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Indrė KUČINSKAITĖ-KODZĖ

PRODUCTION, CHARACTERIZATION AND APPLICATION OF NEW MONOCLONAL ANTIBODIES AGAINST VIRAL ANTIGENS

SUMMARY OF DOCTORAL DISSERTATION

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Indrė KUČINSKAITĖ-KODZĖ

NAUJŲ MONOKLONINIŲ ANTIKŪNŲ PRIEŠ VIRUSŲ ANTIGENUS KŪRIMAS CHARAKTERIZAVIMAS IR TAIKYMAS

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Introduction

Topicality of the problem

Monoclonal antibodies and antibody-based assays are widely used in modern biotechnology, medicine and scientific research. An important field for the use of monoclonal antibodies is immunodiagnostics that dominates over molecular diagnostics within the *in vitro* diagnostics segment. Monoclonal antibodies against viral antigens are valuable diagnostic reagents that contribute to the control and prevention of infectious diseases. Moreover, they represent a useful tool to investigate the structure of viral antigens and immune response to viral infection.

Monoclonal antibodies recognize short amino acid sequences (epitopes) in a complex antigenic structure. Therefore, the investigation of antibody-antigen binding can provide important information on the antigenic similarity between recombinant and native viral proteins, surface-exposed sequences of the antigens, sites of interaction between viral structural proteins. This type of research is relevant to the development of new generation vaccines and immunotherapeutics against viral infections.

The object of research

This dissertation describes development and characterization of monoclonal antibodies against recombinant yeast-expressed antigens: nucleocapsid (N) proteins of human parainfluenza virus type 3, Menangle virus, hantavirus and rabies virus. Antibodies were raised against recombinant N proteins forming nucleocapsid-like particles.

The aim of the dissertation was to investigate the immunogenic properties of recombinant viral proteins and generate monoclonal antibodies in order to use them for virus identification in infected cells and the studies of antigenic structure of viral proteins.

Tasks of the dissertation

1. To investigate the immunogenicity of recombinant viral proteins: hPIV3, Menangle virus, hantavirus and rabies virus N proteins, and generate hybridomas producing monoclonal antibodies against these proteins.
2. To investigate the specificity, cross-reactivity and the ability to recognize native viruses of newly developed monoclonal antibodies and evaluate their diagnostic potential.

3. To investigate the antigenic properties of viral proteins and identify immunodominant epitopes by using newly developed monoclonal antibodies and antisera raised against native viruses.

Methodology of research

The hybridoma technology, different variations of enzyme-linked immunosorbent assay, Western blot analysis, immunofluorescence microscopy, gene engineering and bioinformatics methods were used in this work.

Scientific novelty

Most of the previously described antibodies against viral antigens were produced using inactivated viruses. In the current study, monoclonal antibodies were raised against recombinant N proteins forming nucleocapsid-like particles. Preparation and use of recombinant antigens for immunization is more convenient than the cultivation and purification of viruses, however recombinant and native viral antigens may differ in their antigenic structure. Therefore, it was important to demonstrate that the antibodies raised against recombinant antigens were able to recognize native viral nucleocapsids in virus-infected cells.

Newly developed monoclonal antibodies were employed to study in detail the antigenic structure of the N proteins of two representatives of the paramyxovirus family, hPIV3 and Menangle virus. Our previous study showed that the majority of B-cell epitopes of measles virus N protein are located at the C-terminal part of measles N protein. In the current study, we have investigated the interaction of recombinant hPIV3 and Menangle virus N proteins with monoclonal antibodies and antisera induced by virus infection or vaccination. As a result of this study, new data about the immunodominant regions of paramyxovirus N proteins were obtained. Similarly, the antigenic structure of rabies virus N protein was studied in detail. The results obtained were in line with the previous data on B-cell epitope localization in rabies virus N protein.

The following new results were obtained:

1. Using recombinant yeast-expressed viral antigens, new hybridoma cell lines producing high affinity antibodies against four different viruses: hPIV3, hantaviruses, rabies virus, and Menangle virus N proteins have been generated.
2. For the first time, monoclonal antibodies against Menangle virus N protein have been generated. Their ability to detect Menangle virus in infected tissue specimens has been demonstrated.

3. For the first time, the antigenic structure of the N proteins of two representatives of paramyxovirus family, hPIV3 and Menangle virus, has been explored and the localization of immunodominant regions at the C-terminal domain of N proteins has been demonstrated. The linear epitope of hPIV3 N protein, homologous to the immunodominant epitope of measles virus N protein, has been identified.

Practical value

The results of the present dissertation have a great practical value. The immunochemical analysis of infected cells and tissues has confirmed that antibodies raised against recombinant yeast-expressed viral proteins react with native virus present in infected cells and tissues. Therefore, the antibodies against recombinant viral N proteins can be used in immunodiagnostics of the respective viral infections. Direct determination of the virus is important to identify the infectious agent.

The practical value of the dissertation can be illustrated as follows:

1. Antibodies against Menangle virus N protein have been provided to the CSIRO Livestock Industries, Australian Animal Health Laboratory, where they are used to detect Menangle virus infection and investigate its prevalence in Australia.
2. Antibodies against hantavirus N proteins have been provided to several foreign research institutions which perform investigations of hantaviruses: Friedrich Loeffler Institute (Germany), Centre for Infectious Diseases and Immunity, University of New Mexico School of Medicine (USA), Departamento de Virologia, Instituto Nacional de Enfermedades Infecciosas (Argentina).
3. Antibodies against hPIV3 and hantavirus N proteins have been commercialized and included into the product catalogues of Abcam Ltd (UK) and Santa Cruz Biotechnology (USA). The antibodies are continuously supplied to these companies and distributed worldwide.

