The Effect of Proteolytic Cleavage of La Crosse Virus G1 Glycoprotein on Antibody Neutralization

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SUMMARY

The envelope of the bunyavirus La Crosse contains two glycoproteins, G1 (120,000 mol. wt.) and G2 (38,000 mol. wt.). When incubated with trypsin or plasmin, the G1 glycoprotein of virus grown in cell culture was cleaved, leaving two different sized polypeptides in the envelope (67,000 and 95,000 mol. wt.). Chymotrypsin cleaved G1 leaving polypeptides of 70,000 and 100,000 mol. wt. G2, however, was not altered by these enzymes. When used in antibody neutralization studies, these proteolytically modified viruses were neutralized approximately 1 to 2 log₁₀ units in 60 min while control virus was neutralized by over 4 log₁₀ units in 20 min. Because antibody to G1, but not G2, was involved in La Crosse virus neutralization, cleavage of G1 appeared to be directly responsible for these altered kinetics of neutralization. Antibody did bind to the polypeptides remaining associated with the envelope resulting in infectious virus-antibody complexes. This indicated that a critical site in terms of antibody neutralization was removed from G1 by proteolytic enzymes.

INTRODUCTION

La Crosse (LAC) virus is a member of the family Bunyaviridae and related to the California serogroup of viruses within the family (Porterfield et al., 1975/76). The negative-strand genome of these viruses is composed of three segments of single-stranded RNA (Obijeski et al., 1976; Clewley et al., 1977). It has been shown that the medium-sized RNA codes for the two viral glycoproteins, G1 and G2 (Gentsch et al., 1980), which become an integral part of the virion envelope (Obijeski et al., 1976). Apart from this, and the known mol. wt. of 120,000 (120K) for G1 and 34K for G2 (Obijeski et al., 1976), little is known about the structure and function of the two glycoproteins. Gentsch et al. (1980) have shown that antibody raised against the medium RNA gene products (G1 and G2) does have neutralizing activity when incubated with LAC virions. This is consistent with studies of other viruses that indicate that the viral glycoproteins interact with host cells in the initial steps of virus replication (Fries & Helenius, 1979) and that when reacted with antibody made specifically to these surface glycoproteins, the virus is neutralized (Kelley et al., 1972; Dalrymple et al., 1976). The study with antibody to the medium RNA gene products of LAC, however, does not indicate which of the gene products, G1 or G2 or both, are involved in antibody neutralization. Thus, we report here experiments designed to learn more about the structure of LAC glycoproteins and which ones are involved in attachment to host cells and in antibody neutralization. Our results indicate that G1 is needed for viral infectivity and only antibody made to G1 results in neutralization of LAC virions. In addition, sites critical for antibody neutralization on G1 can be removed by certain proteolytic enzymes. Antibody molecules can bind to the part of G1 left intact after proteolysis, resulting in a high proportion of infectious virus-antibody complexes.
METHODS

Radiochemicals. L-[\textsuperscript{35}S]Methionine (420 Ci/mmol), D-[\textsuperscript{6-\textsuperscript{3}H}(N)]glucosamine, L-[\textsuperscript{6-\textsuperscript{3}H}]fucose and \textsuperscript{14}C-amino acids were purchased from New England Nuclear. \textsuperscript{3}H]Acetic anhydride (2-5 Ci/mmol) was purchased from Amersham/Searle.

Virus and cells. LAC virus was obtained from Dr L. A. Thomas, Rocky Mountain Laboratory, Hamilton, Mt., U.S.A., as a mouse brain suspension at passage level eight. Upon receipt, the virus was passaged in suckling mice and plaque-purified three times. A pool of virus was made by one more mouse brain passage and used as seed stock for all subsequent passages into continuous cell lines.

For growth of LAC in cell culture, baby hamster kidney (BHK) cells were infected by the addition of mouse brain-derived virus at an input multiplicity of 1 to 5 p.f.u./cell. After incubation for 24 to 30 h at 37 °C, the virus suspensions were clarified by centrifugation at 12100 g for 30 min, and stored at −70 °C. Second passage virus added to the appropriate cells at an input multiplicity of 1 was used in all studies reported.

