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Study of Measles Virus Recombinant Proteins and Their Immunobiological Properties

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Abstract—Recombinant proteins rN (nucleocapsid) and rH/Nh (hemagglutinin) of the measles virus strain NovO/96 of genotype A were obtained. The immunobiological properties of the proteins were studied in the reaction with a panel of positive and negative sera. BALB/c mice were immunized with recombinant proteins and native antigen of the measles virus strain NovO/96 in order to obtain hyperimmune serum and its analysis using ELISA (enzyme-linked immunosorbent assay) and PRN (plaque reduction neutralization). The hyperimmune sera against recombinant proteins and native antigen of the measles virus strain NovO/96 were found to be highly active in ELISA. The antibodies against the proteins rN and rH/Nh were found to be capable of neutralizing the virus in titer 1 : 13.5 and 1 : 22.9, respectively. The neutralization titer of the antibodies generated against native antigen of the measles virus strain NovO/96 was 1 : 25.7.

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Measles is one of the most widespread human respiratory viral diseases; it causes approximately 30 million cases and 888000 deaths each year [22]. Measles morbidity in Russia is about 2000–3000 per year [3]. The measles virus genome is a negative single-stranded RNA of 15894 nucleotides, packaged within a helical nucleocapsid. Transcription and replication are carried out on this complex. Virion contains six structural proteins, three of which form the nucleocapsid, i.e., the nucleocapsid protein N (521 amino acids) that binds the viral RNA tightly; the phosphoprotein P (507 aa); and the large protein L (2155 aa), which, along with the P protein, represents a major component of the RNA-dependent RNA polymerase [17]. Three other proteins are associated with the viral envelope, i.e., the membrane protein M (335 aa); the fusion protein F (553 aa), the disulphide-linked heterodimer of the F_1 and F_2 subunits formed after proteolytic cleavage of the precursor protein F_0; and hemagglutinin H/Nh (617 aa) [5].

The measles virus N gene product is a phosphorylated, nonglycosylated protein, the major component of the virion bound to the genomic RNA. Three linear antigenic determinants were localized on the measles virus protein N. Among these determinants recognized by monoclonal antibodies, one is located in the N-terminal region of the protein and conservative among various isolates of measles virus, while two others situated in the C-terminal part are variable and strain-specific epitopes [15]. Using *E.coli*-expressed protein N deletion mutants, the conservative determinant was mapped in the 122–150 region of amino acids, while the first variable epitope was mapped in a region between amino acid 457 and 476, and the second variable was mapped in the 519–525 region of amino acids, respectively [7]. The presence of T- and B-cell linear determinants, along with the fact that protein N does not undergo any complex post-translational modification, enables using a prokaryotic host to produce the protein fully retaining its immunobiological activities [6, 11, 14, 19]. Protein N-specific antibodies can be detected by immunoblotting in virtually all serum samples obtained from vaccinated and reconvalescent patients [14]. Three different expression systems have been used to express recombinant measles virus nucleoprotein, including bacterial (*Escherichia coli* BL21), insect cells (*Spodoptera frugiperda*; Sf9), and mammalian fibroblasts. The nucleoprotein produced in all three hosts was used in ELISA for the detection of IgG and IgM measles virus-specific antibodies [19]. The study of recombinant protein N expression in prokaryotic (*E.coli*) system revealed that only full-length intact protein N can be used as a reagent for ELISA [18]. Since the immunobiological properties of the nucleocapsid protein are thoroughly investigated, the measles virus gene N product is a strong candidate for ELISA antigen. In the present study, we attempt to obtain a composite recombinant protein possessing maximal possible immunobiological activities.

Recombinant hemagglutinin (H/Nh) was produced in a eukaryotic expression system [7]. There are no data available on the measles virus H/Nh protein expression in expression in prokaryotic system. Recombinant H/Nh protein expressed in a eukaryotic system was used in the ELISA for detection of IgM class antibodies in blood and saliva at early stages of the disease, as well

as for the evaluation of postvaccinal immunity through the detection of the level of IgG-class antibodies [8, 9].

The aim of the present study was to subclone the fragments of protein N gene that correspond to the linear antigenic determinants (fragments 64 to 219 aa and 434 to 525 aa), as well as full-length measles virus *H*/*Nh* gene (7271 to 9124 bp), to obtain strains that express the desired recombinant proteins, and to evaluate their immunobiological activities by ELISA and plaque reduction neutralization (PRN) assays.