Defended propositions

1. Recombinant yeast-expressed hPIV3, hantavirus, rabies virus and Menangle virus nucleocapsid proteins can elicit a strong immune response and are suitable antigens for monoclonal antibodies production.

2. Hybridomas against recombinant hPIV3, hantavirus, rabies virus and Menangle virus nucleocapsid proteins secrete specific high-affinity antibodies.
3. Monoclonal antibodies against recombinant virus nucleocapsid proteins can be used to identify native-virus specific epitopes.
4. Monoclonal antibodies against virus nucleocapsid proteins are suitable tools for exploring the antigenic structure of proteins.
5. Monoclonal antibody-based diagnostic systems may be useful tool for direct detection of virus-infected cells.

The scope of the scientific work

The dissertation consists of the introduction, literature overview, materials and methods, results and discussion, the list of references and the list of author's publications. The first Chapter of the dissertation provides literature overview on the genome organization, structural proteins, pathogenesis and epidemiology of parainfluenza viruses, Menangle virus, hantaviruses and rabies viruses. The literature overview also demonstrates the benefits of the expression of recombinant viral proteins in yeast, describes commonly used diagnostic assays for viral infections and application of monoclonal antibodies for the immunodiagnostics of viral infections. In the second Chapter, materials and methods are presented. The third Chapter provides experimental data on the development of new monoclonal antibodies, testing of their specificity and reactivity with different viral N proteins, infected cells and tissues, the use of monoclonal antibodies for studying the antigenic structure of viral N proteins.

The scope of the work is presented in 128 pages, 35 figures and 15 tables. Two-hundred forty sources of literature have been used in the dissertation.

Approval of the results

The material presented in the dissertation was published in four scientific articles in the journals indexed in the list of the Institute for Scientific information ISI Web of Science (Kučinskaite-Kodzė *et al.* 2011; Žvirbliene *et al.* 2010; Žvirbliene *et al.* 2009; Kučinskaitė *et al.* 2007). The results of the dissertation were discussed at six international and national scientific conferences:

1. *"3rd EFIS-EJI Course on Autoimmunity"*. 2007, Tartu, Estonia.
2. 10th scientific conference *"Biochemistry and Systems Biology"*. 2008, Tolieja, Lithuania.
3. *"II Symposium. Standartization in immunology/Standartyzacja w immunologii"*. 2009, Poznane, Poland.

4. *"39th Meeting of Scandinavian Society for Immunology jointly with the Baltic Immunological Society"*. 2010 m. Talinas, Estija.
5. *"14th International Negative Strand Virus Meeting"*. 2010, Bruges, Belgium.
6. *"14th International Congress of Immunology"*. 2010, Kobe, Japan.

1. Literature overview

Human parainfluenza viruses (hPIV) are members of the family *Paramyxoviridae*. Human parainfluenza virus type 3 (hPIV3) is a respiratory tract pathogen and major cause of croup, bronchiolitis and pneumonia in infants and very young children. Approximately 18,000 infants and children are hospitalized each year in the USA because of lower respiratory tract illness caused by hPIV3.

Menangle virus (MenV) is one of several recently discovered RNA viruses of the family *Paramyxoviridae*. MenV was isolated from stillborn piglets with malformations at the piggery in Australia. During the outbreak of MenV infection in 1997, two co-workers of the piggery that had a contact with infected pigs developed an influenza-like illness. Both patients recovered after 10–14 days. The mode of virus transmission from pigs to humans is not yet known.

Hantaviruses represent a separate genus *Hantavirus* of the family *Bunyaviridae*. Hantaviruses are transmitted to humans by a contact with infected rodents and their excreta. The hantaviruses cause two different types of diseases in humans: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). The fatality rate of HFRS varies from <1 % to 12 % depending on viruses. In the case of HPS, the fatality rate is about 40 %.

Rabies virus represents the type species of the genus *Lyssavirus* in the family *Rhabdoviridae*. Rabies virus infects the central nervous system, causing encephalitis and ultimately death. According to the World Health Organization, approximately 55,000 cases of human rabies occur every year worldwide, most of them in developing countries. In Europe three genotypes of genus *Lyssavirus* are present, classical rabies virus (RABV), European bat lyssavirus type 1 (EBLV-1) and European bat lyssavirus type 2 (EBLV-2).

All viruses mentioned above are enveloped, and their genomes are organized on a single negative-sense strand of RNA. Inside the viral envelope there is a ribonucleoprotein consisting of single-stranded genomic RNA to which the N protein is tightly bound. The N protein packs viral RNA genome and form nucleocapsids.

Recombinant N proteins of different viruses have the ability to self assemble into nucleocapsid-like particles in many heterologous systems in the absence of other viral structural proteins. It was demonstrated, that yeast-derived nucleocapsid-like particles of measles and mumps viruses share similar antigenic structures with native viral nucleocapsids. Therefore, the N proteins produced in yeast expression system might provide a valuable material for studying viral protein structure and functions. Also, recombinant N proteins and N protein-specific monoclonal antibodies (mAbs) can be applied for diagnostic tests and seroepidemiological studies.