To radiolabel virus, \textsuperscript{3}H]glucosamine (7 μCi/ml) or \textsuperscript{3}H]fucose (7 μCi/ml) and \textsuperscript{14}C-amino acids (1 μCi/ml) were incorporated into the media added at the end of the 1 h adsorption period. \textsuperscript{35}S]Methionine-labelled virus was prepared as follows. After adsorption of virus to cells for 1 h at room temperature, 10 ml of medium containing, by vol., 48%, Eagle's minimum essential medium (MEM), 50% phosphate-buffered saline (PBS), 1% calf serum and 1% Tryptose (Difco) was added to each 150 cm2 flask of BHK cells. After incubation for 3 h at 37 °C, this medium was removed and replaced with 10 ml of methionine-free MEM with 1% calf serum and 8 to 10 μCi/ml \textsuperscript{35}Smethionine. Virus was harvested at 30 h.

BHK cells were grown in 150 cm\textsuperscript{2} plastic tissue culture flasks for virus propagation or in 60 × 15 mm dishes for plaque assays. All cells were grown in Eagle's MEM with Hanks' salts supplemented with 10% calf serum, 0.2% Trypsone and 0.35 g/l of NaHCO\textsubscript{3}. The medium used for overlay in the plaque assay consisted of MEM with 5% calf serum, 0.2% Tryptose, 0.02 M-Tris base and 0.4% agarose. The medium was adjusted to pH 7.3 with HCl.

Virus purification. Clarified virus (10 ml) was sedimented (2 h at 20000 rev/min at 4 °C in a SW27 rotor) through a 20% (w/v) sucrose (in 0.02 M-Tris, 0.1 mM-EDTA and 10% by vol. of dimethyl sulphoxide) onto a 65% sucrose cushion. Alternatively, virus was centrifuged through a 10 to 30% linear sucrose velocity gradient for 90 min at 20 000 rev/min in a SW27 rotor. Fractions from the sucrose–sucrose interphase or peak fractions from the velocity gradient were pooled and frozen at −70 °C or immediately diluted twofold with buffer and layered onto a 20 to 65% linear sucrose gradient. Following centrifugation for 5 h under the above conditions, 1 ml fractions were collected and an aliquot assayed for infectivity. Virus was stored frozen at −70 °C until needed.

Assay of viral infectivity. Monolayers of BHK cells were washed once with Dulbecco's PBS and an inoculum of 0.2 ml added to each plate. Virus was allowed to adsorb to cells for 1 h at room temperature before the addition of 5 ml of molten overlay medium. Plates were incubated at 37 °C for 45 to 48 h when 200 ml of neutral red solution (0.02% in 0.85% NaCl) was added. Following incubation at 37 °C for 2 h, the excess neutral red solution was removed. Plates were kept in the dark at room temperature for 10 to 15 h before plaques were counted.

Virus neutralization. The virus neutralization procedure utilized was essentially that described by Habel (1969). The appropriate virus inoculum was diluted in PBS plus 0.1% bovine serum albumin (PBS–BSA) to contain 5 × 10\textsuperscript{6} p.f.u./ml. All antisera were heated at 56 °C for 30 min and diluted in PBS–BSA. Aliquots of serum and virus were equilibrated at 37 °C for 5 min and equal volumes mixed at the appropriate times. Zero-time and 60 min controls for heat inactivation were taken from a mixture of virus and normal rabbit serum. (Under these conditions, less than 10% of the virus was inactivated by heat.) Samples of 0.1 ml were taken at appropriate times and diluted immediately into 9.9 ml of cold PBS–BSA. The diluted samples were kept in an ice-bath until all had been taken, further diluted as necessary, and assayed.

Antiserum to the virus were obtained from young adult rabbits immunized with purified virus which had been grown in BHK cells or in suckling mouse brain. Animals were bled to obtain 'normal' serum samples, injected with 2 ml of purified virus (10\textsuperscript{6} p.f.u. from a sucrose cushion) intramuscularly at days 0, 8, 43 and 84 and bled for serum by cardiac puncture at days 52 and 95. The 'hyperimmune' sera obtained at day 95 were used for the experiments described in this paper.