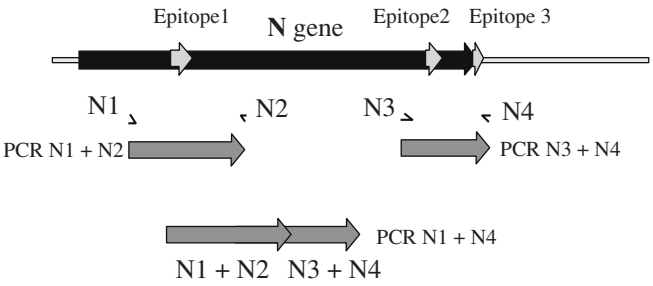
MATERIALS AND METHODS

Virus. The measles virus strain NovO/96 used in this work was obtained from the Vector State Research Center for Virology and Biotechnology Collection.

E.coli strains, kits and materials. E. coli strains BL21 (DE3) and XL-1 blue (Promega, US), restriction endonucleases, T4 DNA ligase, Tte-DNA polymerase and deoxyribonucleotide phosphates (Sibenzyme, Novosibirsk, Russia) were used in this work. All DNA manipulations were performed as previously described [2]. Total RNA isolation was carried out using an RNeasy Mini kit (QIAGEN, Germany). First-strand cDNAs were generated by reverse transcription using random hexamers (dN6) and a Sensiscript RT kit (QIAGEN) according to the manufacturer's protocol. Expression of recombinant proteins was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM for 5 hours. Recombinant proteins were purified on Ni-NTA agarose according to the manufacturer's recommendations (QIAGEN, Germany).

Cloning and expression in E.coli of the measles virus protein N gene fragments. Measles virus strain NovO/96 cDNA nucleotide sequence (GenBank accession number AY504625) and data on measles virus nucleoprotein gene structure were obtained from the GenBank Genetic Sequence Database [16]. Two regions of the protein N gene, corresponding to fragments 64 to 219 aa and 434 to 525 aa, were chosen for subsequent study since all three linear antigenic determinants of the measles virus N protein are located within these regions [12, 17]. The 468-bp and 325-bp N gene fragments were amplified by PCR with cDNA as a template using the primer pairs N1 5'-CCCGGATCCCCCAAACTAACAGGGGCACTA-3', N2 5'-CACCACATCCAACCATTTTCTC-3', generating a 468-bp PCR product (encoding the protein N region between amino acid 64 and 219) and N3 5'-GAGAAAATGGTTGGATGTGGTGGGCAAGGAAGATAGGAGGGTC-3', N4 5'-CCCAAGCTTGTAGGCGGATGTTGTTCTGGT-3', producing a 325-bp PCR fragment (encoding the protein N region between amino acids 434 and 525).

Then, both PCR fragments were mixed together and the mix was used as a template for the second PCR round with the N1 and N4 primers. The 778-bp PCR product (see figure) was digested with *Bam*HI and *Hind*lll restriction endonucleases and cloned at the corresponding sites of pGSDI expression vector carrying 6xHis tag enabling the recombinant protein purification on Ni-NTA agarose [1, 4]. The selected construct pHis-NP encoding the composite measles virus nucleocapsid protein N was transformed into *E. coli* BL21 (DE3) cells. Following induction, the recombinant protein with the expected molecular weight of 27 kDa was expressed in *E. coli* cells. The recombinant protein represented no less than 10% of the total cell protein content as determined by densitometry scans of the stained gels (data not shown). Most of the expressed recombinant protein existed in the soluble fraction of the bacterial lysate.



Scheme used for cloning composite measles virus nucleocapsid protein N gene. Notes: Epitopes 1, 2, and 3 denote the *N* gene antigenic determinants; N1, N2, N3, and N4 are PCR primers; PCR N1 + N2 denotes a fragment amplified using primer pair N1 and N2; PCR N3 + N4 denotes a fragment amplified using primer pair N3 and N4; PCR N1 + N4 is DNA fragment amplified with a mixture of PCR N1 + N2 and PCR N3 + N4 as a template using primer pair N1 and N4.

Cloning and expression in E. coli of measles virus protein H/Nh. The measles virus genome nucleotide sequence from 7271 to 9124 bp encodes the protein H/Nh. This part of the genome was PCR amplified and cloned into a pGEX expression vector and the extra nucleotides that coded for the 6 C-terminal histidine residues were added to the downstream primer sequence, enabling the recombinant protein purification using affine chromatography on Ni-NTA agarose.

