Differential host-dependent expression of α-galactosyl epitopes on viral glycoproteins: a study of eastern equine encephalitis virus as a model

Patricia M. Repik, Julie M. Strizki and Uri Galili*

Department of Microbiology and Immunology, Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129, U.S.A.

The carbohydrate epitope Galα1-3Galβ1-4GlcNAc-R (α-galactosyl) is abundantly expressed on cells of non-primate mammals, prosimians and New World monkeys, where it is synthesized by the enzyme α1,3-galactosyltransferase (α1,3GT). Old World monkeys, apes and humans lack α1,3GT and hence do not synthesize α-galactosyl epitopes. Instead, these species produce α-galactosyl epitopes on viral glycoproteins was investigated using eastern equine encephalitis (EEE) virus as a model to examine the differential expression of α-galactosyl epitopes on the glycoproteins of virus propagated in cells that either produce or lack α1,3GT. As predicted, virus propagated in Vero cells (derived from the African green monkey, an Old World monkey) did not express α-galactosyl epitopes. In contrast, virus propagated in mouse 3T3 cells (EEE3T3) expressed approximately 80 α-galactosyl epitopes per virion on both the E1 and the E2 envelope glycoproteins. Thus, expression of the α-galactosyl epitope on virions paralleled that on host cells. The binding of anti-Gal antibody to these epitopes on EEE3T3 virions partially neutralized virus infectivity, raising the possibility that anti-Gal production in hosts may influence the initial infectious stage of viruses expressing α-galactosyl epitopes.

Asparagine N-linked carbohydrate chains, of the simple high mannose type or of the complex type, are common structures on envelope glycoproteins of viruses that bud from vertebrate cells (Kornfeld & Kornfeld, 1976, 1985; Klenk & Rott, 1980; Schlesinger & Schlesinger, 1986, 1987; Klenk, 1990). It is well established that the oligosaccharide chains of viral glycoproteins are synthesized by host cell glycosylation enzymes i.e. glycosyltransferases (Klenk, 1990). Glycosylation enzymes may vary in different species, as well as in different cells of the same species, so it is possible for one virus to express different types of oligosaccharide structures when grown in different cell lines (Strauss et al., 1970; Burke & Keegstra, 1976; Stollar et al., 1976; Hunt, 1980; Hsieh et al., 1983; Davidson & Hunt, 1985). Sialylated N-linked complex carbohydrate chains, for instance SAα2-3Galβ1-4GlcNAc-R or SAα2-6Galβ1-4GlcNAc-R, are common on viral glycoproteins and on cell surface glycoproteins. This is because sialyltransferases, which are prevalent in the Golgi apparatus of all mammalian cells, cap N-acetyllactosaminyl residues (Galβ1-4GlcNAc-R) of viral carbohydrate chains with sialic acid (Klenk & Rott, 1980; Kornfeld & Kornfeld, 1985; Rademacher et al., 1988).

Here, we have examined the possible expression of the carbohydrate structure Galα1-3Galβ1-4GlcNAc-R (α-galactosyl epitope) on viral proteins. The α-galactosyl epitope is abundant (> 10^6 per cell) on cells of non-primate mammals, prosimians and New World monkeys, where it is synthesized by the glycosyltransferase α1,3-galactosyltransferase (α1,3GT) (Galili et al., 1988; Thall et al., 1991). Humans, apes and Old World monkeys lack α1,3GT and the α-galactosyl epitope (Galili et al., 1988). We have hypothesized that a virus grown in mouse cells would express α-galactosyl epitopes on its envelope glycoproteins, whereas the same virus grown in Old World monkey cells would lack α-galactosyl epitopes. Determination of α-galactosyl epitope expression on viral glycoproteins is of particular interest since humans, apes and Old World monkeys produce large amounts of a natural antibody against the α-galactosyl epitope (Galili et al., 1985, 1987a, b; Galili, 1993). This natural antibody (anti-Gal) constitutes 1% of circulating immunoglobulins in humans (Galili et al., 1984; Davin et al., 1987; Avila et al., 1989) and theoretically has the potential to interact with α-galactosyl epitopes on viral glycoproteins, possibly affecting infectivity.