Monoclonal antibodies against viral antigens are useful tools to detect and quantify viral components in clinical specimens. The viral antigens on the cell surface or inside the infected cell can be detected by an immunofluorescence or immunohistochemistry staining. Different variations of enzyme-linked immunosorbent assay (ELISA) can be used to detect soluble viral antigens. For many viral infections (rabies virus, respiratory syncytial virus, influenza A virus, herpes simplex virus, cytomegalovirus) the direct antigen detection by immunoassays is the most relevant diagnostic test.

2. Materials and methods

Recombinant N proteins of paramyxoviruses, hantaviruses and lyssaviruses used for mAb production and characterization were produced at the Department of Eukaryote Gene Engineering of the Institute of Biotechnology of Vilnius University.

MAbs were generated essentially as described by Kohler and Milstein. BALB/c mice were immunized subcutaneously at days 0, 28, 56 with 50 µg of recombinant N protein. For the primary and second immunizations, the antigen was emulsified in complete and incomplete Freund's adjuvant, respectively.

Specificity of the mAbs was analyzed by an indirect ELISA, Western blot and dot blot analysis. For epitope mapping, competitive ELISA was used. To investigate the reactivity of mAbs with native viral nucleocapsids, an indirect immunofluorescense assay (IFA) was used. The analysis was done using commercial IFA slides with hantavirus- and parainfluenza virus-infected *Vero 6* cells. Indirect immunofluorescence and immunohistochemistry experiments with the infected human or animal tissue specimens were performed at Lithuanian and foreign virology laboratories: Virology section, National Food and Veterinary Risk Assessment Institute (Lithuania); CSIRO Livestock Industries, Australian Animal Health Laboratory (Australia); Department of Pathology, the Center for Infectious Diseases and Immunology, University of New Mexico School of Medicine (USA).

E. coli expressed GST-fused hPIV3 and RABV N proteins fragments were constructed for the localization of mAb epitopes. Identification of the linear immunodominant epitope of hPIV3 N protein was performed using biotin-labeled synthetic peptide spanning the sequence between aa 437–446 of hPIV3 N protein.

3. Results and discussion

3.1. Production and characterization of monoclonal antibodies against hPIV3 N protein and their application for the antigenic characterization of the N protein

Yeast-expressed hPIV3 N protein self-assembled to nucleocapsid-like particles was used as an immunogen for the production of mAbs. Four stable hybridoma cell lines producing mAbs of IgG isotype were obtained. Three mAbs (5F12, 2H1 and 11H7) were of IgG1 subtype and one was of IgG3 (5C9) subtype. The specificity of the mAbs was analyzed by an indirect ELISA and Western blot. Their cross-reactivity with different yeast-expressed N proteins

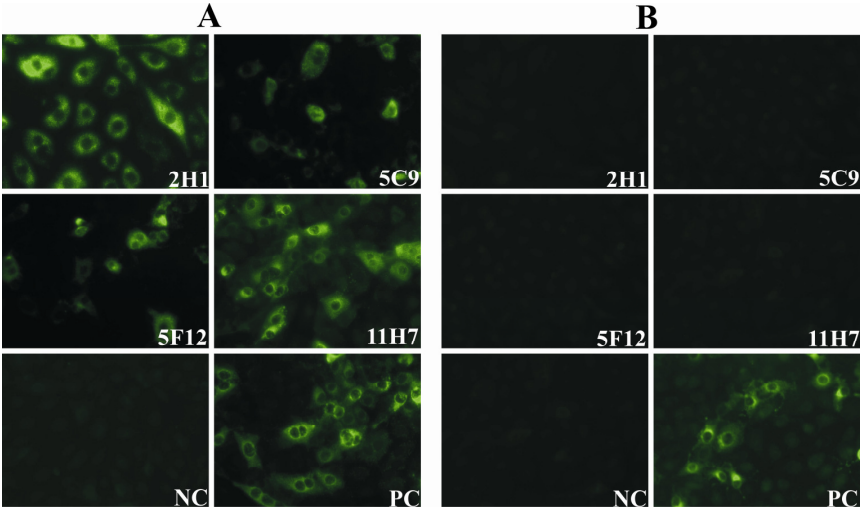

Fig. 1. Indirect immunofluorescence staining of hPIV-infected cells with mAbs raised against hPIV3 N protein. A, hPIV3-infected *Vero 6* cells; B, hPIV1-infected *Vero 6* cells. NC, negative control (slides incubated with human negative serum for hPIV1/3); PC, positive control (slides incubated with human serum containing IgG against hPIV1/3)

was investigated. All mAbs reacted specifically with hPIV3 N protein and did not react with related hPIV1 N protein. As a next step, all mAbs were tested for their reactivity with native nucleocapsids of hPIV3 by indirect IFA. All mAbs showed strong reactivity with mammalian cells infected with wild-type hPIV3 (Fig. 1, A). As expected, the mAbs did not cross-react with hPIV1-infected cells (Fig.1, B). These results demonstrate that the epitopes recognized by the mAbs are exposed in viral N protein thus confirming the antigenic similarity between yeast-expressed and viral nucleocapsids.

In the current study, the mAbs raised against recombinant hPIV3 N protein were employed to study the antigenic structure of hPIV3 N protein. Antigenic sites recognized by mAbs were mapped using recombinant overlapping GST-fused N protein fragments. One major immunodominant site was identified in the C-terminal region (aa 397–486) of hPIV3 N protein. Further studies with smaller N protein fragments and synthetic peptide revealed one liner epitope representing aa 437–446 of the N protein located within this site. This epitope was reactive with 46 % of hPIV3 IgG positive sera. These results suggested that the above antigenic site on the N protein is important in eliciting a humoral immune response against hPIV3.