The measles virus *H*/*Nh* gene of 1854-bp length was amplified by PCR using the primers N5 5'-CCCGGATCCATGTCACCACAACGAGAC-3' and N6 5'-CGAGTCGACTTAGTGATGGTGATGGTGATGTCTGCGATTGGTTCCATC-3'. The PCR product was digested with *Bam*HI and *Sal*l and cloned into the *Bgl*ll and *Sal*l sites of pGEX plasmid. The ligation mix was used to transform competent *E. coli* XLI blue cells, and proper clones carrying plasmid with the measles virus *H*/*Nh* gene insertion were selected by restriction analysis.

Effective titers of hyperimmune sera obtained from animals immunized with the native antigen or rN and rH/Nh recombinant proteins of measles virus strain NovO/96: ELISA- and PRNT-inferred data

Hyperimmune sera against the NovO/96 native antigen and rN and rH/Nh recombinant proteins	ELISA antigens				Sera titer in PRNT with NovO/96 strain
	rN	rH/Nh	NovO/96	rN TOPC	
rN	1 : 6400	N.R.	1 : 3200	N.R.	1 : 13.5
rH/Nh	N.R.	1 : 12800	1 : 1600	N.R.	1 : 22.9
NovO/96	1 : 12800	1 : 6400	1 : 12800	N.R.	1 : 25.7

Note: N.R. signifies no reaction.

Both strands of the *H*/*Nh* gene DNA, as well as vector regions adjacent to the gene, were sequenced by the Sanger method using the genetic analyzer (Beckman Coulter, US). The pGEX-His6-H/Nh construct was chosen for subsequent study because of its 100% similarity to the measles virus strain NovO/96 *H*/*Nh* gene sequence.

The selected plasmid was transformed into *E. coli* BL21 (DE3) cells. Following the induction, the recombinant protein with an expected molecular weight of 96 kDa was expressed in *E. coli* cells. The recombinant protein represented no less than 30% of the total cell protein content as determined by densitometry scans of the stained gels.

Properties of human sera used. Positive and negative measles sera were used in the experiments. Ten sera samples were considered negative according to laboratory tests approved by WHO [20] using two different ELISA-based test systems, Dade Behring Anti-Masern-Virus/IgG (Germany) and IBL Measles virus IgG ELISA (Germany), according to the manufacturer's protocol; in the PRN test following the method described below; and by means of a hemagglutination-inhibition test (HIT) [13]. Positive sera were obtained from 25 patients who had had measles. Clinical diagnosis of measles was confirmed both serologically and by genotyping virus isolates (according to WHO recommendations) [21]. The genotyping revealed 8 cases of genotype A and 17 cases of genotype D6. Patients who had had measles displayed a fourfold increase in antibody titer in the pair sera obtained on days 5 and 35 from the first clinical manifestations of disease.

Preparation of hyperimmune sera against the measles virus rN and rH/Nh recombinant proteins. Five-week-old male BALB/c mice were immunized 3 times at biweekly intervals. The first immunization was carried out subcutaneously using 50 μg of purified protein in complete Freund's adjuvant, while the second immunizations were given in incomplete Freund's adjuvant. Hyperimmune sera collected 2 weeks after the last immunization were subsequently used in ELISA and PRN assays.

ELISA-based determination of antibody titers against the recombinant proteins. A fixed amount (0.5 μg per well) of recombinant protein was adsorbed to Costar 96-well flat-bottom plate (Costar, no. 3369) in carbonate buffer pH 9.6 at 4°C for 14 h. Nonspecific binding sites on the wells were blocked by adding 200 μl of 0.5% casein blocking buffer (Sigma, US), then incubating the plate at 37°C for 2 h with shaking at 50 Hz. Wells were emptied, and murine (at a dilution of 1 : 800–1 : 25 600) or human (at 1 : 231 dilution) sera in the 100-μl volume in PBS-T were added, after which the plates were incubated at 37°C for 1.5 h with shaking at 50 Hz (BIS shaker-thermostat model 207). Wells were emptied, then washed and rinsed 5 times with PBS-T. 100 microliters of anti-mouse IgG alkaline phosphatase-conjugated Abs (Anti-Mouse-IgG, Sigma, US) at 1 : 30000 dilution was added, then the plates were incubated at 37°C for 1.5 h with shaking at 50 Hz; for detecting human sera, anti-human IgG (Anti-Human IgG (H + L); ICN, US) alkaline phosphatase-conjugated Abs at 1 : 50000 dilution were used. Wells were emptied, then washed and rinsed as described above. Bound Abs were detected by adding alkaline phosphatase substrate solution (Sigma Fast, Sigma, US), then incubating the plate at room temperature for 30 min. The reaction was stopped by adding 50 μl of 0.9 M H_2SO_4, after which the absorbance at 492 nm was evaluated with the E1 × 800 spectrophotometer (Bio-Tec Instruments, US). The SARS coronavirus recombinant nucleocapsid protein obtained in the same way [4] was used as a negative control.