In this study, the putative differential expression of α-galactosyl epitopes on viral glycoproteins was investigated using eastern equine encephalitis (EEE) virus, a member of the Alphavirus genus within the Togaviridae family. The E1 and E2 glycoproteins of EEE virus are predicted to have one and two potential N-linked glycosylation sites respectively (Chang & Trent, 1987).
Fig. 1. Immunoreactivity of EEE_vero and EEE_3T3 virus structural proteins with anti-Gal antibody and BS lectin. Reactivity of proteins from EEE_vero (lane 1) and EEE_3T3 (lane 2) viruses with anti-Gal. Reactivity of proteins from EEE_vero (lane 3) and EEE_3T3 (lane 4) viruses with BS lectin. Note that reactivity was demonstrated only with virus propagated in 3T3 cells (lanes 2 and 4). The positions of the viral proteins are indicated on the left. The binding to the C protein is non-specific.

Since EEE virus, like other alphaviruses, is capable of infecting a large variety of mammalian cells (Strauss & Strauss, 1977; Schlesinger & Schlesinger, 1987; Morris, 1988), this virus was selected as a model for the study.

EEE virus strain 215-85 was grown in mouse 3T3 fibroblasts which produce an abundance of α-galactosyl epitopes (Santer et al., 1989) and in Vero cells, which originate from the African green monkey and lack α-galactosyl epitopes (Galili et al., 1988; Joziasse et al., 1989). Monolayers of Vero cells or mouse 3T3 fibroblasts were inoculated with the virus at a multiplicity of 0.05 to 0.1 p.f.u. per cell, after which the virus was propagated and purified as described by Weaver et al. (1991). It was then suspended in 20 mM-Tris-HCl pH 7.4 and 2.0 mM-EDTA prior to use.

The occurrence of α-galactosyl epitopes on EEE viral glycoproteins was determined by a Western blot assay using purified anti-Gal antibody, and the lectin Bandeiraea simplicifolia IB₅ (BS lectin; Vector Laboratories) which also interacts with the α-galactosyl epitope (Wood et al., 1979). Anti-Gal antibody was isolated from normal human blood group AB sera by affinity chromatography on synthetic Galα1-3Galβ1-4GlcNAc-R linked to silica beads (Synsorb 115; Chembiodi) and biotinylated as previously described (Galili et al., 1987a, b; Thall & Galili, 1990). Biotinylated and non-biotinylated antibody preparations were stored at −20°C in PBS containing 1% BSA.

For Western blot analysis, virus proteins derived from 50 µg of purified 3T3 cell-propagated EEE virus (EEE_3T3) and Vero cell-propagated EEE virus (EEE_vero) preparations were resolved by SDS-PAGE with a 13% polyacrylamide resolving gel and a 3-6% polyacrylamide stacking gel, using the Mini-PROTEAN II gel system (Bio-Rad) at 200 V for 30 min, as described by Schmaljohn et al. (1983). EEE proteins were subsequently assayed for expression of α-galactosyl epitopes by immunostaining with anti-Gal solution (10 µg/ml) and with horseradish peroxidase (HRP)-conjugated BS lectin (20 µg/ml) for 2 h at 24°C. Anti-Gal-reactive proteins were visualized by staining with HRP-conjugated rabbit anti-human IgG and the substrate 3,3′-diaminobenzidine tetrahydrochloride (DAB). Detection of HRP–BS lectin-reactive proteins was performed by direct addition of DAB. As shown in Fig. 1, anti-Gal antibody readily interacted with both the E1 and E2 glycoproteins of EEE_3T3, indicating expression of α-galactosyl epitopes on these molecules. In addition, anti-Gal interacted with the high molecular weight (HMW) protein which is consistently detected in purified preparations of EEE virus and thought to be composed primarily of E2 molecules (J. M. Strizki & P. M. Repik, unpublished results). In contrast, anti-Gal antibody did not bind to the E1 or E2 glycoproteins or to the HMW protein of EEE_vero, implying the lack of α-galactosyl epitopes on these proteins. These data suggest that, for viruses propagated in mouse 3T3 cells, a proportion of the single carbohydrate chain linked to the E1 protein, as well as the two potential chains on the E2 protein, are of the complex type with some chains possessing α-galactosyl epitopes. BS lectin bound to the E2 and HMW proteins of EEE_3T3, but binding to the E1 protein was not detected. As expected, BS lectin did not bind to the glycoproteins of EEE_vero. The inability of the BS lectin to bind to the E1 protein of EEE_vero could be due to a paucity of α-galactosyl epitopes on this molecule which has only one potential N-glycosylation site (Chang & Trent, 1987). The BS lectin, being a tetramer, must interact with at least two epitopes on a glycoprotein molecule in order to bind effectively enough to withstand repeated washing of the blot. Such low-affinity interactions between BS lectin and monomeric ligands have previously been demonstrated (Blake & Goldstein, 1980). Thus binding of the BS lectin to relatively few α-galactosyl epitopes via only one of its four binding sites would be of low affinity and hence the lectin would be removed from the blotted protein during the process of washing. In contrast, the affinity of anti-Gal antibody binding to single α-galactosyl epitopes is sufficient to withstand repeated washing of the blot. The binding of both the antibody and the BS lectin to the capsid (C) protein of EEE virus in the two viral preparations appeared to be non-specific, and could not be eliminated with any blocking solution used (horse serum, casein or BSA).

