Rabies Group-Specific Ribonucleoprotein Antigen and a Test System for Grouping and Typing of Rhabdoviruses

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Received for publication 30 November 1972

Cell-associated ribonucleoprotein (RNP) was isolated from BHK-21 cells infected with several strains of rabies and rabies-related viruses. The RNP-antigen from rabies and related viruses induced the formation of complement-fixing, precipitating, and immunofluorescent antibodies, and proved to be the group-specific antigen common to all rabies viruses. Antigens of the envelope which induce virus-neutralizing antibodies are apparently determinative for the serotype of a virus as evidenced by two-way neutralization tests. A combination of these methods seems to be a useful approach to the serological grouping and typing of rhabdoviruses.

Disruption of rabies virus particles and separation of the components yielded preparations which contained the nucleocapsid and envelope constituents (16, 19). These preparations were not sufficiently pure to obtain clear-cut serological results. For example, ribonucleoprotein (RNP) preparations still induced the formation of virus neutralizing antibody, apparently due to adhering envelope components (16, 23). We have succeeded in isolating RNP from virus-infected cells which serologically proved to be a single antigen free from contaminating envelope constituents. Moreover, this isolated RNP is shown to be the group-specific core antigen of the rabies group of rhabdoviruses. The isolation procedure and the properties of cell-derived rabies virus RNP are described in this paper.

MATERIALS AND METHODS

Virus strains. Virus strains used for isolation of cell-associated RNP were the clone-purified Flury high-egg passage (HEP) strain and the challenge virus standard (CVS) strain of rabies virus. Mokola virus (MOK), formerly named Ibadan shrew virus 27377 (17), and Lagos bat virus (LBV) were obtained from R. E. Shope, New Haven, Conn., as early mouse-passage material and adapted to tissue culture.

Further rabies strains tested were the plaque-purified ERA strain obtained from T. J. Wiktor, Philadelphia, Pa.; the field strains W239/69, W37/71, W48/71, W56/71, and W131/71 isolated from *Microtus arvalis* in southern Germany (L. G. Schneider and Ute Schoop, 1973. Rabies-like viruses. Symp. Series Im-

munobiol. Scand. in press). Two bat isolates, SVF (22) and Stade; two fox isolates, S-1763 and Frankfurt 2; two human isolates, SVM₁ and SVM₂; and Nigerian horse virus (13) were obtained from J. S. Porterfield, Great Britain. The rodent, bat, fox, and human isolates were taken from the strain collection of our institute and were used as early-mouse passage material.

Other rhabdoviruses tested in this study were vesicular stomatitis virus (VSV) strain New Jersey, VSV-Indiana, VSV-Brazil, VSV-Argentina, VSV-Cocal, Piry, Chandipura, Flanders, Kern Canyon, and Mt. Elgon Bat virus contributed by R. E. Dierks, Ames, U.S.A.; *Microtus* 1056 virus obtained from H. Johnson, Berkeley, Calif.; and Bovine Ephemeral Fever virus obtained from C. A. Mims, Canberra, Australia. Marburg virus was tested at the Institute of Hygiene, University of Marburg, with the assistance of S. Szlenska. Lymphocytic choriomeningitis (LCM) virus, strain Armstrong, was obtained from F. Lehmann-Grube, Hamburg, Germany.

Virus production and purification. The viruses (except the rabies field strains) were propagated in roller-cultures of BHK-21 C13 cells (21). Culture conditions and media were described before (15). Infectious cell culture fluids and infected cells were harvested between 72 and 96 h postinfection and were separated by low-speed centrifugation. The virus from supernatant fluids was concentrated and purified by batch adsorption onto and elution from aluminum phosphate gel as formerly described (15), followed by rate-zonal centrifugation in a linear sucrose gradient (10-48% wt/vol) at 24,000 rpm for 1 h.

Infectivity assay. Viruses were titrated in three-day old suckling mice (HEP, LBV), or in weanling mice (CVS, MOK). Virus dilutions were made in

distilled water containing 2% (vol/vol) horse serum and were inoculated intracerebrally (0.02 or 0.03 ml) in five animals per dilution. The mean lethal dose (LD_{50}) was calculated using the method of Kärber (7).

