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Antigenic Comparison of Equine Arteritis Virus (EAV) and Lactic Dehydrogenase Virus (LDV); Binding of Staphylococcal Protein A to the Nucleocapsid Protein of EAV

By

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With 2 figures and one table

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Introduction

Equine arteritis virus (EAV) causes a febrile infection in horses which is usually accompanied by oedema and petechiae and may induce abortion in pregnant mares (1). Lactic dehydrogenase virus (LDV) is ubiquitous in wild and laboratory mice where it causes a lifelong infection accompanied by viraemia. Infected animals show no signs of illness and the infection goes unnoticed. It has become clear, however, that LDV may cause more pronounced pathological effects in mice (2) and even polioencephalomyelitis (3).

The genome of both viruses is an infectious single-stranded RNA molecule of about 4×10^6 mol. wt. (4, 5). A similar protein composition of EAV and LDV has been reported (6, 7, 8); the virions contained a nucleocapsid protein (VP 1) with a molecular weight of 12—15 K, a non-glycosylated membrane protein (VP 2) of 14—18 K daltons and a glycosylated membrane protein (VP 3) of 21—44 K daltons. This latter variation was due to the heterogenous migration (24—44 K) during SDS-PAGE of VP 3 of LDV (8). Both viruses have been classified as non arthropod-borne togaviruses (9, 10), but they do not fit into one of the established genera of the family Togaviridae (11).

The genera alphavirus (12) and flavivirus (13) of the Togaviridae show cross-reactions in serology and this antigenic relationship is mirrored by a close resemblance in the polypeptide patterns. Within each genus a further subdivision in clusters of related viruses can be made on the base of cross-neutralization and haemagglutination-inhibition tests (14). Determinants on the envelope (glyco)proteins are important in the latter case (13, 15), whereas the nucleocapsid proteins contain group-reactive determinants, a situation analogous to e. g. influenza virus A (16) and rhabdoviruses (17).

Since EAV and LDV seemed to have a similar protein composition (6, 7), we have investigated whether they are also antigenically related. An interesting and unexpected finding in the course of this study was that VP 1 of EAV binds directly to staphylococcal protein A.

Material and Methods

Cells and viruses. Growth of BHK 21, Vero and RK 13 cells and propagation and titration of EAV on Vero cells have been described before (4, 6, 18). LDV stocks were prepared in Swiss mice infected intraperitoneally with 10^5 ID₅₀ units. Infectious plasma was collected 24 h later and stored at -70°C . LDV titres were estimated by titration in mice, as described previously (19). Labelled EAV was isolated from the medium of infected BHK 21 cells (200 PFU/cell) which were incubated from 5 to 20 h p.i. with ^{35}S -methionine (25 $\mu\text{Ci}/\text{ml}$, 800 Ci./mmol., The Radiochemical Centre, Amersham, Bucks., England) described previously (20). Labelled LDV was obtained from 24 h old macrophage cultures (21) infected with infectious mouse plasma (500 ID₅₀ units/cell) and labelled from 4 to 16 h p.i. at 37°C with 50 $\mu\text{Ci}/\text{ml}$. ^3H -leucine (136 Ci./mmol.) or ^{35}S -methionine. Culture media were clarified and layered over a 10–60 % sucrose gradient made up in TES-buffer (0.02 M Tris-HCl, 1 mM EDTA and 0.1 M NaCl, pH 7.4) supplemented with 0.1 % FCS. Gradients were run for 6 h at 5°C at 27,000 rpm. Infectious virus peaks were found at a density of 1.14 to 1.15 g./ml.

Sera. Antibodies against EAV were raised in rabbits, mice, hamsters and in a horse. Rabbits were injected subcutaneously with a mixture of 1 ml. infectious tissue culture fluid from RK 13 cells (10^7 PFU) and 1 ml. of complete Freund's adjuvant. Two and 4 weeks later the procedure was repeated with mixtures containing Freund's incomplete adjuvant and serum was collected at six weeks. Mice were immunized following the same protocol, using 0.3 ml. volumes of EAV grown in BHK 21 cells. Hamster anti-EAV was prepared as described (18) and horse anti-EAV was obtained from a mare that had recovered from an experimental infection. Anti-LDV serum came from mice chronically infected with LDV for at least 4 months. It was heated for 60 min at 58°C to inactivate endogenous virus. Anti-species IgG sera were obtained from Miles-Yeda Ltd., Rehovot, Israel.

Virus neutralization test. Sera were tested for anti-EAV activity by incubating EAV-dilutions in PBS-DEAE-2 % calf serum with antiserum for 1 h at 37°C , before 0.5 ml. of guinea pig complement was added. After an additional 30 min at 37°C samples of 0.2 ml, corresponding to 80–120 PFU of input virus, were assayed for infectivity. Sera were tested for anti-LDV activity by incubating 10^4 ID₅₀ units of LDV with antiserum for 3 h at 37°C ; infectious immune complexes were neutralized by adding 0.15 vol. of goat anti-rabbit or goat anti-mouse serum, after which the incubation was continued for 1 h. Samples containing about 150 ID₅₀ units of neutralized virus were titrated in mice.

