ORIGINAL ARTICLE

Optimization of the Conditions for Preparation of Antirabic Diagnostic Globulins

Marina A. Efimova[1,2,3], Antonina G. Mukhamedzhanova[2], Kamil S. Khaertynov[2,4], Nikolai D. Shamaev[1], Eduard A. Shuralev[1,2,4]

[1]Federal Center for Toxicologic, Radiative, and Biologic Safety of Animals, Nauchniy Gorodok 2, Kazan, Republic of Tatarstan, 420075, Russia, [2]Federal Center for Toxicological, Radiation and Biological Safety of Animals, Nauchniy Gorodok-2, Kazan, 420075, Russian Federation, [3]Bauman Kazan State Academy of Veterinary Medicine, 35 Sibirskiy Trakt St., Kazan, 420029, Russian Federation, [4]Kazan State Medical Academy – Branch Campus of Russian Medical Academy of Continuous Professional Education, 36 Butlerova St., Kazan, 420012, Russian Federation

Abstract

Aim: The objective of this research was to obtain highly purified antirabic immunoglobulins to diagnostically significant fractions of the rabies virus antigen obtained by buoyant density separation in a step gradient of sucrose by ultracentrifugation at 30,000 g. **Materials and Methods:** As an immunogen, the rabies virus antigen of the "Ovechiy" GNKI strain with infectious titer Lg $10^{-5.25}$ was used. Purification of the virus was carried out by separation in a stepwise gradient of sucrose 10–50% on Optima L-90K ultracentrifuge (Beckman) followed by gel filtration through the ENrich™ SEC 70 columns. **Results:** As a result of the separation, the fractions of the viral material, selected from the sucrose zone 10–20%, with the localization range of the antigenic determinants of 50–65 kDa were the most active, which was confirmed by the results of electrophoresis in the separating polyacrylamide gel and immunoblot. These characteristics of the obtained drug served as the basis for its use as a material for the hyperimmunization of clinically healthy sheep were carried out according to the generally accepted scheme. Hyperimmune serum with a titer of 1:6400–1:12800 in ELISA was used to isolate γ-globulin fractions by three-fold reprecipitation with a saturated solution of ammonium sulfate, followed by concentration in dialysis bags, resulting in 5 final fractions of antirabic globulins. **Conclusion:** According to the results of ELISA and electrophoresis, globulin fractions were characterized by the greatest activity and specificity, the distribution of polypeptides in which corresponded to the location of antigenic determinants. The immunochemical properties of the obtained globulin drugs make them suitable in the future for use as specific components of improved ELISA-based express test systems for the diagnosis of rabies.

Key words: Antirabic globulin, electrophoresis, ELISA, immunoblot, rabies virus

INTRODUCTION

Over the past two decades, epizootic situation with rabies in the territory of the Russian Federation has remained extremely tense,[1] and the expansion of the disease noso area is observed annually.[2] Domestic and wild animals participate in the spread of rabies, as well as other zoonotic infections.[3,4] As a lethal anthropozoonotic infection, rabies causes significant economic damage, resulting from the costs of specific prevention among wild and farm animals[5] and diagnostic tests. Therefore, to increase the effectiveness of antiepizootic measures, it is necessary not only to conduct extensive monitoring studies[6] but also to improve methods for diagnosing rabies,[7,8] in particular, ELISA-based test systems using highly purified specific components - antigens and globulins. However, the existing test systems are characterized by insufficient sensitivity and specificity, which are determined by the degree of purification of their components. This dictates the need for search for an optimal technical solution that facilitates the

Address for correspondence:
Marina A. Efimova, Immunology Laboratory Manager of the Federal Center for Toxic and Radiation Safety of Animals, Nauchniy Gorodok 2, Kazan, Republic of Tatarstan. E-mail: marina-2004r@mail.ru

Received: 22-11-2018
Revised: 09-12-2018
Accepted: 14-12-2018

production of highly purified antigenic and globulin drugs free of secondary impurities. The effectiveness of this approach was demonstrated earlier when developing diagnostic tests for other infections.[9] The question of increasing the declared properties of specific drugs can be solved using globulins obtained for diagnostically significant antigens of the rabies virus and polymethodic evaluation of their properties.[10,11]

The purpose of this study was to optimize the conditions for the production of antirabic diagnostic immunoglobulins.