To obtain antibody specific for LAC proteins (G1, G2 and N), purified BHK-derived virus was electrophoresed in a 10% SDS–polyacrylamide gel. The G1, G2 and N protein bands were 3 to 5 cm apart, so that each one could be cut without any possible cross-contamination. A vertical strip was cut from each gel and stained to determine the location of the virus proteins. The rest of the gel was kept at 4 °C overnight. By staining a strip of a gel which had been refrigerated overnight, it was determined that the proteins did not diffuse. With allowance for swelling of the stained gel strip, the position of each protein in the unstained gel was determined by relative migration. A 3 to 4 mm horizontal strip containing each protein was cut out and the gel ground with a mortar and pestle and mixed with PBS. Young female rabbits received weekly intramuscular injections of one of the LAC proteins. Each injection contained purified G1, G2 or N from approximately 2 × 10\textsuperscript{10} p.f.u. of virus. Rabbits were bled after six
weekly injections and sera collected and frozen at \(-20^\circ \text{C}\). Antibodies to LAC and G1 were also obtained in ascitic fluids in mice using the procedure of Qureshi & Trent (1973). Each mouse received 10^6 p.f.u. on day 1 and 10^6 p.f.u. on days 14 and 35, or G1 cut from gels (see above) on days 1, 14, 25 and 35. Injections were given intraperitoneally except those on day 35 which were given by the intravenous (LAC) or subcutaneous (G1) routes. Sarcoma 180 cells were injected intraperitoneally on day 28. Ascites fluids collected on days 40 to 45 were centrifuged to remove cells and clots and stored frozen at \(-20^\circ \text{C}\).

**Polyacrylamide gel electrophoresis.** Proteins were electrophoresed in 1.5 mm slab gels consisting of a 10% acrylamide resolving gel and a 3.5% acrylamide stacking gel (O’Farrell et al., 1973). The SDS gel and buffer solutions used are those described by Laemmli (1970). Electrophoresis was carried out at 30 V until the bromophenol blue dye reached the resolving gel, then at 30 mA until the dye reached the bottom of the gel. The gel was stained in 0-2% Coomassie Brilliant Blue in 50% methanol and 7% acetic acid for 2 to 4 h and then destained with repeated washings of a solution containing 7% acetic acid and 5% methanol. Gels were dried by vacuum and heat in an apparatus designed by Maizel (1971) and autoradiographed on Kodak RP Royal O-Mat medical X-ray film. Fluorography (Bonner & Laskey, 1974) was used to detect the radioactivity of gels containing proteins with low amounts of ^35S label.

LAC proteins were also electrophoresed in cylindrical phosphate-urea gels and analysed for radioactive content as previously described (Kingsford et al., 1980).

**Proteolytic cleavage of LAC virus glycoproteins.** A stock solution of trypsin (Sigma, type III) containing 400 µg/ml was made in Tris-buffered saline (TBS) with CaCl₂ (0.1 M-NaCl, 0.01 M-Tris-HCl pH 7.5, 0.01 M-CaCl₂) and stored at \(-70^\circ \text{C}\). The enzyme activity of this trypsin was equal to 0.68 units/µg of protein. The trypsin was diluted to 20 µg/ml in TBS-CaCl₂ and mixed with an equal volume of virus also diluted in TBS-CaCl₂. The virus–trypsin mixture was incubated for 90 min at 35°C. Calf serum was added to the virus–trypsin mixture to a final concentration of 10%. The addition of heated calf serum stopped the trypsin activity as well or better than soybean trypsin inhibitor. Control virus was treated the same way with the exception that TBS–CaCl₂ was used instead of the trypsin solution.

Although data are not presented in this paper, trypsin preparations from several sources were used with the same results: trypsin, types III and XII (Sigma), acetylated trypsin type V (Sigma), trypsin 1:250 and 1:300 (Nutritional Biochemicals), diphenylcarbamyl chloride-treated trypsin (Sigma), and TPCK–trypsin (Worthington).