In conclusion, the current study enhances the knowledge of the antigenic structure of hPIV3 N protein and may facilitate the development of better diagnostic methods for hPIV3 infection.

3.2. Production and characterization of monoclonal antibodies against Menangle virus N protein and their application for the antigenic characterization of the N protein

The development of mAbs against MenV antigens has not yet been reported. The N protein is the most abundant structural protein of paramyxoviruses and essential component of viral nucleocapsid. Therefore, N protein-specific antibodies is a suitable tool for virus detection in infected tissues. To generate MenV-specific mAbs, we have used recombinant yeast-expressed MenV N protein as an immunogen. Following immunizations with the N protein, three mAbs (2D1, 4G11 and 10G8) of IgG1 subtype were produced and their specificity was investigated in detail. One mAb (clone 10G8) was cross-reactive with recombinant N protein of Tioman virus. The epitopes of mAbs were mapped using a series of truncated MenV N proteins comprising of aa 1–400, aa 1–430, aa 1–460 and aa 1–490. The epitopes of two mAbs (2D1, 4G11) were mapped at aa 430–460 of MeV N protein while the epitope of mAb (10G8) was mapped at aa 460–490. These results provide new

information on the surface-exposed, B cell accessible regions of MenV N protein. The epitopes recognized by the mAbs are located in the same C-terminal part of MenV N protein as the main antigenic region recently identified in the N proteins of measles virus and hPIV3, other members of the family *Paramyxoviridae*.

All three mAbs raised against MenV N protein specifically recognized MenV by immunohistochemical staining of MenV-infected brain tissue isolated from stillborn piglet (Fig. 2).

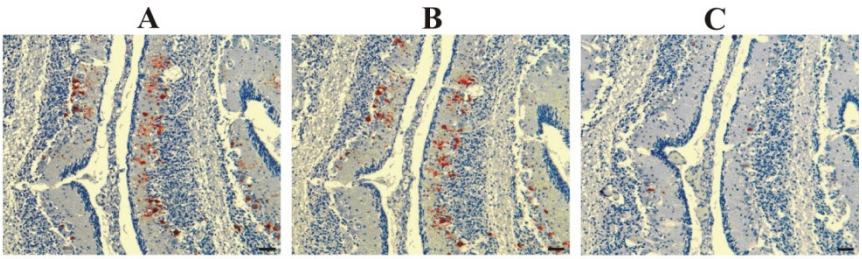

Fig. 2. Immunohistochemistry of MeV infected tissues. A-C, brain of MeV-infected stillborn piglet. Tissue samples were probed with primary antibodies: A, mAb of clone 2D1; B, mAb of clone 4G11; C, mAb of clone10G8

In summary, the mAbs described in the current study may be useful for studying the antigenic structure of MenV N protein and facilitate the development of laboratory diagnostic tools for MenV infection.

3.3. Production and characterization of monoclonal antibodies against hantavirus N proteins

Recombinant yeast-expressed N proteins of hantaviruses (Andes, Sin Nombre, Puumala-Vranica) were used as immunogens for the production of mAbs. Six mAbs (4H3, 7G2, 2C6, 5C5, 5E11 and 7A5) of IgG isotype were produced. The cross-reactivities of the mAbs were analyzed by an indirect ELISA and Western blot analysis using various hantavirus N proteins. MAbs 4H3 and 2C6 reacted exclusively with the SNV and ANDV N proteins (Fig. 3). In contrast, the mAbs 7G2 and 5E11 demonstrated a broad cross-reactivity with various hantavirus N proteins (Fig. 3).

MAbs 5C5 and 7A5 reacted almost exclusively with the N proteins of Sigmodontinae-, Neotominae- and Arvicolinae-associated hantaviruses. This broad cross-reactivity pattern reflects the fact that the N protein is highly conserved hantavirus antigen.

Previous investigations of the immune response in natural infection of hantaviruses demonstrated that the N-terminal region of N protein is highly immunogenic and cross-reactive. In line with these data, the mAbs developed in the current study recognized different epitopes in the N-terminal region of the Puumala-Vranica hantavirus N protein. To localize the approximate regions of epitopes recognized by mAbs, we employed chimeric proteins harboring different segments of Puumala-Vranica hantavirus N protein: aa 1–45, aa 1–80, aa 1–120. The regions of epitopes of three mAbs (2C6, 5C5, 5E11) were localized between aa 1–45 whereas the epitope of mAb 7A5 was localized between aa 81–120 of Puumala-Vranica N protein. MAb 7G2 recognized the epitope located at aa 121–433. The mAb 4H3 raised against Andes virus and Sin Nombre virus N proteins did not react with Puumala Vranica N protein, therefore it was not possible to localize its epitope.

