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# Cellular immune response in infected mice to NSP protein encoded by the negative strand NS RNA of influenza A virus

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## ABSTRACT

Influenza A virus belongs to a family of enveloped viruses with an RNA genome of negative polarity consisting of 8 RNA segments. The transcription of this RNA genome results in the synthesis of positive-sense mRNAs that translate up to 16 unique viral proteins with the help of splicing and translational shift mechanisms. The 8th *NS* segment encodes the NS1 protein (27 kDa), which is an active interferon antagonist, and the nuclear export protein NEP (14 kDa) through the standard negative polarity pathway. In addition, an alternative open reading frame for the synthesis of a third viral protein (NSP, negative-strand protein) by means of a direct translation of genome polarity RNA (the so-called positive polarity genome strategy) was identified in the *NS* segment. Since it is unknown as to whether the NSP protein can be synthesized in the infected organism post viral infection, the generation of spleen leucocytes specific to this protein was studied in mice after two sequential infections with influenza A viruses of H1N1 and H3N2 subtypes. It was found that leucocyte clones specifically recognizing a peptide domain in the central region of the NSP protein (amino acid positions 82-119) were generated in mice infected with influenza A viruses. *In silico* prediction has shown strong major histocompatibility complex-1 (MHC-I) and MHC-II specific epitopes in this central domain of the NSP. Comparative analysis of the influenza H3N2 viruses circulating in humans during 1968-2018 has shown high NSP variability, which was similar to that shown for the hemagglutinin (HA) and neuraminidase (NA) proteins. The highest variability was found to be in the N- and C-terminal parts of the NSP. These observations suggest that synthesis of the NSP protein occurs in infected animals and further support a bipolar (ambisense) strategy of the RNA genome of human influenza A virus.

## INTRODUCTION

Influenza A virus (*Orthomyxoviridae* family, alphainfluenzavirus genus) is an enveloped virus with an RNA genome that has a negative-sense strategy for replication and expression in infected cells. The virus genome consists of 8 negative-sense RNA segments; each of them serves as a template for the synthesis (transcription) of the positive-sense mRNAs, which are translated in the infected cells to produce 16 viral proteins, using splicing and a translational frame shift [1, 2]. The eighth *NS* RNA segment encodes for two proteins by means of classical negative-sense strategy – the nonstructural protein NS1 (27 kDa), which is an active interferon (IFN) antagonist, and nuclear export protein NEP (14 kDa) [1].

It was previously reported that an alternative pathway for the synthesis of the third viral protein by the direct translation of virion negative-sense vRNA is encoded in the *NS* segment as shown in Fig. 1 [3-5]. An open reading frame (ORF) for an additional viral protein, the so-called negative-strand protein (NSP), was identified in the *NS* segment of the overwhelming majority of human influenza A viruses [6]. Study of the evolution of the *NSP* gene over time by the method of multi-metric analysis of gene variability in different viral strains showed that the *NSP* gene appeared in a population of human influenza A viruses in the early twentieth century [6]. In the vast majority of avian influenza A viruses, the *NSP* gene in the *NS* segment is blocked by a series of nonsense stop codons. Based on the analysis of the primary structure of the *NSP* gene of various virus strains, the corresponding protein can be considered as transmembrane since it has two distinct hydrophobic domains at the N- and C-termini of the polypeptide molecule and one N-glycosylation site [4].

According to our previous results, the *NSP* gene of influenza A virus protein inserted into the insects' baculovirus genome (nuclear polyhedrosis virus) can be expressed in the virus-infected ovarian cells of the cabbage looper (H5 cell line) [7]. The synthesized protein had perinuclear localization and, possibly, was associated with the Golgi apparatus membranes due to its transmembrane properties. Recently, we have shown that the full-size RNA of

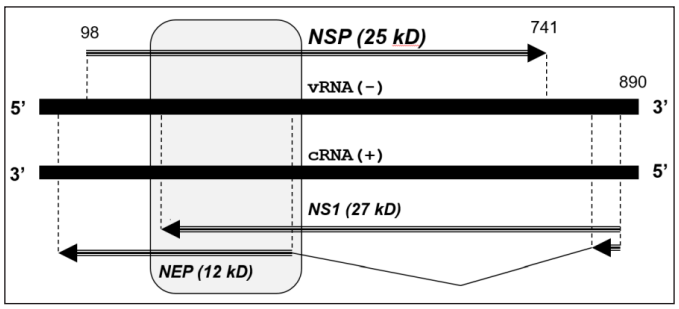


**Fig. 1.** The scheme of transcription and translation of the *NS* vRNA segment of influenza A virus.

Schematic representation of genes in the *NS* segment of influenza A/Aichi/2/68 (H3N2) virus is shown. vRNA – negative-sense virion RNA; cRNA – positive-sense complementary RNA (replicative form). Numbers indicate the positions of the nucleotides from the 5'-end of the vRNA. The exons of the genes encoding for the NS1, NEP and NSP proteins are indicated by arrows. The broken line shows the splicing zone of the mRNA *NEP* gene. The grey field indicates the overlapping region of three viral genes: *NS1*, *NEP*, and *NSP*.