Solutions. STE-buffer, pH 7.4, is 0.15 M sodium chloride, 0.01 M Tris-hydrochloride and 0.001 M ethylenediaminetetraacetic acid. PBS is phosphate buffered saline.

Protein determination. Proteins were determined by the Lowry method (8) with bovine serum albumin as a reference standard.

Isolation of RNP from intact virus particles. Virus-derived RNP was obtained by disruption of purified rabies virus with 1% (vol/vol) Nonidet P-40 (NP-40), followed by equilibrium centrifugation in CsCl as described before (16). The detergent to protein ratio (vol/wt) was 10.

Procedure for isolation and purification of intracellular RNP. Virus-infected and noninfected cells were trypsinized, washed twice with cold PBS, and frozen. After thawing, about 4×10^8 cells were suspended in 200 ml of STE buffer. After the addition of 20 ml of a 10% (vol/vol) solution of NP-40, the suspension was incubated for 4 min at 37 C, then immediately cooled in an ice-bath and shaken for 30 min at 4 C. The homogenate was centrifuged at $1,000 \times g$ for 10 min. The pellet containing nuclei and cell membranes was discarded and the supernatant fluid was extracted with 30% (vol/vol) trichloro-trifluoroethane (Arcton, p. a., Serva, Heidelberg, Germany). After centrifugation for 10 min at $2,200 \times g$ the aqueous phase was once more extracted by arcton and centrifuged as above followed by sedimentation through CsCl of the density of 1.30 on a cushion of CsCl of the density 1.40 g/ml.

Sephacrose gel filtration. For column gel filtration Sepharose 4 B (Pharmacia, Uppsala, Sweden) was equilibrated with STE buffer. Column size was 5 by 55 cm. Assay material was eluted by ascending flow with STE buffer and fractions were screened at optical density ($OD_{260}^{1\%}$) using a LKB constant flow ultraviolet recorder (Uppsala, Sweden).

Polyacrylamide gel electrophoresis. Acrylamide gel electrophoresis was carried out as described by Bolognesi and Bauer (1) with the following modifications. The protein samples were treated with sodium dodecylsulfate (SDS) and dithiothreitol (DTT) to give a final concentration of 1.0% and 0.1 M, respectively, and heated for 1 min at 100 C. Samples of 50 μ g containing approximately 100 μ g of protein were applied to the gels.

Sedimentation experiments. Equilibrium centrifugation was carried out in CsCl, with the initial density of 1.33 as described before (15). Zonal centrifugations using sucrose gradients 15 to 30% (wt/vol) were carried out in a Spinco model L ultracentrifuge using the SW25 rotor at 24,000 rpm for 2.5 h at 4 C. Sedimentations through a CsCl solution of the density 1.30 g/ml onto a cushion of CsCl (density 1.40 g/ml) were done with the SW25 rotor at 24,000 rpm for 4 h. The sedimentation coefficient $s_{20,w}$ was estimated according to the tables of McEwen (11) using foot-and-mouth disease virus as the sedimentation marker.

Buoyant density determination. The visible band of RNP from equilibrium centrifugation was diluted with CsCl of the density 1.33 resulting in an optical density of $OD_{260}^{1\%} = 0.1$. A 12-mm Kel F-centrifuge cell of the analytical ultracentrifuge, Beckman Instruments, model E, was filled with this solution and centrifuged at 44,770 rpm for 18 h at 25 C using the An H rotor. Photographs were taken with Schlieren and ultraviolet optics.

The refractive index of the CsCl solution was determined with an Abbé-refractometer (Zeiss) and the buoyant density was calculated according to the equation of Schildkraut et al. (14) as follows: $\rho_{25\text{ C}} = 10.860 \times n_D - 13.4974$.

Electron microscopy. Dialyzed material from isopycnic centrifugations was negatively stained with 1% (wt/vol) uranyl acetate or 1% (vol/vol) phosphotungstic acid and examined immediately in a Siemens Elmiskop 101 at an instrumental magnification of $\times 60,000$.

Production of antiserum. Anti-RNP sera were prepared by immunization of rabbits with cell-derived purified RNP. Virus concentrated by adsorption onto and elution from aluminum phosphate gel and purified by sucrose gradient centrifugation was used to produce antiviral sera. BHK-21 cell extract was obtained by the freeze-thawing of cells in PBS for production of anti-BHK serum. Immunization schedule: three injections were applied during the first week, one intraperitoneally with complete Freund adjuvant followed by one intradermal and one subcutaneous injection without adjuvants. A subcutaneous booster inoculation was given 4 weeks later. Blood was obtained by heart puncture and tested after thermal inactivation (three times 30 min, 56 C) for specific activity.