Using the same protocol as for trypsin, incubations of LAC virus were also performed with equal volumes of each of the following enzymes: 20 µg/ml alpha-chymotrypsin (52.9 units/mg, Worthington), 10 µg/ml bromelain (420 units/g solid with 50% activity, Sigma), 10 µg/ml thermolysin (Calbiochem-Behring), 10 µg/ml Pronase (B grade, Calbiochem-Behring), 50 µg/ml plasminogen, 500 units/ml streptokinase, 500 units/ml plasminogen activator, or plasm in which was prepared by mixing equal volumes of plasminogen (0.1 mg/ml) with streptokinase (1000 units/ml) in a 0.1 M-citrate buffer (pH 4.5). The assay was read by eye 30 min after addition of substrate.

**Enzyme-immunosorbent assay (EIA).** Purified LAC virus or trypsinized LAC virus was diluted in 50 mM-carbonate–bicarbonate buffer pH 9.5, to give the equivalent of 10^9 p.f.u./ml. One-tenth ml of 0.1 m-phenylmethylsulphonyl fluoride (PMFS) in dry isopropanol was mixed with 2 ml of 0.1 m-trypsin (type XII, Sigma). This mixture was kept in an ice-bath for 3 h then desalted on a Sephadex G-25 column equilibrated with TBS–CaCl₂ at pH 7.5. The peak fractions were determined by absorbance at 280 nm, pooled and frozen in aliquots at \(-70^\circ \text{C}\). PMSF–inactivated trypsin had a residual enzymic activity of 7.5 × 10^-5 units/ml as compared with stock trypsin with 4.05 units/ml used at a final concentration of 10 µg/ml. Enzyme activity was measured by the spectrophotometric method of Hummel (1959).

**Haemagglutination inhibition assay.** The procedure for the haemagglutination inhibition assay was essentially that of Clarke & Casals (1958) except that formalin-treated erythrocytes (Ito & Iwasa, 1980) from day-old male chicks were used in microtitre U-well plates (Dynatech, Immulon I). Antigen was sucrose cushion-purified virus with an approximate titre of 10^10 p.f.u./ml which was diluted to give the appropriate haemagglutinating units.
Fig. 1. Polyacrylamide gel electrophoresis of LAC virions after treatment with trypsin or chymotrypsin. Autoradiograph of a gel containing (a) structural proteins of LAC virions or cleavage products of virions incubated for 90 min at 35 °C with (b) 10 μg/ml trypsin or (c) 20 μg/ml chymotrypsin is shown.

RESULTS

Effect of trypsin and chymotrypsin on LAC virus

Trypsin and chymotrypsin were initially chosen to determine their effects on the infectivity and structure of LAC virus. Purified LAC virions were incubated with 10 μg/ml trypsin or 20 μg/ml chymotrypsin at 35 °C for 90 min. This protease treatment resulted in a consistent 10-fold loss of infectivity.

To determine what effect trypsin might have on the envelope glycoproteins G1 and G2, purified [35S]methionine-labelled LAC virus was incubated with trypsin or chymotrypsin, immediately boiled for 2 min in gel sample buffer and electrophoresed in 10% SDS-polyacrylamide gels along with untreated virus. As seen in Fig. 1, LAC virus has four structural proteins: L (170K), G1 (120K), G2 (38K) and N (23K). These mol. wt. values are in good agreement with those assigned to proteins of bunyaviruses by Obijeski et al. (1976). With trypsin treatment, the majority of the G1 glycoprotein had disappeared and two major polypeptides of
Proteolytic cleavage of LAC glycoprotein 2151

95K and 67K were apparent (Fig. 1). Small peptides below N and at the dye front were also observed. When trypsinized LAC was pelleted or centrifuged through a gradient before electrophoresis, the only G1 cleavage products remaining with the virions were the 67K and 95K polypeptides. Cleavage products of chymotrypsin-treated virus were similar to those seen for trypsin, except that both of the peptides were larger: 100K and 70K. The other structural proteins, L, G2 and N, were not affected by either enzyme. It thus appeared that trypsin and chymotrypsin cleaved only the G1 glycoprotein in situ and this cleavage resulted in two smaller major components.