METHODS

As an immunogen, the rabies virus antigen of the "Ovechiy" GNKI strain with infectious titer Lg $10^{-5.25}$ was used. Purification of the virus was carried out by separation in a stepwise gradient of sucrose 10–50% on Optima L-90K ultracentrifuge (Beckman) followed by gel filtration through the ENrich™ SEC 70 columns.

The activity of the antigen fractions was analyzed by electrophoresis in 12.5% polyacrylamide gel (PAGE) in the presence of 0.1% sodium dodecyl sulfate, stained with Coomassie G-250 colloids, immunoblotting using hyperimmune rabies sera, and sandwich ELISA using the hyperimmune (titer 1:3200) and negative sheep serums.

The most active fractions of highly purified antigens were used to immunize clinically healthy sheep by sequential 4-fold administration of inactivated antigen at intervals of 14 days in a mixture with Incomplete Freund's Adjuvant (Thermo Fisher Scientific) intramuscularly into the medial thigh area in a volume of 16 cm^3. The blood sampling was made from the vascular vein when the serum titers reached 1:6400–1:12800 in ELISA.

The γ-globulin fraction was isolated by precipitation with a 2.78 M solution of ammonium sulfate according to the previously described procedure,[12] followed by dialysis against a starting 0.025 M Tris-HCl buffer (pH=7.8). The primary fraction emerged as a free peak; the yield of the subsequent fractions was initiated by changing the linear molar gradient by introducing 0.150 M Tris-HCl buffer into the system, which allowed separating the globulins from the carrier. The final fractions of the globulins were concentrated in closed dialysis bags. Chromatographic purification of the drug was carried out in the presence of DEAE cellulose at an elution rate of 0.7 ml/min.

Separation of primary globulins into fractions was performed by gel filtration on an NGC Discovery (Bio-Rad) chromatograph followed by measuring the protein concentration on a UV5 spectrophotometer (Mettler Toledo) at a wavelength of 280 nm. The protein fraction profiles were also determined by analytical electrophoresis in 12.5% PAG by Laemmli method[13] with Coomassie G-250 staining. Electrophoresis was performed in a Mini-PROTEAN$_{®}$ Tetra chamber for vertical electrophoresis (Bio-Rad) with a voltage of 210 V for 60–80 min. The results of electrophoresis were recorded on a Gel Doc XR+ (Bio-Rad) and processed using Image Lab Software 6.0.

The serological activity of the resulting globulin was assessed by sandwich ELISA at protein concentrations of 100–500 μg/ml in five replicates. Controls for the production of ELISA were as follows: Positive rabies virus antigen obtained from the brain tissue of mice infected with the Ovechiy GNKI production strain of the rabies virus, negative antigen from the brain tissue of intact mice, and heterologous antigen from the brain tissue of mice infected with the Aujeszky's disease virus ("Arsky" strain with infectious titer Lg 10^{-3}). Antigens were used 1:800 diluted, and the antirabic peroxidase conjugate (production of the Federal Center for Toxicological, Radiation and Biological Safety, Kazan, Russia) - 1:3200. The results of the reaction were processed with a BioRad 680 spectrophotometer at a wavelength of 490 nm.

RESULTS AND DISCUSSION

Analysis of the activity of the obtained fractions of highly purified rabies virus antigens

As a result of concentration and purification of the virus in a step gradient of sucrose density with ultracentrifugation at 30,000 g, five fractions of the rabies antigen were obtained. The electrophoretic analysis of antigenic fractions in 12.5% PAG showed that the protein fraction extracted from the sucrose zone of 10–20% is maximally purified, corresponds to a molecular weight of 56–66 kDa, and is characterized by homogeneity of the main antigen and the absence of secondary proteins [Figure 1], which is also confirmed by a densitogram [Figure 2].