Agar gel precipitation. The test has been described before (20).

Immunoelectrophoresis. The formerly described technique (10) was used which includes 1% (wt/vol) special Noble agar (Difco) in veronal buffer, pH 8.6, at an ionic strength of 0.05. Proteins were stained with amido black, nucleic acids with acridine orange (3). The "reversed" immunoelectrophoresis technique (9) was used to identify the type of antibody which caused precipitation. By this technique the serum is electrophoresed first and then allowed to diffuse towards the antigen.

CF test. The Laboratory Branch complement fixation method as adapted to microplates (2) was used for block titration of sera and antigens. The reciprocal of the highest dilution giving 25 to 30% hemolysis for a volume of 0.025 ml was taken as the complement fixation (CF) activity of the sample.

FA procedures. The direct immunofluorescent (FA) technique (L. G. Schneider, Chapter 6, Laboratory techniques in rabies. WHO monogr., 3rd ed., in press) was employed using infected coverslip cultures or brain smears as a source of antigen.

SN test. Serum neutralization (SN) tests were performed in suckling or weanling mice as indicated for infectivity tests. Increasing serum dilutions were tested against constant virus dilutions. Equal vol-

umes of serum and virus were mixed, incubated at 37 C for 90 min, and kept in crushed ice until used for inoculations. Signs of disease and death were recorded daily during the two-week observation period. Virus neutralizing activity of the serum was calculated by the method of Kärber (7).

RESULTS

Purification of intracellular RNP. The most important step during purification is Sepharose gel filtration, since it eliminates all of the cellular antigens (Table 1, Fig. 1). In several experiments, between 5 and 15% of intracellular RNP was recovered as measured by CF-activity. The amount of purified RNP was between 2 and 5 mg.

Physicochemical characterization of intracellular RNP: buoyant density. Figure 2 represents photographs by Schlieren and ul-

traviolet-absorption optics of purified RNP following centrifugation in the analytical centrifuge. From these results the buoyant density of the RNP was calculated to be $\rho_{\text{RNP}} = 1.314$ g/ml.

Sedimentation constant. Figure 3 shows the sedimentation profile of purified RNP following rate-zonal centrifugation. Foot-and-mouth disease virus with a sedimentation constant of 140S served as sedimentation marker. The sedimentation coefficient $s_{20, w}$ of the intracellular RNP was estimated to be 150 to 180S according to the tables of McEwen (11).

Protein composition. Three bands were ob-

TABLE 1. Recovery rate of cell-derived RNP and ratios of protein/CF activities during purification

Sample	Total protein	RNP-CFU per mg of protein	BHK-CFU per mg of protein
NP-40 cell-extract	354 mg	10^3	1.6×10^4
Before Sepharose gel filtration	46 mg	4.3×10^3	2.1×10^3
After Sepharose gel filtration	8.3 mg	7.3×10^3	ϕ
Purified RNP after CsCl centrifugation	1.7 mg	1.3×10^4	ϕ
Recovery	1:208	6.3%	0%

FIG. 2. Band of cell-derived rabies RNP in the analytical centrifuge. The initial density of CsCl was 1.3662 g/cm^3 and the solution had an optical density of $\text{OD}_{260 \text{ nm}}^{1 \text{ cm}} = 0.097$. Centrifugation time was 18 h at 44,770 rpm; the temperature 25 C. The buoyant density according to Schildkraut et al. (14) was $\rho_{\text{RNP}} = 1.314_s \text{ g/cm}^3$.

FIG. 1. Gel filtration of NP-40-arcton extract of rabies-infected BHK-21 cells on a Sepharose 4 B column, 5 by 55 cm, eluted with STE buffer. Symbols: (O), optical density at 260 nm; (●), CF titer of sample per 0.025 ml.