Distribution of oligosaccharides on G1

It has been reported that both the G1 and G2 glycoproteins of LAC are of the type 'A' serum glycoproteins containing glucosamine, galactose, fucose, mannose and neuraminic acid (Vorndam & Trent, 1979). As part of G1 is proteolytically removed by trypsin, it was of interest to determine whether all of the sugar moieties would be found on the trypsin-sensitive part or distributed along the G1 glycoprotein. Thus, control and trypsinized [3H]glucosamine- and 14C-amino acid-labelled LAC were electrophoresed in phosphate–urea gels. As shown in Fig. 2(a), the 3H label migrated with the G1 and G2 glycoproteins as expected. When LAC was trypsinized, only 55 to 60% of the label originally migrating with G1 remained, but was now found migrating where the 67K and 95K polypeptides would be expected (Fig. 2b). The amount of 3H label migrating with G2 was not altered by trypsinization. When this experiment was repeated using [3H]fucose instead of [3H]glucosamine, the relative amounts of label in G1, G2 and the G1 cleavage products were the same as shown for the [3H]glucosamine-labelled virus.

Proteolytic cleavage of LAC glycoproteins by other proteases

Because trypsin and chymotrypsin produced such unique cleavage products of the G1 glycoprotein, it was of interest to determine what effect other proteolytic enzymes might have on the glycoproteins. Thus, 35S-labelled LAC was incubated with various proteases and electrophoresed in a 10% SDS–polyacrylamide gel. Each virus sample was also assayed for residual infectivity. As seen in Fig. 3, three patterns of proteolytic action on the G1 glycoprotein were observed, i.e. complete cleavage, no cleavage or partial cleavage. Bromelain and Pronase completely digested G1 and reduced the infectivity of 10^8 p.f.u. by over 6 log\_10 units. Thermolysin had no visible proteolytic effect on the glycoproteins or on infectivity. Although not shown, plasminogen, plasminogen activator and streptokinase, like thermolysin, had no effect. Yet these enzymes were active as determined by appropriate assays. Plasmin, trypsin and chymotrypsin only partially cleaved the G1 glycoprotein, resulting in relatively little loss in infectivity (approx. 1 log\_10). Like trypsin, plasmin cleaved G1 to give two polypeptides of 95K and 67K. As shown before, chymotrypsin cleaved G1 resulting in polypeptides of 100K and 70K. Because these protease-treated samples contained all of the degradation products and not just those remaining with the virions, additional peptides are present in the gel.

The G2 glycoprotein was resistant to degradation by all the proteolytic enzymes tested except bromelain. It should be noted that Pronase completely cleaved the G1 molecule, but left G2 intact (see Fig. 3). Yet this virus was non-infectious. This implicates G1 in attachment of LAC virions to host cell receptors and/or penetration into the cell. This also indicates that G2 alone is not capable of this function.

Neutralization of LAC virions

The neutralizing capacity of antibody made to infectious LAC or to G1, G2 and N proteins was demonstrated in an assay of the kinetics of neutralization. As seen in Fig. 4, anti-LAC and anti-G1 gave similar degrees and rates of neutralization while antibody to G2 or N did not neutralize the virus. The presence of specific antibody molecules in all four antisera was demonstrated in an immunofluorescence assay using whole BHK cells infected with LAC virus. The titres of anti-LAC, anti-G1, anti-G2 and anti-N sera were 1:64, 1:16, 1:16 and 1:16, respectively. Titres of normal sera obtained by pre-bleeding the same rabbits were all less than 1:4. In addition, both anti-G1 and anti-G2 sera had haemagglutination inhibition titres of 1:40.
L. KINGSFORD AND D. W. HILL

Fig. 2. Polyacrylamide gel electrophoresis of \([3H]\)glucosamine- and \(^{14}\)C-amino acid-labelled virus. LAC virus was grown in BHK cells with 7 \(\mu\)Ci/ml \([3H]\)glucosamine (©) and 1 \(\mu\)Ci/ml \(^{14}\)C-amino acids (●) and then purified. Untreated virus (a) or virus incubated with trypsin (b) as described in the legend of Fig. 1 was electrophoresed in a 10\% phosphate–urea cylindrical gel. Each gel was cut into 1 mm fractions and analysed for radioactivity by liquid scintillation counting.

while anti-N serum was negative. In the neutralization assay shown in Fig. 4, the anti-LAC serum was diluted 1:200 and the other three antisera diluted 1:10. G2 antiserum did not neutralize the virus even when used undiluted. Anti-LAC and anti-G1 antibodies obtained in mouse ascitic fluid gave the same results as the rabbit antisera. These data indicate that only the G1 glycoprotein contains sites which react with antibody to result in neutralization.
Proteolytic cleavage of LAC glycoprotein