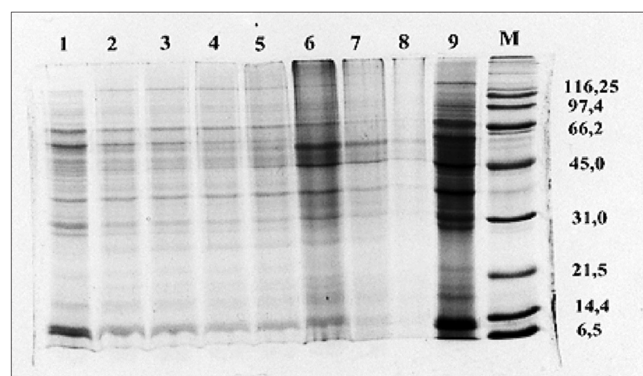

Figure 1: Electrophoretic analysis of the obtained rabies virus antigen fractions. 1 - fraction No 1 (viral material selected from the sucrose zone 10-20%); 2, 3 - fraction No 2 (viral material selected from the sucrose zone 30%); 4, 5 - fraction No 3 (viral material selected from the sucrose zone to 10%); 6 - fraction No 4 (viral material selected from the sucrose zone 40%); 7 - fraction No 5 (viral material selected from the 50% sucrose zone); 8 – supernatant after ultracentrifugation; 9 - original antigen of the rabies virus (before ultracentrifugation); 10 - marker of molecular masses broad range (BioRad)

Serological activity of antigenic drugs in ELISA and immunoblot using hyperimmune serum of sheep blood is presented in Table 1.

The data in Table 1 indicate that the most active and specific was the fraction of viral material selected from the sucrose zone of 10–20%. The diagnostic significance of this fraction of rabies virus polypeptides was confirmed by us on the model of the collection of sera obtained from the animals vaccinated against the rabies virus.

Immunization of sheep with the most active fraction of rabies virus antigen in a mixture with incomplete Freund's adjuvant allowed us to obtain hyperimmune serum with 1:6400–1:12,800 activity in ELISA.

Production of highly purified immunoglobulins

As a result of reprecipitation and chromatographic purification of the initial hyperimmune serum, we identified five main immunoglobulin fractions characterized by different protein concentrations, depending on the degree of dilution and intensity of air concentration. In the process of three-fold reprecipitation of the initial hyperimmune serum with a saturated solution of ammonium sulfate, we obtained fractions conventionally designated Ig No.1, Ig No.2, and Ig No.3, after which a combined sample of supernatants was formed during centrifugation at 4200 g, and then, as a result of additional concentration, the fractions such as Ig No.4 and Ig No.5 were isolated. The highest concentration of protein, approximated to that in the initial serum, is characterized by fractions obtained after the third cycle of reprecipitation; less saturated fractions were obtained from the supernatant by further processing.

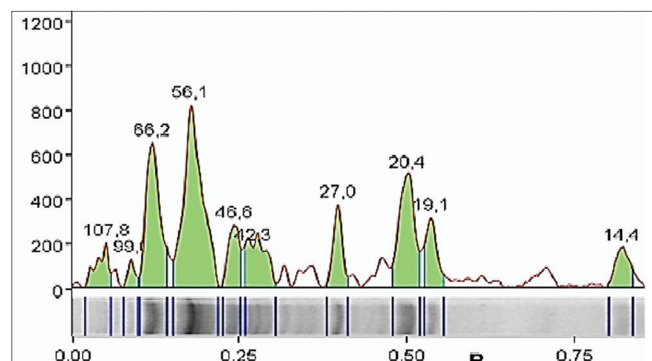

Figure 2: Densitogram of fraction №1 - viral material selected from the sucrose zone 10–20%

Further, the samples obtained were subjected to analytical electrophoresis in a 12.5% separating PAG [Figure 3].

The distribution zone of the serological activity of the investigated fractions (Ig No.1 - Ig No.5) covers the range of 30–70 kDa, while the content of the greatest number of polypeptides in this region is common for the Ig No.1, Ig No.2, and Ig No.3 fractions, whereas in Ig No.4 and Ig No.5, this trend was not observed. Presumably, this may be due to a lower content of active material in the original supernatant. The ranges of distribution of serological activity in the studied preparations are also confirmed by densitograms [Figure 4a-f].