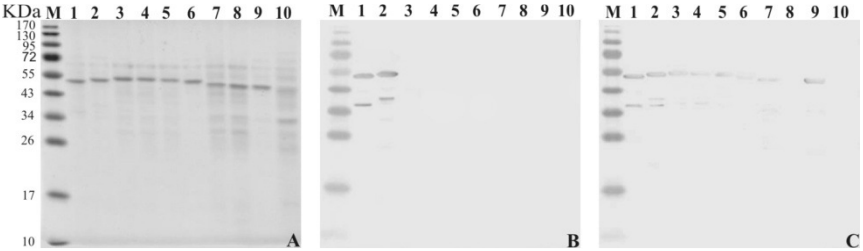

Fig. 3. Western blot analysis of the cross-reactivities of the mAbs 4H3 (B) and 7G2 (C) with N proteins of Sin Nombre virus (lane 1), Andes virus (lane 2), Puumala virus (PUUV), strain Kazan (lane 3), PUUV, strain Sotkamo (lane 4), PUUV, strain Vranica/Hällnäs (lane 5), Tula virus (lane 6), Hantaan virus (lane 7), Dobrava-Belgrade virus, strain Slovenia (lane 8), Seoul virus (lane 9). Lane 10, crude lysate of non-transformed yeast cells. Lane M, prestained protein ladder (UAB Fermentas, Lithuania). A, as a control, the same yeast cell crude lysates were run in a SDS polyacrylamide gel and stained with Coomassie blue

The reactivity of mAbs with native viral nucleocapsids was also confirmed by their reactivity in immunohistochemistry assay and indirect IFA. All mAbs reacted with native viral nucleocapsids in hantavirus-infected cells.

Therefore, the novel mAbs represent useful tools for the detection and investigation of hantavirus infection by new serological and immunohistochemistry-based diagnostic methods.

3.4. Production and characterization of monoclonal antibodies against rabies virus N protein and their application for the antigenic characterization of the N protein

In Europe, three genotypes of the genus *Lyssavirus*, family *Rhabdoviridae* are present, classical rabies virus (RABV), European bat lyssavirus type 1 (EBLV-1) and European bat lyssavirus type 2 (EBLV-2). The N proteins of RABV, EBLV-1 and EBLV-2 were expressed in yeast. The purified N proteins revealed formation of nucleocapsid-like structures. The antigenic structure of the N proteins was investigated according to heir reactivity with previously generated mAbs raised against different lyssaviruses and sera from vaccinated animals. The reactivity pattern of each mAb was virtually identical between IFA using virus-infected cells, ELISA and dot blot assay using corresponding N proteins. These observations lead us to summarize that yeast-expressed lyssavirus N proteins share antigenic properties with naturally expressed lyssavirus proteins.

Recombinant RABV N protein was used as an immunogen for the production of mAbs. Four hybridoma cell lines producing mAbs (3G8, 4A12, 5F12 and 6G7) of IgG isotype were generated. The specificity of the mAbs was analyzed by an indirect ELISA and Western blot. Their cross-reactivity with other lyssavirus yeast-expressed N proteins (EBLV-1 and EBLV-2) was studied. ELISA and Western blot studies revealed that all mAbs were cross-reactive with EBLV-1 and EBLV-2 N proteins and two of them (3G8 and 5F12) recognized conformational epitopes.

The reactivities of all mAbs with RABV nucleocapsids were tested by an indirect IFA using brain tissue specimens from fox infected with RABV. The IFA data revealed that the mAbs that recognize conformational epitopes (3G8 and 5F12) are able to detect native viral nucleocapsids (data not shown).

To localize the epitopes recognized by mAbs, the attempts were made to express in *E. coli* three overlapping N protein fragments and investigate their interaction with mAbs. Two recombinant fragments were expressed that corresponded to the N-terminal and C-terminal parts of N protein (1–186 and 367–450 aa). By using a combination of two different mapping strategies – overlapping N protein fragments and competitive ELISA – we have located the major antibody binding site at the N-terminus of RABV N protein, between aa 1 and 186. These results demonstrate that the terminal-N region of RABV N protein is immunogenic and induces antibody formation.

These data suggested that two conformation-specific mAbs and two mAbs against liner epitope recognized epitopes located within the N-terminal region of lyssavirus N protein. In summary, the mAbs against recombinant lyssavirus

N protein are suitable for laboratory diagnosis of rabies as well for structure-function studies of RABV N protein.

General conclusions

1. Four hybridoma cell lines producing high affinity antibodies against yeast-expressed hPIV3 N protein have been generated. It was determined that the antibodies specifically recognize viral nucleocapsids in hPIV3-infected cells and do not react with related hPIV1.
2. New data on the antigenic structure of hPIV3 protein have been obtained using monoclonal antibodies and antisera from patients with natural hPIV3 infection. The immunodominant sequences of hPIV3 N protein have been located at the C-terminal domain of the N protein, between aa 397 and 446. The linear epitope spanning aa 437–446 of hPIV3 N proteinhas has been identified that is homologous to the previously identified imunodominant epitope of measles virus N protein.
3. For the first time, monoclonal antibodies against Menangle virus N protein have been generated by using yeast-expressed N protein. One antibody (clone 10G8) cross-reacts with N protein of related Tioman virus. All three antibodies recognize Menangle virus in infected tissues and are suitable for the laboratory diagnosis of Menangle virus. The epitopes recognized by the antibodies are located in the C-terminal domain of N protein, between aa 431 and 490.
4. Studies on the antigenic structure of paramyxovirus N proteins demonstrated that the C-terminal domain of N proteins is immunodominant and located on the surface of viral nucleocapsid.
5. Monoclonal antibodies against recombinant hantavirus N proteins have been produced and characterised in detail. Their different cross-specificity with N proteins of various hantaviruses, confirms a close phylogenetic relationship between hantaviruses. The antibodies recognize hantaviruses in the specimens of infected human and animal tissues and may be useful for the diagnostics of hantavirus infection.
6. Four monoclonal antibodies against recombinant classical rabies virus (RABV) N protein have been produced. Three antibodies cross-react with EBLV-1 and EBLV-2 virus N proteins. Two antibodies recognize linear epitopes located in the terminal domain of RABV N protein, between aa 1 and 186. Two antibodies recognize conformational epitopes and recognize viral nucleocapsids in the infected brain tissue.