To determine the number of α-galactosyl epitopes per virion, EEE_3T3 and EEE_vero viruses were analysed by a radioimmunoassay previously developed for this purpose (Thall & Galili, 1990). Briefly, bovine thyroglobulin,
which has 11 α-galactosyl epitopes per molecule (Spiro & Bhoyroo, 1984), was diluted to 50 µg/ml and used as the solid-phase antigen to coat microtitre wells (Falcon 3912; Becton Dickinson). Samples of biotinylated anti-Gal solution (1 µg/ml), mixed with various concentrations of EEE virus, were incubated for 20 h at 4 °C and subsequently placed in the coated wells for 2 h at 24 °C. Under these conditions, glycoproteins lacking the α-galactosyl epitope do not inhibit binding of anti-Gal antibody to the solid-phase α-galactosyl epitopes, whereas those containing the epitope neutralize various proportions of the biotinylated antibody, thus decreasing anti-Gal antibody binding to the solid-phase α-galactosyl epitopes. Anti-Gal antibody binding to the epitopes was assessed by the subsequent binding of 125I-streptavidin (Amersham; 10⁶ c.p.m./50 µl) to the biotinylated antibody. Bovine thyroglobulin was used to generate a standard curve for quantification of α-galactosyl epitopes on virions.

As shown in Fig. 2, EEEvero lacked α-galactosyl epitopes and thus did not inhibit interaction of anti-Gal antibody with the α-galactosyl epitopes on bovine thyroglobulin (solid-phase antigen). The 10% inhibition detected at a viral concentration of 2 mg/ml is likely to be a non-specific effect due to the high concentration of virions in the well. In contrast, EEE3T3 exerted a 20% inhibition at a viral concentration of 0.06 mg/ml and a 50% inhibition at a concentration of 1 mg/ml. Bovine thyroglobulin (M₉ 670000), which has 11 α-galactosyl epitopes per molecule (Spiro & Bhoyroo, 1984), inhibited 50% of anti-Gal binding to the solid-phase antigen at a concentration of 0.07 mg/ml, which corresponds to 100 nmol/ml. These data suggest that the EEE3T3 virus expresses an average of 80 α-galactosyl epitopes per particle, according to the following calculation. The 50% inhibition obtained with bovine thyroglobulin is the neutralizing effect of 6.6 x 10¹⁴ α-galactosyl epitopes present on 100 nmol of thyroglobulin, as 1 nmol thyroglobulin is 6 x 10¹ⁱ molecules and each molecule contains 11 α-galactosyl epitopes. A 50% inhibition of anti-Gal antibody binding is achieved by the same number of epitopes on 1 mg/ml of EEE3T3 virus particles. The average M₉ of the two EEE viral envelope glycoproteins is approximately 50000 (E₁ is 53000; E₂ is 42000 to 43500; P. M. Repik & J. M. Strizki, unpublished results) and it is estimated that the envelope glycoproteins constitute approximately 50% of the virus weight (Strauss & Strauss, 1977; Koblet, 1990). Thus the 50% inhibition achieved by the EEE3T3 virus corresponds to 0.5 mg of EEE3T3 glycoproteins, which in turn corresponds to 10 nmol, or approximately 4 x 10¹⁵ molecules. This number of molecules expresses a total of 6.6 x 10¹⁴ α-galactosyl epitopes, suggesting that one out of six EEE3T3 viral envelope glycoprotein molecules expresses the α-galactosyl epitope. Since each EE virus is assumed to have a total of 480 E₁ and E₂ molecules [the values obtained for Sindbis virus by Fuller (1987)], the average number of α-galactosyl epitopes was calculated to be approximately 80 per EEE3T3 virion. These findings suggest that α-galactosyl epitope expression may be widely distributed on enveloped viruses propagated in non-primate mammalian cells. The presence of this carbohydrate structure on viral glycoproteins has been previously reported by Geyer et al. (1984), who identified α-galactosyl epitopes as a major carbohydrate structure on the Friend murine leukaemia virus using proton nuclear magnetic resonance and methylation analysis.