PRN-based determination of antibody titers. All the sera were incubated at 56°C for 30 min and diluted two-fold starting at dilutions of 1 : 8. Each dilution was mixed with an equal volume of virus suspension (the measles virus strain NovO/96) and incubated at 37°C for 90 min. The serum–virus mixture was transferred onto Vero cell monolayer prepared in 24 well plates (3 iterations) and incubated for 1 h before the mixture was aspirated and overlay medium was added to the VERO monolayer. The overlay medium was double Eagle's MEM containing 0.5% agar and 5% fetal serum. Plates were incubated at 37°C in 5% CO_2 for 6 days, then cells

were covered with the second agar overlay containing neutral red. Plaques were counted the next day. The antibody titer was defined as the serum dilution showing a 50% reduction of the plaque number below that of the virus control containing 30 pfu in 100 μl.

RESULTS AND DISCUSSION

Recombinant rN (nucleocapsid) protein containing N protein linear antigenic determinants formed by amino acids 64–219 and 434–525 and rH/Nh (hemagglutinin) protein of the measles virus strain NovO/96 were obtained in this work, and the immunobiological properties of recombinant proteins were studied.

Detection of antibodies against the measles virus strain NovO/96 recombinant proteins in the animal hyperimmune sera by ELISA and PRNT. As is evident from the table, serum obtained from a protein rN-immunized animal produced an ELISA signal at a titer of 1 : 3200 when added to the measles virus strain NovO/96 native antigen, as well as at a titer 1 : 6400 when added to rN recombinant protein. No interactions of the serum with the rH/Nh protein were observed. The serum obtained from the animal immunized with protein rH/Nh displayed interactions with both the corresponding NovO/96 native antigen (at a titer of 1 : 1600) and the rH/Nh recombinant protein (at a titer of 12800), while no interactions with rN protein were observed. The hyperimmune serum against the NovO/96 native antigen interacted in ELISA with rN, rH/Nh proteins, and NovO/96 native antigen at titers 1 : 12800, 1 : 6400 and 1 : 12800, respectively. None of the hyperimmune sera reacted with the SARS coronavirus rN antigen (see table). The serum obtained from intact mice was not reactive to any of the antigens used in ELISA.

Sera against recombinant proteins rN and rH/Nh were able to neutralize the measles virus (see table). The data obtained reveal the high immunobiological activity of proteins studied.

Interactions of rN and rH/Nh recombinant proteins with human sera in ELISA. A panel of human sera was used to evaluate the specificity of recombinant proteins by ELISA. Data on the specificity inferred by ELISA were compared with those obtained using the Dade Behring test system (Siemens, Germany), the standard WHO-approved test for detection of IgG against the measles virus. The analysis of negative sera using the Dade Behring kit revealed optical density (OD) values of less than 0.1, while the positive sera showed OD readings of 0.48 ± 0.06. The negative sera did not react with rN and rH/N recombinant proteins showing OD values of less than 0.1. The positive sera interacted with the rN protein (OD readings of 0.36 ± 0.05) supporting specificity of the recombinant protein. Thus, not only the full-length polypeptide, as was shown earlier in [18], but also its fragments containing linear antigenic determinants, can be used for producing recombinant rN protein in *E. coli*. Furthermore, the C-terminal 6xHis tag can be used to purify recombinant protein, whereas the use of large affinity tags like β-galactosidase and maltose binding protein led to a loss of immunobiological activities of the recombinant protein [18]. Finally, the recombinant protein rN obtained in this work has a molecular weight 27 kDa less than that of intact protein (60 kDa) and can be used as an antigen for detecting IgG against the measles virus by ELISA.