7. Monoclonal antibodies raised against recombinant viral N proteins can be used to develop new virus diagnostic systems. The reactivities of the antibodies with virus-infected cells confirm the antigenic similarity between recombinant yeast-expressed N proteins and viral nucleocapsids.

List of author's publications on the topic of the dissertation

Kučinskaitė-Kodzė, I.; Petraitytė-Burneikienė, R.; Žvirblienė, A.; Hjelle, B.; Medina, R. A.; Gedvilaitė, A.; Ražanskienė, A.; Schmidt-Chanasit, J.; Mertens, M.; Padula, P.; Sasnauskas, K.; Ulrich, R. G. 2011. Characterization of monoclonal antibodies against hantavirus nucleocapsid protein and their use for immunohistochemistry on rodent and human samples, *Archives of Virology* 156: 443-456. ISSN 0304-8608 (ISI Web of Science).

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NAUJŲ MONOKLONINIŲ ANTIKŪNŲ PRIEŠ VIRUSŲ ANTIGENUS KŪRIMAS, CHARAKTERIZAVIMAS IR TAIKYMAS

Mokslo problemos aktualumas

Monokloniniai antikūnai ir jų pagrindu sukurti imunocheminiai metodai plačiai taikomi šiuolaikinėje biotechnologijoje, medicinoje bei moksliniuose tyrimuose. Svarbi monokloninių antikūnų panaudojimo sritis yra imunodiagnostika – įvairių infekcinių ir sisteminių ligų nustatymas, tiriant biologinius mėginius imunocheminiais metodais. Šie metodai užima dominuojančią padėtį šiuolaikinėje laboratorinėje medicinoje, lyginant su kitais *in vitro* diagnostikos metodais. Todėl gerai charakterizuotų, aukšto specifiškumo ir afiniškumo antikūnų ir naujų imunodiagnostinių metodų poreikis nuolat auga.

Monokloniniai antikūnai, sukurti prieš virusų baltymus, yra ne tik vertingi diagnostiniai reagentai, bet ir patogus įrankis virusinių antigenų struktūros bei imuninio atsako į virusinę infekciją tyrimams. Monokloniniai antikūnai atpažįsta trumpas aminorūgščių sekas (epitopus) sudėtingoje antigeno struktūroje, todėl šie tyrimai gali suteikti svarbios informacijos apie rekombinantinių ir natyvių virusinių baltymų antigeninį panašumą, baltymo paviršiuje eksponuojamas sekas, sąveikos vietas su kitais viruso struktūriniais baltymais. Tokio pobūdžio tyrimai svarbūs, kuriant naujos kartos vakcinas ar kitas virusinių infekcijų prevencijos ar imunoterapijos priemones.

Tyrimų objektas

Šiame disertaciniame darbe aprašomi nauji monokloniniai antikūnai, sukurti prieš žmogaus paragripo trečiojo tipo viruso (hPIV3) nukleokapsidės (N) baltymą, bei gyvūnų platinamų virusų – hantavirusų, pasiutligės viruso, Menangle viruso – N baltymus. Monokloninių antikūnų kūrimui buvo panaudoti rekombinantiniai mielėse susintetinti virusų antigenai, formuojantys į virusus panašias struktūras.

Darbo tikslas – ištirti rekombinantinių virusinių baltymų imunogenines savybes ir sukurti monokloninius antikūnus prieš šiuos baltymus, siekiant juos pritaikyti virusų nustatymui infekuotose ląstelėse bei virusinių baltymų antigeninei struktūrai tirti.

Darbo uždaviniai

1. Ištirti rekombinantinių virusinių baltymų – hPIV3, Menangle viruso, hantavirusų bei pasiutligės viruso nukleokapsidės (N) baltymų – imunogeniškumą ir sukurti hibridomas, gaminančias monokloninius antikūnus prieš šiuos baltymus.
2. Ištirti sukurtų monokloninių antikūnų specifiškumą, kryžmines reakcijas, sugebėjimą atpažinti natyvius virusus infekuotose ląstelėse ir įvertinti jų diagnostinį potencialą.
3. Naudojant sukurtuosius monokloninius antikūnus ir antiserumus, susidariusius natūralios infekcijos metu, ištirti virusinių N baltymų antigenines savybes ir identifikuoti imunodominuojančius epitopus.

Tyrimų metodika

Darbe buvo naudojama hibridomų technologija, įvairūs imunofermentinės analizės variantai, imunoblotingo ir imunofluorescencinės mikroskopijos, genų inžinerijos ir bioinformatikos metodai.

Mokslinis naujumas

Literatūroje aprašyta nemažai monokloninių antikūnų prieš virusų antigenus. Tačiau dauguma anksčiau aprašytųjų antikūnų buvo sukurti, naudojant nukenksmintus virusus. Mūsų sukurtieji antikūnai skiriasi nuo anksčiau aprašytųjų tuo, kad jie buvo gauti prieš rekombinantinius virusų baltymus, formuojančius į virusų nukleokapsides panašias daleles. Rekombinantinių antigenų paruošimas ir naudojimas imunizacijai yra paprastesnis ir saugesnis, nei virusų kultivavimas ir gryninimas, tačiau rekombinantinių ir natyvių virusinių antigenų struktūra gali skirtis. Todėl buvo svarbu pademonstruoti, kad mūsų sukurtieji antikūnai geba atpažinti natyvius virusus infekuotose ląstelėse.