Fig. 3. Polyacrylamide gel electrophoresis of LAC virions after incubation with various proteolytic enzymes. The autoradiograph is of a 10% SDS gel containing (a) structural proteins of LAC virus or cleavage products of virions incubated at 37 °C for 60 min with (b) 10 µg/ml trypsin, (c) 20 µg/ml chymotrypsin, (d) 10 µg/ml bromelain, (e) 10 µg/ml thermolysin, (f) 10 µg/ml Pronase or (g) 5 µg/ml plasmin.

Effect of trypsin and chymotrypsin treatment on neutralization kinetics of LAC virus

After 5 × 10⁷ p.f.u. of LAC had been incubated with 10 µg/ml (4.05 units/ml) trypsin or 20 µg/ml chymotrypsin for 90 min at 35 °C, calf serum was added to stop the enzymic activity. The titre of each virus preparation was consistently 1 log₁₀ lower at this point. Enzyme-treated virus or 5 × 10⁶ p.f.u. of untreated LAC were equilibrated at 37 °C for 5 min and anti-LAC antibody (rabbit or mouse) immediately added for a neutralization assay. Results shown in Fig. 5 indicate that proteolytic treatment of virus resulted in virions that reacted very differently with antibody. The rate of neutralization was greatly decreased, resulting in a much larger persistent fraction as compared with untreated virus. Increasing the concentration of antibody added to virus which had been pre-incubated with trypsin or chymotrypsin did not increase the amount of neutralized virus.

Controls showed that calf serum effectively inhibited the effect of trypsin on the virus, as no further reduction in viral infectivity occurred over a 2 h period. In addition, antibody pre-incubated with 10 µg/ml of trypsin for 60 min at 37 °C retained its full activity as demonstrated by its ability to neutralize over 4 log₁₀ units of LAC virus in 20 min. This indicated that any residual trypsin would not be deleterious for the antibody molecules during the kinetics of neutralization assay.

To ensure that the trypsin degradation products freed from G1 or trypsin itself did not interfere with the antibody neutralization reaction, LAC virus was incubated as usual with [³H]acetylated trypsin that had been labelled with [³H]acetic anhydride by the method of Montelaro & Rueckert (1975). The virus–enzyme mixture was then layered onto a 20 to 65% sucrose gradient and centrifuged to equilibrium. All of the ³H-labelled trypsin remained at the
Fig. 4. Neutralization kinetics of LAC virus. Aliquots of purified LAC virions containing $5 \times 10^6$ p.f.u. were mixed with equal volumes of diluted antiserum containing antibodies to LAC virus ($\bigcirc$) or to polyacrylamide gel-purified G1 ($\bigtriangleup$), G2 ($\triangleleft$) or N ($\bigtriangledown$) proteins. Samples were taken and assayed as described in Methods.

Fig. 5. Neutralization kinetics of virus preincubated with trypsin, PMSF-inactivated trypsin or chymotrypsin. Virus was preincubated with a final concentration of $10 \mu$g/ml PMSF-trypsin (equal in activity to $7.5 \times 10^{-5}$ units/ml) ($\bigtriangleup$), $10 \mu$g/ml trypsin (4.05 units/ml) ($\trianglehat$) or $20 \mu$g/ml chymotrypsin ($\bigtriangledown$) for 90 min at 35 °C. After addition of 10% calf serum, antiserum containing anti-LAC antibody was added for studies of the kinetics of neutralization. Untreated LAC ($\bigcirc$) was used in the neutralization reaction as a control.

Trypsin inactivated with PMSF was used as a control to determine whether the cleavage of G1 was directly related to the altered neutralization. An equivalent of $10 \mu$g/ml PMSF–trypsin contained $7.5 \times 10^{-5}$ units/ml of activity. Virus was first reacted with this inactivated enzyme preparation, then pelleted and electrophoresed in polyacrylamide gels to determine whether the G1 glycoprotein was cleaved. This preparation of PMSF–trypsin left most of the G1 intact, but some cleavage products could be seen.