Immunofluorescence test. Immunofluorescence with FITC-labelled protein A (50 $\mu\text{g}/\text{ml}$. in PBS; Pharmacia, Uppsala, Sweden) was done on acetone-fixed coverslip cultures of infected and mock infected cells (18).

Immunoprecipitation of viral proteins. Virus was lysed in TES-buffer, containing 0.5 % Triton X-100 and 0.5 % 1,5-naphthalenedisulphonate disodium salt (final concentrations). Reaction mixtures containing 150 μl . of disintegrated radioactive virus were incubated for 16 h at 4°C with 20 μl . of antiserum, which had been centrifuged for 10 min at $10,000 \times g$ before use. Non-specific precipitation was reduced by adding 3 M KCl to a final concentration of 0.5 M immediately before 400 μl . of a 10 % (v/v) suspension of *Staphylococcus aureus* suspension in TES-buffer-0.1 % Triton X-100 (24) was added. After 30 min at 4°C immune complexes adsorbed to *S. aureus* were spun down (2 min, $10,000 \times g$). The pellet was washed three times with TES-buffer-0.1 % Triton X-100 and prepared for electrophoresis.

Alternatively, the optimal concentrations of rabbit serum directed against hamster IgG (40 μl .) or goat serum directed against rabbit IgG (100 μl .) were added instead of *S. aureus* and these mixtures were incubated at 4°C for 24 h. Immune precipitates were washed twice with TES-buffer-0.1 % Triton X-100 and analysed by SDS-polyacrylamide slab gel electrophoresis (22, 23).

Table 1
Lack of cross-neutralization between EAV and LDV

serum	neutralization titre	
	EAV	LDV
mouse anti-LDV	< 20	100
rabbit anti-EAV	1000	< 20
mouse anti-EAV	200	< 20
horse anti-EAV	1000	< 20

Neutralization titres are expressed as the reciprocal of the highest antiserum dilution, which caused a 90 % reduction (EAV) or complete inactivation (LDV) of infectivity.

Results

Cross-neutralization between EAV and LDV

To detect a possible antigenic relationship between EAV and LDV on the level of the viral surface, neutralization experiments with homologous and heterologous antisera were done (Table 1). Anti-LDV serum from chronically infected mice, heat-inactivated to destroy infectious immune complexes, did neutralize LDV. The infectivity of EAV tested in this system, however, remained unchanged. Also several potent anti-EAV sera were unable to neutralize LDV.

Fig. 1. Analysis by SDS-PAGE of immune precipitates of detergent-treated ^{35}S -methionine-labelled EAV and LDV; direct binding of EAV's VP1 to *S. aureus*

A) EAV protein markers (1); immunoprecipitate with rabbit anti-EAV serum and *S. aureus* (2); precipitate with *S. aureus* (3). B) EAV protein markers (1); immunoprecipitate with rabbit anti-EAV serum and goat anti-rabbit IgG serum (2); immunoprecipitate with hamster anti-EAV serum and rabbit anti-hamster IgG serum (3). C) LDV protein markers (1); immunoprecipitate with hamster anti-EAV serum and rabbit anti-hamster IgG serum (2); immunoprecipitate with normal hamster serum and rabbit anti-hamster IgG serum (3)

Immunoprecipitation of EAV- and LDV-structural proteins; direct binding of VP1 to protein A

To make an overall antigenic comparison immunoprecipitation experiments were carried out in which disintegrated radioactive EAV or LDV was precipitated with anti-EAV antiserum. Using a rabbit anti-EAV serum, VP1 and VP2 of EAV were precipitated (Fig. 1 A, lane 2). VP3 became visible only after extended fluorography.

Surprisingly, when a second antibody was used instead of *S. aureus* no precipitation of VP1 was observed (Fig. 1 B, lane 2). The explanation was that our rabbit anti-EAV serum did not contain antibodies directed against VP1, but VP1 does bind directly to *S. aureus* in the absence of antibodies (Fig. 1 A, lane 3). This was confirmed by an

experiment in which FITC-labelled protein A was incubated with EAV-infected and mock-infected BHK 21 cells; the infected cells showed distinct cytoplasmic fluorescence, whereas the mock-infected cells were negative (Fig. 2). VP 1 of EAV also bound directly to protein A-Sepharose (not shown). In the procedure finally adopted a hamster anti-EAV serum was employed in conjunction with a second antibody directed against hamster IgG. Using this method, more than 70 % of the radioactive protein, predominantly VP 1 and VP 2, was precipitated (Fig. 1 B, lane 3). Using the same conditions only 2 %, a background level (the same amount was found with normal hamster serum) of ^{35}S -methionine labelled LDV was precipitated (Fig. 1 C, lanes 2 and 3). Anti-LDV sera caused only insignificant homologous precipitation and could therefore not be used in EAV anti LDV immunoprecipitation experiments.