Naudojant sukurtuosius monokloninius antikūnus, buvo išsamiai apibūdinta dviejų paramiksovirusų šeimos atstovų – hPIV3 ir Menangle viruso – N baltymų antigeninė struktūra. Anksčiau mūsų atlikti tyrimai parodė, kad tymų viruso N baltymo C galinėje dalyje sutelkta dauguma B ląstelių epitopų. Šiame disertaciniame darbe, ištyrus rekombinantinių hPIV3 ir Menangle viruso N baltymų sąveiką su monokloniniais antikūnais bei antiserumais, susidariusiais natūralios infekcijos arba vakcinacijos metu, buvo gauti nauji duomenys apie minėtų virusų N baltymų B ląstelių epitopus, identifikuotos šių baltymų imunodominuojančios sekos. Panašiai buvo ištirta pasiutligės viruso N baltymo antigeninė struktūra. Gauti rezultatai sutapo su anksčiau publikuotais duomenimis apie B ląstelių epitopų lokalizaciją pasiutligės viruso N baltymo N galinėje dalyje.

Buvo gauti šie nauji rezultatai:

1. Naudojant rekombinantinius mielėse susintetintus virusų antigenus, buvo sukurtos naujos hibridomų linijos, sekretuojančios aukšto afiniškumo antikūnus prieš 4 skirtingų virusų – hPIV3, hantavirusų, pasiutligės viruso ir Menangle viruso – N baltymus.
2. Pirmą kartą buvo sukurti monokloniniai antikūnai prieš Menangle viruso N baltymą ir pademonstruota, kad jie tinka viruso nustatymui infekuotame audinyje.
3. Pirmą kartą buvo ištirta paramiksovirusų šeimos atstovų – hPIV3 ir Menangle viruso – N baltymų antigeninė struktūra ir parodyta, kad imunodominuojančios sekos yra lokalizuotos N baltymų C galinėje dalyje. Identifikuotas hPIV3 N baltymo linijinis epitopas, homologiškas tymų viruso N baltymo imunodominuojančiam epitopui.

Praktinė vertė

Šio disertacinio darbo rezultatai turi didelę praktinę reikšmę. Atlikti infekuotų ląstelių ir audinių tyrimai patvirtino, kad antikūnai, sukurti prieš rekombinantinius mielėse susintetintus virusų baltymus, reaguoja su natyviais virusais infekuotose ląstelėse ir audiniuose. Todėl sukurtieji antikūnai gali būti naudojami minėtų virusinių infekcijų imunodiagnostikai. Tiesioginis viruso nustatymas yra svarbus, norint patvirtinti virusinę infekciją ar tiksliai nustatyti infekcijos sukėlėją.

Disertacinio darbo praktinę reikšmę iliustruoja šie pavyzdžiai:

1. Antikūnai prieš Menangle viruso N baltymą perduoti Australijos veterinarijos laboratorijai (*CSIRO Livestock Industries, Australian Animal Health Laboratory*), kur jie naudojami Menangle viruso nustatymui ir šios infekcijos paplitimo tyrimams.
2. Antikūnai prieš hantavirusų N baltymą perduoti kelioms užsienio mokslo įstaigoms, kuriose atliekami hantavirusų tyrimai: *Friedrich Loeffler Institute* (Vokietija), *Center for Infectious Diseases and Immunity, University of New Mexico School of Medicine* (JAV), *Departamento de Virologia, Instituto Nacional de Enfermedades Infecciosas* (Argentina).
3. Antikūnai prieš hPIV3 ir hantavirusų N baltymus komercializuoti ir įtraukti į kompanijų *Abcam Ltd* (D. Britanija) bei *Santa Cruz Biotechnology* (JAV) katalogus. Jie nuolat tiekiami šioms kompanijoms ir platinami visame pasaulyje.

Ginamieji teiginiai

1. Rekombinantiniai mielėse susintetinti hPIV3, hantavirusų, pasiutligės viruso ir Menangle viruso nukleokapsidės baltymai sukelia stiprų imuninį atsaką ir yra tinkami antigenai monokloniniams antikūnams kurti.
2. Hibridomos, gautos naudojant rekombinantinius hPIV3, hantavirusų, pasiutligės viruso ir Menangle viruso nukleokapsidės baltymus, sekretuoja specifiškus ir afiniškus antikūnus.
3. Monokloniniai antikūnai, sukurti prieš rekombinantinius virusų nukleokapsidės baltymus, atpažįsta epitopus, būdingus natyviems virusams.
4. Monokloniniai antikūnai prieš virusų nukleokapsidės baltymus gali būti naudojami šių baltymų antigeninei struktūrai tirti.
5. Monokloninių antikūnų pagrindu gali būti kuriamos diagnostinės sistemos, skirtos tiesioginiam virusų nustatymui infekuotose ląstelėse.