FIG. 3. Sedimentation profile of rabies virus RNP after zonal centrifugation in sucrose 15 to 30% (wt/vol), rotor SW25 at 24,000 rpm for 2.5 h at 4°C. Symbols: (O), optical density at 260 nm; (●), complement fixation titer of sample per 0.025 ml.

served when RNP isolated from trypsin-treated cells was subjected to SDS polyacrylamide gel electrophoresis (Fig. 4A). The molecular weights of the three polypeptides N_1 , N_2 , N_3 were estimated to be 61,000, 48,000, and 10,000, respectively. RNP from cells which were mechanically harvested showed only one band corresponding to a molecular weight of 61,000 (Fig. 4B). Purified RNP was not found to be contaminated with other polypeptides.

Electron microscopy. In the electron microscope, purified, cell-derived rabies virus RNP appeared as loosely coiled strands of varying length. The strands were not different from those obtained from disrupted virions. The preparations contained only RNP, and no other structures were observed.

Serological properties of intracellular RNP: agar-gel precipitation. The double diffusion test revealed a single precipitation line when intracellular RNP reacted with anti-RNP or anti-virion serum. The joining lines of cell-derived and of virion-derived RNP indicated serologically identical antigens; RNP from trypsinized or mechanically harvested cells was undistinguishable (Fig. 5A). RNP-antisera from HEP, CVS, MOK, and LBV precipitated with homologous and heterologous RNP antigens indicating a close serological relationship (Fig. 5B). Staining with acridine orange caused a faint brick-red fluorescence of the precipitation line indicating the presence of a ribonucleoprotein.

Immunoelectrophoresis. When immunoelectrophoresed at pH 8.6, both, virus- and

cell-derived RNP migrated towards the anode and precipitated at identical positions (Fig. 6A). Compared to a normal bovine serum the position of RNP was that of α_2 -globulins. When undiluted RNP antiserum was used, a broad and diffuse precipitation line appeared. Serum dilution improved and sharpened the line indicating a Liesegang phenomenon of excess antibody. At a 1:5 dilution of serum only a single sharp line was present. By the "reversed" immunoelectrophoresis it was shown that the precipitation lines were reaction products between one antigen and different types of antibody. Fig. 6B shows several joining lines caused by an early immune serum which were located in the region from α_2 - to γ_2 -globulins. Following a booster inoculation 3 months later, the same rabbit serum contained RNP-specific immunoglobulin G (IgG) only and precipitated as a single, sharp line in the region of γ_2 -globulins (Fig. 6B).

CF test. In CF tests RNP-antisera were reacted with cell-derived RNP preparations of HEP, CVS, MOK, and LBV (Table 2). Each value given is the antisera endpoint over the antigen endpoint in a block titration. The results from repeated tests with the same reagents did not differ more than twofold with regard to endpoints. Table 2 indicates that HEP- and CVS-RNP are undistinguishable. Differences among the individual antisera were maximally eightfold, which indicates a close relationship of the four RNP-antigens tested. No cross-reactions were observed with BHK-21 cell-extract and BHK-21 antiserum, or with RNP-antigen and RNP-antiserum of the Indiana strain of VSV. Cell-derived RNP, therefore, is virus specific.

Immunofluorescence. Fluorescein-conjugated RNP antibody was reacted with infected

FIG. 4. Polypeptide pattern of purified rabies virus RNP denatured with 1% SDS and electrophoresed in 7.5% polyacrylamide gel. Drawing represents optical density of the Coomassie brilliant blue-stained gels measured using a Beckman Analytrol photometer. A, RNP from infected, trypsinized cells. B, RNP from infected cells harvested by mechanical action.

FIG. 5. Agar-gel double diffusion test. A, HEP-RNP from virions (3 and 6), from trypsinized (5) and from mechanically harvested (2) cells against homologous RNP-antiserum (1, 4 and center well). Antiserum diluted 1:2. B, HEP-RNP from mechanically harvested cells (center well) against homologous and heterologous RNP-antisera: 1 and 4, Anti-HEP RNP, 1:2; 2 and 6, anti-CVS RNP, 1:2; 3, anti-MOK RNP, undiluted; 5, anti-LBV RNP, undiluted.