Fig. 2. Binding of FITC-labelled protein A to EAV-infected BHK 21 cells. The photomicrographs show infected cells (left) and mock-infected cells (right). The cells were incubated at 40 °C for 8 h after infection and then fixed with acetone and stained

Discussion

Our data indicate that EAV and LDV are not antigenically related; this conclusion is based on cross-neutralization studies with heterologous antisera and radioimmune precipitation experiments with antiserum to the structural proteins of EAV in which no cross-reactivity was found with LDV proteins. Especially important is the lack of cross-reactivity between the nucleocapsid proteins, which carry antigenic determinants with broader (subgroup or genus) specificity (13, 15). In all cases where an antigenic relationship has been found between the nucleocapsid protein of togaviruses it was readily detectable.

The data support the view that EAV belongs to a separate genus of the Togaviridae 9a comparable taxonomic position to that of rubella virus (25). Also our recent data on the replication mechanism of EAV (20) which show that it appears to be completely different from that of the alpha- or flaviviruses support this view.

More insight into the replication mechanism of LDV, of which now it is known only that in infected cells a heterologous RNA population (10—40 S) is found in addition to the genome sized 48 S RNA (26), could still reveal a similarity in the mode of replication of LDV and EAV.

Staphylococcal protein A is widely used as an immunological reagent since it binds specifically and with high affinity to IgG species. To our knowledge there are no reports of specific binding of protein A to proteins other than immunoglobulins. The binding of VP 1 of EAV to protein A is therefore exceptional. In addition to the positive immunofluorescence assay on EAV-infected cells with FITC-labelled protein A, this reaction could be used to precipitate VP 1 from infected cells with *S. aureus* in the absence of anti-EAV serum.

Summary

The proteins of equine arteritis virus (EAV) and lactic dehydrogenase virus of mice (LDV), both non-arthropod-borne togaviruses, are of very similar molecular weight, which might indicate a common evolutionary origin. Cross-neutralization tests and electrophoretic analysis of heterologous radioimmunoprecipitates in polyacrylamide gels showed, however, that there was no antigenic relationship at the level of the envelope proteins or nucleocapsid proteins. During this study it was found that VP 1, the nucleocapsid protein of EAV, binds directly to staphylococcal protein A.

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Zusammenfassung

Die Eiweißanteile des equinen Arteritisvirus (EAV) und des Laktatdehydrogenase-Virus (LDV) der Maus besitzen etwa gleiches Molekulargewicht, was entwicklungs-geschichtlich gesehen einen gemeinsamen Ursprung vermuten läßt. Kreuzneutralisationstests und die Untersuchung heterologer Radioimmunopräzipitate in der Polyacrylamid-Gel-elektrophorese zeigten jedoch, daß keine Antigenverwandtschaft auf der Basis der Hüll- oder Nukleokapsidproteine besteht. Im Verlauf dieser Untersuchungen wurde festgestellt, daß VP 1, das Nukleokapsidprotein von EAV, direkt an das Staphylokokken-Protein A bindet.

Résumé

Comparaison antigénique du virus de l'artérite équine (EAV) et du lactate-déhydrogénase-virus (LDV); liaison de la protéine A staphylococcique à la protéine de nucléocapside de EAV.

Les parties protéiques du virus de l'artérite équine (EAV) et du lactate-déhydrogénase-virus (LDV) de la souris possèdent un poids moléculaire à peu près égal, ce qui laisse supposer une origine commune du point de vue de leur développement dans le temps. Des tests de neutralisation croisés et l'examen de précipités radioimmunologiques hétérologues dans l'électrophorèse sur gel de polyacrylamide ont montré qu'il n'y avait aucune parenté antigénique sur la base des protéines de l'enveloppe ou de la nucléocapside. Il a été établi au cours de ces recherches que la protéine de nucléocapside d'EAV (VP 1) se lie directement à la protéine A staphylococcique.

Resumen

Comparación antigénica del virus de la arteritis equina (EAV) y del virus de la dehidrogenasa láctica (LDV); fijación de la proteína estafilocócica A a la proteína del nucleocápside del EAV

Las porciones proteicas del virus de la arteritis equina (EAV) y del virus de la lactatodehidrogenasa (LDV) del ratón poseen aprox. el mismo peso molecular, lo cual, ontogénicamente visto, hace sospechar un origen conjunto. Sin embargo, las pruebas de neutralización cruzada y el estudio de los radioinmunoprecipitados heterólogos en la electroforesis en poliacrilamida-gelosa mostraron que no existe ningún parentesco antigénico sobre la base de proteínas de envoltura o de nucleocápside. En el curso de esta investigación se comprobó que el VP 1 fija directamente la proteína del nucleocápside del EAV a la proteína estafilocócica A.

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