The specific interaction of anti-Gal antibody with α-galactosyl epitopes on EEE3T3 virus raised the possibility that this natural antibody may neutralize virions expressing α-galactosyl epitopes by inhibiting their ability to bind to cells. To assess the neutralizing capability of anti-Gal, plaque reduction neutralization tests were performed essentially as described by Russell et al. (1967). Briefly, serial twofold dilutions of anti-Gal antibody, ranging from 1:2.5 to 1:80 (40 µg/ml to 1:25 µg/ml antibody respectively), were added to virus that had been previously diluted to yield 100 to 150 p.f.u./0.1 ml. The virus–antibody mixtures were then incubated at 37 °C for 30 min, followed by 4 °C for 18 h. Residual infectious virus was measured by plaque assay on Vero cell monolayers as previously described (Repik et al., 1983) in the absence or in the presence of anti-Gal antibody in the overlay. When present, anti-Gal was again serially diluted from 1:2.5 to 1:80 in the overlay medium. This was then added to wells containing the corresponding dilutions of antibody in the inocula. The percentage plaque reduction was calculated by comparison of wells containing virus–antibody to control.
Percentage plaque reduction curves obtained following incubation of viruses with increasing concentrations of anti-Gal. Plaque assays were performed without the antibody in the overlay medium. The addition of anti-Gal to the overlay had no further effect on the number of plaques formed by EEE3T3 virus. In contrast, anti-Gal antibody had no effect on the number of plaques formed by EEEvero virus, which lacks α-galactosyl epitopes. In three separate experiments, incubation of EEE3T3 virus with 20 μg/ml anti-Gal solution resulted in a 20 to 50% decrease in plaque formation (Fig. 3). The presence of anti-Gal antibody in the overlay medium did not further reduce the number of plaques formed by EEE3T3. In contrast, and as expected, anti-Gal antibody had no effect on the ability of EEEvero virus to infect Vero cells and form plaques (Fig. 3).

The neutralizing activity of anti-Gal at 20 to 40 μg/ml may be of potential significance since the physiological concentration of anti-Gal in human sera is around 50 μg/ml (Galili et al., 1984; Davin et al., 1987; Avila et al., 1989). These findings suggest the possibility that anti-Gal antibody in humans is theoretically capable of partially neutralizing virions expressing α-galactosyl epitopes, such as viruses originating from non-primate mammals, prior to the first round of replication in the host. The fact that the inhibition was incomplete suggests that the α-galactosyl epitope is not an integral part of the binding site of the virion. This is supported by the fact that EEEvero virus, which lacks α-galactosyl epitopes, is infectious. It may be that the inhibition of EEE3T3 by anti-Gal is the result of steric hindrance caused by antibody molecules binding to sites on glycoprotein spikes adjacent to those important for cell attachment, thereby partially interfering with virion attachment to cellular receptors. Alternatively, since the number of α-galactosyl epitopes calculated per EEE3T3 virion represents an average figure, some of the virions in the preparation may contain more or fewer epitopes. Virions containing fewer epitopes would be neutralized to a lesser extent than those with a greater number of epitopes.

Overall, the data obtained in this study suggest that envelope glycoproteins of viruses grown in cells of non-primate mammals and New World monkeys express α-galactosyl epitopes, and that the binding of anti-Gal antibody to these epitopes may theoretically limit virus infection at the initial stage, prior to one round of replication in the host. Studies in non-human primate species, however, would be required to evaluate the potential in vivo protective capability of this antibody circulating in the intact host.

We thank Dr Robert Shope of the Yale Arbovirus Research Unit, New Haven, Conn., U.S.A. for providing the EEE virus strain, and Christine Hamilton for careful preparation of the manuscript. This work was supported by Public Health Service (NIH) grants AI 24989 to P.M.R. and GM 40205 and AG 06299 to U.G.

References


(Received 26 August 1993; Accepted 10 December 1993)