brain-smears and infected coverslip cultures. At a 1:4,000 dilution the FA-conjugate still gave a 3⁺ intense fluorescence. A total of 30 rhabdoviruses and LCM virus were tested with the RNP conjugate. The rabies viruses included three plaque-purified laboratory strains (HEP, CVS, ERA), 6 rodent-isolates (including MOK), 3 bat-isolates (including LBV), 2 fox-, 1 horse-, and 2 human-isolates. All strains gave 3⁺ to 4⁺ brilliant fluorescence which could be inhibited by adsorption of the conjugate with rabies virus-infected mouse-brain suspension. No fluorescence was observed with 5 strains of VSV, or with Pirý, Chandipura, Flanders, Kern Canyon, Mt. Elgon bat, *Microtus* 1056, bovine ephemeral fever, Marburg, and LCM viruses. Rabies virus RNP, therefore, is an antigen common and specific for all strains of rabies virus tested and is mainly responsible for the immunofluorescent reaction in rabies-infected tissues.

Serum neutralization. RNP-antisera against

HEP-, CVS-, MOK-, and LBV-RNP were tested for virus-neutralizing activity. Between 10 and 300 LD₅₀ of the challenge viruses were not neutralized by undiluted homologous or heterologous RNP-antisera. Cell-derived, purified RNP is an internal antigen free from envelope proteins of the virus.

By using the constant virus-diluted serum method, two-way neutralization tests were performed (Table 3). The viruses listed were closely related in the complement fixation and fluorescent antibody tests; however, in the virus neutralization test obvious differences were observed. Whereas HEP and CVS showed strong cross-reactions, MOK and LBV gave weak cross-reactions. To define the degree of overlap the typing procedure for human rhinoviruses (see Discussion) was applied to the system. Accordingly, HEP and CVS belong to the same serotype, whereas MOK and LBV represent different serotypes. The usefulness of the described test systems for grouping and typing of

FIG. 6. Immunoelectrophoresis of purified rabies virus RNP. A, Immunoelectrophoresis of cell-derived (upper line) and virion-derived (lower line) rabies RNP. Below, Normal bovine serum as a control for the electrophoretic mobility. B, "Reversed" immunoelectrophoresis of rabies virus RNP against early (upper line) and late immune serum (lower line).

TABLE 2. CF test with RNP antisera and RNP antigens of rabies virus strains

RNP antigen	Antiserum dilution/antigen dilution			
	HEP ^a	CVS ^a	MOK ^a	LBV ^a
HEP	640/64	640/32	40/32	10/32
CVS	640/20	640/20	40/20	10/20
MOK	80/8	80/8	160/8	20/8
LBV	160/32	160/32	80/32	80/32

^a RNP antisera.

rabies viruses and of other rhabdoviruses will be discussed.

DISCUSSION

By the described method, intracellular rabies virus RNP was isolated from infected BHK-21 cells and purified in milligram amounts. The physical, chemical, and serological properties of cell-associated RNP are similar to those of virus-derived RNP.

The density (1.314) as measured in the analytical ultracentrifuge corresponds well to the values obtained by preparative procedures (19). Compared to the sharp RNP-band after isopycnic centrifugation a broad band was observed after zonal centrifugation which indicates a heterogeneity in the length distribution of the RNP-particles. We believe that this does not reflect the situation in the cell but was caused by breakage of RNP-molecules during purification. Since these breakage products, present in the starting material, are lost during sedimentation and Sepharose gel filtration, as shown by CF testing, only a 10-fold increase in RNP-specific CF activity was achieved during purification.

Nucleocapsids prepared from disrupted rabies virus were first shown by Sokol et al. (18) to be composed of two polypeptides of molecular weights 62,000 and 55,000. Neurath et al. (12) also found two nucleocapsid proteins NP₁ (58,000 mol wt) and NP₂ (47,000 mol wt). These values compare favorably to our findings of 61,000 (mol wt) for N₁ and 48,000 for N₂. In addition we found a third polypeptide, N₃, of approximately 10,000 mol wt. Recently, it was suggested by Sokol (personal communication) that the rabies nucleocapsid is composed of only one protein which might be cleaved by trypsin into major and minor polypeptides. In fact, we could show that RNP derived from mechanically harvested cells contained only one polypeptide (N₁). Apparently, under the action of trypsin, the N₁ polypeptide is partially cleaved into two polypeptides (N₂ and N₃) of smaller molecular weights (Fig. 5). However, RNP from cells harvested either by trypsin or by mechanical action were shown to be serologically identical (Fig. 6).

