

Acknowledgments

This is publication #16451 from the Department of Neuropharmacology, the Scripps Research Institute. The authors thank Dr. Kevin Campbell for materials graciously supplied and Dr. Esteban Domingo for helpful discussions. This research was supported by US Public Health grant AI 45927. S.K. received fellowships from Swiss National Science Foundation.

References


Klimstra, W.B., Ryman, K.D., Johnston, R.E., 1998. Adaptation of Sindbis


Teng, M.N., Borrow, P., Oldstone, M.B., de la Torre, J.C., 1996. A single amino acid change in the glycoprotein of lymphocytic choriomeningitis virus is associated with the ability to cause growth hormone deficiency syndrome. J. Virol. 70 (12), 8438–8443.