segment 8 (*NS*) of human influenza A/Aichi/2/68 (H3N2) virus can be recognized by the ribosomes of mammalian cells *in vitro*. This RNA was able to initiate the synthesis of 2 viral polypeptides with a molecular weight (MW) of 23 and 13 kDa, which specifically reacted with antibodies specific to the $NSP_{82-119}$ peptide representing the central part of the NSP protein of influenza A virus [8]. These results confirm that the vRNA of segment 8 of human influenza A virus has a messenger translation function that is in accordance with the concept of bipolar (ambisense) influenza A viral genome strategy. Currently, the significance of the detected gene and the possibility of NSP protein expression during virus replication have not yet been established [9-12]. The importance of the positive-polar gene in the influenza A virus has been proved by the fact that, since the beginning of the 20th century until the present time, this gene remains fully functional in the genome of human viral strains despite the pronounced drift variability of the viral genome [6]. It is not yet known how and in what cells the NSP protein of the influenza A virus can be synthesized, but over the course of its synthesis in the host organism, specific humoral and/or cellular response to this viral protein may be generated. This specific immune response will serve as the confirmation of the synthesis of this viral protein in the infected host organism.

In order to check this hypothesis, we investigated the formation of the lymphocytes specific to the $NSP_{82-119}$ peptide, after the double infection of mice with influenza A virus.

## MATERIALS AND METHODS

### Viruses

Influenza virus A/WSN/33 (H1N1) and the reassortant of A/Aichi/2/68 (H3N2) virus with A/WSN/33, containing one *NS* segment from A/WSN/33 virus and the rest of the genes from A/Aichi/2/68 (H3N2) (named as A/Aichi/2/68-WSN) were used in this study. The reassortant A/Aichi/2/68-WSN (H3N2) was obtained by the reassortment method at the Gamaleya Scientific Research Institute of Epidemiology and Microbiology. Both viruses were propagated in the allantoic cavity of 9-day-old chicken embryos.

### Virus titration

The infectious titer of viruses was determined by the method of infectious foci formation in the Madin Darby canine kidney (MDCK) cells, as described previously [6, 13]. The infectious activity of virus preparations was expressed in focus-forming units (FFU).

### Production of recombinant NSP protein in bacterial cells

For the synthesis of NSP protein in the *E. coli* bacterial system (BL21/DE3)pLysS, IPTG-inducible vector pET30a and the *NSP* gene sequence (amino acid positions 27-158) of the influenza A/WSN/33 (H1N1) virus strain (the GenBank accession number M12597.1) were used. In order to reduce the toxicity of the synthesized NSP protein in *E. coli* cells, the *NSP* gene sequence was altered in such a way that its translation resulted in the protein lacking the N-terminal hydrophobic peptide of 26 amino acids. The shortened *NSP* gene was amplified by PCR using NSP (-sp)/Bgl/fo (tat aag atc tcc aag cga atc tct gta g) and NSPwsnR1/re (tat cga att cgg aac tga cat gac tct tga gg) primers adding two 6xHis sequences at each N and C ends of the *NSP*. The obtained *NSP* gene fragment was integrated into pET30a between the Bgl-II and Eco-R1 sites. Competent (BL21/DE3)pLysS cells were transformed with pET30a-NSP(-26)-his+ plasmid using transformation TSS buffer with the heat shock at 42°C by the standard procedure. Positive colonies were collected post overnight cultivation in kanamycin Luria-Bertani (LB) medium. For NSP protein synthesis (calculated MW 19.5 kDa), the resulting culture was mixed with 4 parts of LB, maintained for 1.5 h at 37°C, and after the addition of IPTG to a final concentration of 1 mM incubated for 3.5 h at 25°C. The cells were pelleted, destroyed by sonication, and centrifuged at 12,000 g for 20 min at + 4°C. The precipitates were dissolved in 5M urea solution prepared with 50 mM phosphate buffer (PBS) (pH 7.3) containing 0.3% of NP-40 detergent and protease inhibitors cocktail (Calbiochem). Protein purification was performed on Ni-NTA agarose (Qiagen) according to the manufacturer's protocol. The elution of the protein was carried out with buffer containing 0.3 M NaCl, 0.4 M imidazole, and 50 mM PBS (pH 6.0). NSP protein fraction was dialyzed against 50 mM PBS (pH 7.3). The NSP protein content in the obtained preparations was about 60-80%.