The highest protein content in the isolated fractions Ig No.1, Ig No.2, and Ig No.3 is concentrated in the regions of 27–30, 45–50, and 65–70 kDa and in the fractions Ig No.4 and Ig No.5 - 50–65 kDa, which correspond to polypeptide determinants revealed in the analysis of the antigen obtained from the rabies virus.

Assessment of serological activity of the main fractions of highly purified immunoglobulins

A further stage of the study was the assessment of serological activity in sandwich ELISA globulins with electrophoretic profiles most satisfying the requirements (Ig No.1–Ig No.5) and establishment of the dependence of the protein concentration in the drug on its immunochemical characteristics [Table 2].

The results of the sandwich ELISA tests found that immunoglobulins Ig No.2 and Ig No.3, which are also characterized by the highest concentration of protein, were the most active. However, the less saturated fractions Ig No.4 and Ig No.5, in which optical density in the smallest dilution 1.27 times exceeded that of hyperimmune sheep serum ($P < 0.001$), were also satisfactory.

SUMMARY

In the course of the research work, the following conclusions were drawn:

Table 1: Serological activity of antigenic fractions of the rabies virus

Number of fraction/sucrose zone (%)	Protein concentration (C_p), mg/ml	Activity	
		Sandwich ELISA	Immunoblot
No.1 (10–20)	29.7	412.39	412.39
No.2 (30)	12.7	412.39	412.39
No.3 (10)	9.203	412.39	0
No.4 (40)	7.659	412.39	412.39
No.5 (50)	7.918	412.39	412.39

Table 2: Serological activity of immunoglobulin fractions in sandwich ELISA

Ig fractions	Protein concentration (C_p), mcg/ml	Optical density at the wavelength 490 nm at the highest dilution, OU		
		Rabies virus antigen	Control negative antigen	Aujeszky's disease virus antigen
Original sheep hyperimmune serum	100	0.913±0.003	0.041±0.002	0.057±0.002
	200	1.056±0.002	0.043±0.001	0.061±0.001
	300	1.128±0.004	0.047±0.003	0.059±0.001
	400	1.167±0.005	0.049±0.002	0.060±0.003
	500	1.205±0.001	0.051±0.001	0.062±0.002
Ig No.1	100	0.736±0.001	0.046±0.001	0.061±0.002
	200	0.837±0.001	0.047±0.001	0.063±0.003
	300	0.905±0.002	0.049±0.002	0.062±0.002
	400	1.098±0.009	0.051±0.001	0.065±0.001
	500	1.247±0.001	0.050±0.003	0.067±0.002
Ig No.2	100	1.075±0.006	0.057±0.002	0.062±0.003
	200	1.452±0.004	0.059±0.001	0.063±0.001
	300	1.634±0.008	0.061±0.003	0.065±0.002
	400	1.833±0.003	0.063±0.002	0.067±0.003
	500	1.832±0.003	0.064±0.002	0.066±0.002
Ig No.3	100	1.031±0.007	0.051±0.002	0.068±0.003
	200	1.236±0.006	0.058±0.001	0.071±0.001
	300	1.494±0.002	0.059±0.002	0.070±0.002
	400	1.636±0.004	0.061±0.001	0.072±0.003
	500	1.931±0.003	0.062±0.001	0.074±0.005
Ig No.4	100	0.830±0.002	0.051±0.002	0.059±0.002
	200	1.054±0.001	0.052±0.003	0.061±0.001
	300	1.231±0.002	0.054±0.002	0.060±0.002
	400	1.317±0.004	0.054±0.001	0.063±0.003
	500	1.531±0.002	0.055±0.002	0.065±0.002
Ig No.5	100	0.796±0.001	0.047±0.001	0.059±0.001
	200	0.879±0.002	0.047±0.001	0.061±0.003
	300	1.016±0.004	0.048±0.003	0.063±0.002
	400	1.278±0.003	0.051±0.002	0.064±0.002
	500	1.534±0.002	0.053±0.002	0.066±0.001