The results of double diffusion tests and of

TABLE 3. Two-way neutralization tests with antiviral sera and virus from cell culture fluid

Virus	LD ₅₀ per inoculum	Reciprocal of serum titer ^a			
		HEP ^a	CVS ^a	Lagos bat ^b	Mokola ^a
HEP	20	92,000	> 40,000	10	30
CVS	50	> 100,000	> 40,000	4	0
Lagos bat	50	10	5	300	20
Mokola	20	5	0	6	> 40,000

^a Zero indicates titer of <40.

^b Antiviral serum.

immuno-electrophoresis clearly indicate that cell-derived RNP represents one single antigen. In both tests a diffuse broad line was observed when undiluted early or late immune sera were used. This was considered to be the result of the Liesegang phenomenon of excess antibody (4) since a slight dilution of antiserum resulted in a single, sharp line of precipitation. Using early immune serum, different types of antibody precipitated with antigen, whereas late immune serum contained IgG only.

Immune sera prepared against RNP from several virus strains contained virus-specific complement-fixing, precipitating, and immunofluorescent antibodies, but were completely free from virus-neutralizing capacity. The sera did not react with cellular antigens.

Staining of infected cell cultures and of infected animal tissues with fluorescein-conjugated RNP-antibody demonstrated that only classical rabies viruses and some newly detected rabies-related rhabdoviruses from Africa (17) reacted with the RNP-antibody. No other rhabdoviruses or LCM gave this reaction. We therefore considered the core protein of the rabies virus, similar to influenza viruses (5), to be the group-specific antigen. CF and precipitation tests with RNP-antisera supported this assumption. RNP from several virus strains was closely related though not completely identical.

Since the fluorescence technique using this antiserum has been found to be much more sensitive than that using antisera against the complete virion, it would be advisable in the future to use RNP prepared in this manner for the production of high-titering diagnostic antisera.

In contrast to CF and FA testing, striking differences among several rabies virus strains were observed when tested in neutralization tests with antivirion sera. In order to define the degree of cross-reactions we used as a model the typing procedure for human rhinoviruses as proposed by Kapikian et al (6). Different serotypes, A and B, were thought to be established if 20 antibody units or more of virus A (that is at least 20 times the limiting concentration of a specific antiserum against virus A) failed to neutralize 20 to 300 LD₅₀ of strain B, and vice versa. Three serotypes were found for the rabies group of rhabdoviruses (Table 3). Serotype 1 is represented by the HEP and CVS strain of rabies virus and probably includes the majority of field and laboratory strains of the classical rabies virus. Serotypes 2 and 3 are represented by LBV and MOK, respectively.

We therefore propose that isolated RNP determines the group specificity, whereas the

TABLE 4. *Model for serological classification of rhabdoviruses*

Term	Characteristics	Test procedures
Genus	Similar structural, chemical and physical properties	Various
Group Serotype	Same group antigen Same group antigen. Surface antigens related Strong overlap = same serotypes Weak overlap = different serotypes ^a	CF, FA 2-way SNT
Variant	Physical or biological differences within same serotype, or one-way Sn-relationship	Various

^a Different serotypes, A and B, are established if 20 antibody units of A (that is, 20 times the limiting concentration of a specific antiserum A) fails to neutralize 20 to 300 LD₅₀ of virus B, and vice versa.

envelope-associated antigen is specific for the serotype. The combination of two serological procedures allows the classification of rhabdoviruses as outlined above and summarized in Table 4.

Genus. The genus of rhabdoviruses includes all viruses with similar structural, chemical, and physical properties.

Group. The groups are characterized by the RNP group-antigens. Grouping of a candidate virus is accomplished by reacting it with known group-specific, non-neutralizing RNP sera in CF or FA tests. Only positive reactions, weak or strong, account for the group membership of a candidate virus.

Serotype. The group members are divided into serotypes according to their envelope antigens. Typing is established by two-way neutralization of the candidate strain with known serotypes. The degree of cross-reactions is decisive for the serotype.

Variants. Variants are members of the same serotype which show biological or antigenic differences of a minor degree.

ACKNOWLEDGMENTS

We thank M. Mussgay for helpful discussions and his support in the course of this work. The skillful technical assistance of M. Genić and of G. Meyer is gratefully acknowledged.

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