An analysis of positive sera interactions with rH/N recombinant protein revealed the OD value of 0.26 ± 0.04. Negative sera produced lower readings of –0.1 in the same test. These data indicates that the interaction of sera with rH/N recombinant protein was rather specific. It has been shown previously that full-length H/Nh protein expressed in a eukaryotic system had a high specific reactivity with sera from measles-infected or vaccinated patients in ELISA, which makes it a good candidate for detecting specific IgM and IgG against the measles virus [7, 8, 10]. No data on the expression of H/Nh protein in a prokaryotic system are available at this juncture.

Thus, in the present study, we demonstrated the production of the rN and rH/N recombinant proteins of the measles virus in the prokaryotic expression system. The immunobiological properties of these proteins make them suitable antigens for ELISA.

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REFERENCES.

1. Belavin, P.A., Netesova, N.A., Reshetnikov, S.S., et al., *Biotekhnologiya*, 1997, no. 3, pp. 3–9.
2. Maniatis, T., Fritsch, E.F., and Sambrook, J., *Molecular cloning: a laboratory manual*, Cold Spring Harbour, 1982, 1st edition.
3. *Materialy VIII Vserossiiskogo S"ezda epidemiologov, mikrobiologov i parazitologov: sbornik statei* (Proceedings of VIII Russian Conference on Microbiology, Epidemiology and Parasitology), Moscow, 2002, vol. 2, p. 288.
4. Netesova, N.A., Belavin, P.A., Seregina E.V., et al., *Dokl. Acad. Nauk.*, 2004, vol. 397, no. 4, pp. 4–10.
5. *Manual for the laboratory diagnosis of measles virus infection*, *WHO*, Geneva, 2001.
6. Amann, E., Ochs, B., and Abel, K.J., *Gene*, 1988, vol. 69, no. 2, pp. 301–315.
7. Bouche, F., Ammerlaan, W., Berthet, F., et al., *J. Clin. Microbiol.*, 1998, vol. 36, no. 3, pp. 721–726.
8. Bouche, F., Ammerlaan, W., Fournier, P., et al., *J. Virol. Meth.*, 1998, vol. 74, no. 1, pp. 77–87.
9. Bouche, F.B., Brons, N.H., Houard, S., et al., *J. Clin. Microbiol.*, 1998, vol. 36, no. 12, pp. 3509–3513.

10. Buckland, R, Giraudon, P., and Wild, F., *J. Gen. Virol.,* 1989, vol. 70, part 2, pp. 435–441.
11. Fooks, A.R., Stephenson, J.R., Wames A., et al., *J. Gen. Virol.*, 1993, vol. 74, part 7, pp. 1439–1444.
12. Giraudon, P., Jacquier, M.F., Wild, T.F., *Virus Res.*, 1988, vol. 10, nos. 2–3, pp. 137–152.
13. Hartter, H.K., de Swart, R.I., Hanses., F., et al., *J. Virol. Meth.,* 2000, vol. 84, no. 2, pp. 191–200.
14. Hummel, K.B., Erdman, D.D., Heath, J., and Bellini, W.J., *J. Clin. Microbiol.,* 1992, vol. 30, no. 11, pp. 2874–2880.
15. Norrby, E. and Oxman, N.N., *Fields Virology*, Fields, B.N. and Knipe, D.M., Eds., New York: Raven Press, 1990, 2nd edition, pp. 1013–1014.
16. Parks, C.L., Lerch, R.A., Walpita, P., et al., *J. Virol.*, 2001, vol. 75, no. 2, pp. 910–920.
17. Rima, B.K., *J. Gen. Virol.*, 1983, vol. 64, pp. 1205–1219.
18. Warnes, A., Fooks, A.R, Stephenson, J.R., *J. Virol. Meth.*, 1994, vol. 49, no. 3, pp. 257–268.
19. Warnes, A., Fooks, A.R., Dowsett, A.B., et al., *Gene*, 1995, vol. 160, no. 2, pp. 173–178.
20. *Wkly Epidemiol. Rec.*, 2000, vol. 50, p. 411.
21. *Wkly Epidemiol. Rec.*, 1998, vol. 74, pp. 429–440.
22. Wiesmuller, K.H., Spahn, G., Handtmann, D., et al., *J. Gen. Virol.,* 1992, vol. 73, part 9, pp. 2211–2216.