OU: Optical units

1. During the purification of the rabies virus antigen in a stepped sucrose gradient with ultracentrifugation at 30,000 g, we obtained five fractions of the purified antigen, with the most homogeneous fraction No.1, which is a viral material selected from the sucrose zone of 10–20%. Its electrophoretic analysis in 12.5% PAGE confirmed the content of antigenic determinants in the range of 56–66 kDa, which allowed using the fraction as an immunogen.
2. As a result of triplicate reprecipitation with ammonium sulfate and chromatographic purification of the hyperimmune serum obtained on the above antigen fraction, we obtained five purified globulin drugs. According to the results of electrophoresis and sandwich ELISA, the fractions Ig No.2, Ig No.3, Ig No.4, and Ig No.5 showed the greatest serological activity, and the main concentration of polypeptides of which is concentrated in the range of 50–65 kDa. The high activity of these fractions, which do not correlate with the protein concentration, is related to the matching of their polypeptide distribution to the antigenic determinants of the rabies virus fraction used for immunization.
3. The conditions for conducting the sandwich ELISA test with the obtained globulins have been optimized, which will allow efficiently to complete the corresponding test systems with them.

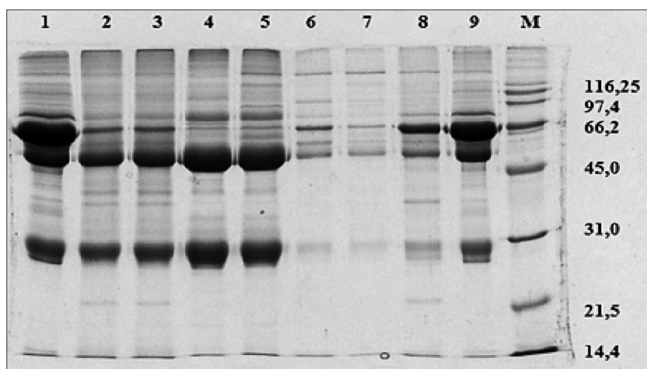

Figure 3: Electrophoretic analysis of protein profiles of isolated immunoglobulin fractions: 1, 9 - original sheep hyperimmune serum (C_p = 38.9 mg/ml); 2, 3 - Ig No.1 (C_p= 35.0 mg/ml); 4 – Ig No.2 (C_p = 49.0 mg/ml); 5 - Ig No.3 (C_p = 46.0 mg/ml); 6 - Ig No.4 (C_p = 6.7 mg/ml); 7 - Ig No.5 (C_p = 4.7 mg/ml); 8 - final supernatant; 10 - marker of molecular masses broad range (BioRad)

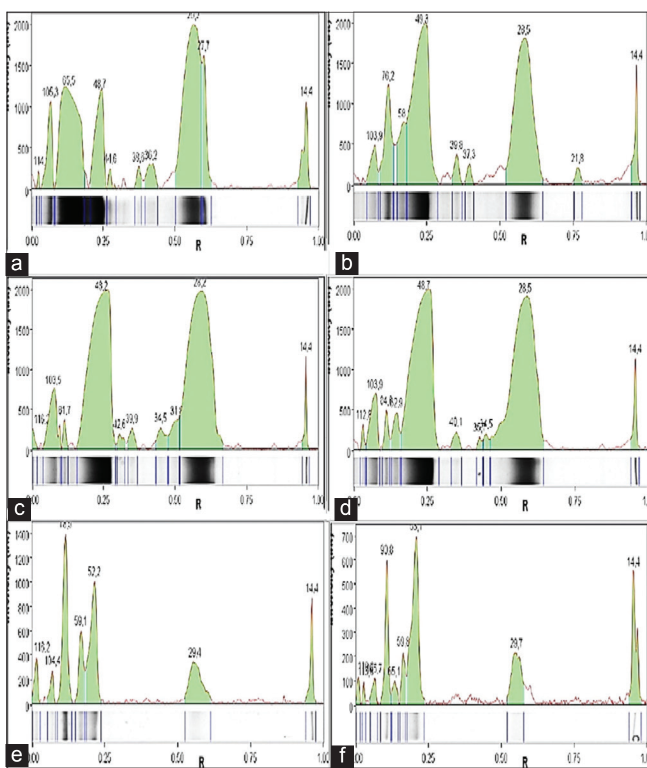

Figure 4: Densitograms of the fractions: (a) Original sheep hyperimmune serum, (b) Ig No.1, (c) Ig No.2, (d) Ig No. 3, (e) Ig No. 4, (f) Ig No. 5