Because the PMSF–trypsin-treated virus did have some intact G1 glycoprotein left, as observed in gels, treatment of virus with this ‘inactivated’ enzyme should give greater levels of neutralization than trypsin-treated virus. As seen in Fig. 5, neutralization of PMSF–trypsin-treated virus gave neutralization kinetics more like the control virus.

These experiments indicate that trypsin does not interfere with neutralization by binding to virus or by hydrolysing antibody molecules. They also suggest that the degree of neutralization of LAC virus depends on the amount of intact G1 present in the envelope.

**Binding of anti-G1 antibody to trypsinized virus**

To determine whether antibody binding sites were still present on the trypsin-resistant part of G1, mouse anti-LAC and anti-G1 were titrated by EIA using LAC and trypsinized LAC as
antigens. Anti-G1 and anti-LAC antibody did bind to trypsinized virus. In fact, the titre of each antiserum was 1:800 on both untreated and trypsin-treated LAC antigen.

**DISCUSSION**

Incubation of LAC virions with trypsin, plasmin or chymotrypsin resulted in the loss of approximately 1 log₁₀ unit of infectivity and the cleavage of the G1 glycoprotein to two polypeptides. This unique cleavage of G1 but not of G2, together with the high degree of infectivity remaining, allowed us to learn certain things about the structure and biological properties of these two glycoproteins. The infectivity of LAC virus seems to be dependent upon the presence of the G1 glycoprotein. When it was removed by Pronase, leaving only G2 intact, the virions were non-infectious. Thus, G1 is probably the glycoprotein needed for attachment to host cell receptor sites and/or for penetration and uncoating.

Antibody raised against the G2 glycoprotein did exhibit haemagglutination inhibition activity. This is probably due, however, to a steric blocking of haemagglutinin sites on G1 rather than the presence of receptor sites for erythrocytes on G2. A similar situation has been described for influenza virus by Webster et al. (1982), who proposed that the anti-haemagglutination activity of antibody to the neuraminidase glycoprotein was due to a steric blocking of sites on the haemagglutinin glycoprotein.

The neutralization data together with the infectivity of trypsinized virus indicate that there are multiple recognition sites for host cell receptors and multiple antibody-binding sites on G1. When 25K to 53K mol. wt. of protein are removed, a significant amount of infectivity remains, indicating the presence of cell receptor sites. Anti-G1 antibody binds to the remaining 67K and 95K polypeptides but does not neutralize the virus. In addition, anti-G2 and anti-N antibodies do not neutralize the virus, the critical sites for antibody neutralization as well as some host cell receptor sites must be on the part(s) of G1 removed by trypsin. The presence of multiple antibody-binding sites as well as non-neutralizing antibody-binding sites has been shown for other viruses such as Sindbis (Chanas et al., 1982), measles (ter Meulen et al., 1981) and mouse mammary tumour (Massey & Schochetman, 1981) viruses by the use of monoclonal antibodies.

The study with proteolytic enzymes raises questions about how G1 and G2 are oriented in the lipid bilayer of the envelope. At first it was thought that trypsin might have cleaved a single G1 glycoprotein molecule into two pieces (67K and 95K). However, the total mol. wt. of the two peptides would be equal to 162,000, much higher than that of G1. These two cleavage products migrated similarly in reducing or non-reducing gels (L. Kingsford & D. W. Hill, unpublished data), indicating they are not disulphide-linked. Thus, cleavage of G1 does not appear to be analogous to the cleavage of glycoproteins of viruses such as influenza (Skehel & Waterfield, 1975), mouse mammary tumour (Sheffield et al., 1976), and Sendai (Homma & Ohuchi, 1973) viruses. When LAC virus was treated with chymotrypsin, two cleavage products were also found, both larger than the polypeptides resulting from trypsin treatment. In addition, trypsin and chymotrypsin degrade part of G1 into small peptides which are found between N and the dye front, or at the dye front (Fig. 1 and 2b). These data indicate that cleavage of two different G1 molecules has to occur. Perhaps two G1 molecules or G1 and G2 interact in such a way that one of two trypsin- (or chymotrypsin-) susceptible sites are exposed on different G1 molecules. G2 was highly resistant to proteolytic enzymes either because of its association with and protection by G1 and/or its association with the lipid bilayer.

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