Darbo apimtis. Disertaciją sudaro: įvadas, literatūros apžvalga, metodinė dalis, rezultatai ir jų aptarimas, naudotos literatūros sąrašas, publikacijų disertacijos tema sąrašas. Pirmasis disertacijos skyrius skirtas literatūros apžvalgai: jame apibūdinamos Menangle viruso, hantavirusų ir pasiutligės virusų šeimos ir gentys, genomai, struktūriniai baltymai, patogenezė ir epidemiologija. Literatūros apžvalgoje taip pat aprašomi rekombinantinių virusinių baltymų sintezės mielėse privalumai, dažniausiai naudojami virusinių infekcijų diagnostikos metodai bei monokloninių antikūnų taikymas virusinių infekcijų diagnostikai. Antrajame skyriuje aprašomi disertaciniame darbe naudoti metodai. Trečiajame skyriuje pateikiami eksperimentiniai duomenys apie naujų monokloninių antikūnų kūrimą, jų specifiškumo tyrimus, sąveiką su įvairių virusų N baltymais, infekuotų ląstelių ir audinių tyrimus, virusų N baltymų antigeninės struktūros tyrimus.

Darbo apimtis yra 128 puslapių, tekste pateikti 35 paveikslai ir 15 lentelių. Rašant disertaciją, buvo panaudota 240 literatūros šaltinių.

Darbo rezultatų aprobavimas

Disertacijos tema yra atspausdinti keturi moksliniai straipsniai, įtraukti į ISI Web of Sciense sąrašą (Kučinskaite-Kodzė *et al.* 2011; Žvirbliene *et al.* 2010; Žvirbliene *et al.* 2009; Kučinskaitė *et al.* 2007).

Disertacijoje atliktų tyrimų rezultatai buvo paskelbti 6 mokslinėse konferencijose Lietuvoje ir užsienyje:

1. Tarptautinėje konferencijoje *„3rd EFIS-EJI Course on Autoimmunity“* 2007 m. Tartu, Estijoje.

2. Lietuvos Biochemikų draugijos konferencijoje *„Biochemija ir Sistemų Biologija“* 2008 m. Tolėja.
3. Tarptautinėje konferencijoje *„II Symposium. Standartization in Immunology/Standartyzacja w Immunologii“* 2009 m. Poznanė, Lenkija.
4. Tarptautinėje konferencijoje *„39th Meeting of Scandinavian Society for Immunology jointly with the Baltic Immunological Society“* 2010 m. Talinas, Estija.
5. Tarptautinėje konferencijoje *„14th International Negative Strand Virus Meeting“* 2010 m. Briugė, Belgija.
6. Tarptautinėje konferencijoje *„14th International Congress of Immunology“* 2010 m. Kobė, Japonija.

Bendrosios išvados

1. Sukurtos 4 hibridomų linijos, sekretuojančios aukšto afiniškumo IgG klasės antikūnus prieš mielėse susintetintą hPIV3 N baltymą. Nustatyta, kad antikūnai specifiškai atpažįsta viruso nukleokapsides hPIV3 infekuotose ląstelėse ir nereaguoja su gimiringu hPIV1.
2. Naudojant monokloninius antikūnus ir infekuotų žmonių antiserumus, gauti nauji duomenys apie hPIV3 N baltymo antigeninę struktūrą. Nustatyta, kad hPIV3 N baltymo imunodominuojantys rajonai yra išsidėstę baltymo C galinėje dalyje, tarp 397 ir 446 ar. Identifikuotas hPIV3 N baltymo linijinis epitopas (437–446 ar.), homologiškas tymų viruso N baltymo epitopui.
3. Naudojant mielėse susintetintą Menangle viruso N baltymą, pirmą kartą sukurti monokloniniai antikūnai prieš Menangle virusą. Vienas iš antikūnų (klonas 10G8) kryžmiškai reaguoja su gimiringo Tioman viruso N baltymu. Visi 3 sukurtieji antikūnai atpažįsta Menangle virusą infekuotuose gyvūnų audiniuose ir tinka šio viruso laboratorinei diagnostikai. Antikūnų atpažįstami epitopai išsidėstę N baltymo C galinėje dalyje, tarp 431 ir 490 ar.
4. Paramiksovirusų N baltymų antigeninės struktūros tyrimai patvirtina, kad šių baltymų C galinė dalis yra imunodominauojanti ir lokalizuota nukleokapsidės paviršiuje.
5. Sukurti ir detaliai charakterizuoti monokloniniai antikūnai prieš rekombinantinius hantavirusų N baltymus. Jie pasižymi skirtingu kryžminiu specifiškumu su įvairių hantavirusų N baltymais, kas patvirtina hantavirusų glaudų filogenetinį ryšį. Sukurtieji antikūnai

atpažįsta hantavirusus infekuotuose žmogaus ir gyvūnų audiniuose ir gali būti panaudoti, kuriant naujas laboratorines diagnostikos sistemas hantavirusų infekcijai nustatyti.

6. Sukurti 4 monokloniniai antikūnai prieš rekombinantinį klasikinio pasiutligės viruso (RABV) N baltymą. Trys antikūnai kryžmiškai reaguoja su EBLV-1 ir EBLV-2 virusų N baltymais. Du antikūnai atpažįsta linijinius epitopus, kurie lokalizuoti RABV N baltymo N galinėje dalyje, tarp 1 ir 186 ar. Du antikūnai atpažįsta konformacinius epitopus ir sąveikauja su viruso nukleokapsidėmis infekuotose smegenų ląstelėse.
7. Monokloniniai antikūnai prieš rekombinantinius virusų N baltymus gali būti panaudoti naujoms virusų diagnostikos sistemoms kurti. Antikūnų sąveika su virusais infekuotose ląstelėse patvirtina rekombinantinių mielėse susintetintų N baltymų ir virusų nukleokapsidžių antigeninį panašumą.

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Summary of Doctoral Dissertation
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