CONCLUSION

The studies that we conducted are initiated by the need to improve express test systems for the diagnosis of rabies caused by an extremely strained epizootic situation with this disease in the territory of the Russian Federation. Highly purified antirabic immunoglobulins isolated by immunochemical, immunological, and serological methods and produced in industrial-scale volumes in the future can be used as specific components of improved express test systems for the diagnosis of rabies based on sandwich ELISA, which will improve the effectiveness of epizootic monitoring of rabies.

ACKNOWLEDGMENTS

The work is performed according to the Russian Government Program of Competitive Growth of Kazan Federal University.

REFERENCES

1. Movsesiants AA. Rabies: Features of the modern epizootic and epidemiological situation in Russia. Epidemiol Prev Vaccin 2011;5:4-5.
2. Metlin AE, Chernyshova EV, Rybakov SS. Rabies of animals: Epizootology, control measures and prospects. Vet Med Kuban 2009;6:2-4.
3. Khismatullina NA, Karimov MM, Khaertynov KS, Shuralev EA, Morzunov SP, Khaertynova IM, *et al*. Epidemiological dynamics of nephropatia epidemica in the republic of Tatarstan, Russia, during the period of 1997-2013. Epidemiol Infect 2016;144:618-26.
4. Shuralev EA, Shamaev ND, Mukminov MN, Nagamune K, Taniguchi Y, Saito T, *et al*. *Toxoplasma gondii* seroprevalence in goats, cats and humans in Russia. Parasitol Int 2018;67:112-4.
5. Cherkasskii BL, Khadartsev OS, Movsesiants AA. Epidemiological surveillance of rabies in the Russian federation. Bull Vaccin News Prev Vaccin 2005;1:7-8.
6. Elakov AL, Zaikova ON, Kochergin-Nikitskii KS, Grebennikova TV, Aliper TI. Monitoring of rabies in wild animals in Bryansk region. Vet Med 2015;1:11-4.
7. Chacko K, Parakadavathu RT, Al-Maslamani M, Nair AP, Chekura AP, Madhavan I. Diagnostic difficulties in human rabies: A case report and review of the literature. Qatar Med J 2017;2016:15.
8. Maxwell MJ, De Carvalho MH, Hoet A, Vigilato MA, Pompei JC, Cosivi O, *et al*. Building the road to a regional zoonoses strategy: A survey of zoonoses programmes in the Americas. PLoS One 2017;12:e0174175.
9. Khaertynov KS, Valeeva AR, Ivanov AV, Mukminov MN, Urazov NG, Khaertynova IM, *et al*. Extraction and serological properties of mycobacterium cell surface and excreted proteins. Bio Nano Sci 2018;8:459-66.
10. Eggerbauer E, De Benedictis P, Hoffmann B, Mettenleiter TC, Schlottau K, Ngoepe EC, *et al*. Evaluation of six commercially available rapid immunochromatographic tests for the diagnosis of rabies in brain material. PLoS Negl Trop Dis 2016;10:e0004776.
11. Faye M, Dacheux L, Weidmann M, Diop SA, Loucoubar C, Bourhy H, *et al* Development and validation of sensitive real-time RT-PCR assay for broad detection of rabies virus. J Virol Methods

2017;243:120-30.
12. Efimova MA, Khaertynov KS, Arslanova AF, Akhmadeev RM, Nikitin AI, Gumerov VG, *et al*. Isolation, purification and evaluation of serological activity of rabies virus antigens. Probl Highly Dangerous Infect 2017;4:27-30.
13. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.

Source of Support: Nil. **Conflict of Interest:** None declared.