### Infection of mice with influenza A viruses

The Balb/c mice (weighing 10-12 g) were used for the experiments. All of the animal experiments were approved by the Ethics Committee of the Gamaleya Scientific Research Institute of Epidemiology and Microbiology. Experimental procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory

Animals. All of the painful procedures were performed under anesthesia. Animals were infected first with influenza A/WSN/33 (H1N1) virus, and in 4 weeks with the reassortant virus A/Aichi/2/68-WSN (H3N2). Mice were infected intranasally by inhaling virus aerosol ($10^5$ FFU/ml) obtained by the sonication of virus suspension for 2 min using a Musson-1 ultrasonic nebulizer (Russia). The infective dose was 10-100 FFU/mouse. On the 21st day after infection, the blood sera of animals were collected. The presence of specific antibodies to the influenza virus was determined using the hemagglutination inhibition assay (HAI) carried out according to the standard procedure. To determine the influenza virus in the mouse lungs, the broncho-alveolar washes were collected by washing the lungs with 1 ml of PBS solution through the trachea on the 3rd day after the second infection. The presence of the virus in the washes was determined by virus titration according to the method of infectious foci formation in the MDCK cells, as described hereinabove.

### Fractionation of leukocytes from the spleen of mice infected with influenza virus

The spleen of each mouse was mechanically disrupted to obtain a single cell suspension and clarified by centrifugation at 170 g for 10 min (Eppendorf 5804/R, FL 100 rotor). The level of viable cells in the supernatant was not lower than 80% according to trypan blue staining. The cells from each mouse were added to a 96-well plate in the amount of 1.1-1.6 x $10^6$ cells/well and cultured for 24 h at 37°C in RPMI 1640 medium supplemented with 10% of fetal bovine serum (Gibco BRL). Then, the studied preparations: peptide $NSP_{82-119}$ (1 μg/ml), NSP protein (2.5 μg/ml), or virus A/Aichi/2/68-WSN (H3N2) with the multiplicity of infection of 0.01 FFU/cell were added. Bovine serum albumin (BSA) with the final concentration of 3 μg/ml was added to the control cells. After the subsequent incubation for 20 h, cells from each well were harvested and pelleted. RNA was isolated from the pellets followed by analyzing the level of IFNγ mRNA and ribosomal 28S RNA using real-time PCR (RT-PCR).

### Determination of the IFNγ level in leucocytes by RT-PCR

In order to study the cellular immunity of mice after double infection with influenza viruses, the degree of leucocyte immune reactivity to NSP protein in the mice was assessed. The polypeptide of 38 amino acids with the sequence *CTSSSVCPGREGSGEISPTIVPSSVKALSNIRVSSRSK* corresponding to the central part of the NSP protein of influenza A/WSN/33 (H1N1) (amino acid positions 82-119) and common for the most viral strains, was synthesized at Quyun (China) (purity 90%) and was used as a target antigen. The first amino acid leucine (L) was replaced with cysteine (C) for conjugation with the keyhole limpet hemocyanin (KHL) carrier protein during immunization [7]. The degree of the leukocyte's reactivity was evaluated by the level of mRNA of IFNγ measured by RT-PCR with the primers specific for murine IFNγ (Table 1) after the contact with polypeptide $NSP_{82-119}$, purified recombinant protein NSP or influenza virus. The RNA from leukocytes was isolated using the Proba-NK kit according to the manufacturer's protocol (DNA-Technology LLC, Russia). The cDNA corresponding to this mRNA was synthesized using a random primer according to the Reverta-1 kit protocol (AmpliSense LLC, Russia). The obtained cDNA was used for PCR with primers to the IFNγ gene. To normalize the obtained values for IFNγ mRNA, the level of murine ribosomal 28S RNA was evaluated simultaneously using the primers specific for ribosomal 28S RNA (Table 1). The mRNA IFNγ content was expressed in arbitrary units (AU), normalized by the number of ribosomal 28S RNA in the analyzed sample.

### Electrophoresis in polyacrylamide gel and Western blot (WB) analysis

The proteins were fractionated in a polyacrylamide gel (PAAG) containing sodium dodecyl sulphate (SDS). Then, the proteins were transferred from the gel to a nitrocellulose membrane Protran 0.45 μm (Schleicher & Schull) by semi-dry method [13]. The membrane was washed with PBS, incubated for 2 h in 3% skim milk, and then overnight at +5°C in PBS containing 0.5% BSA (Sigma) and specific primary antibodies. The primary antibodies were obtained in guinea pig against $NSP_{82-119}$ oligopeptide according to the procedure described earlier [7]. The membrane was then washed with PBS and treated with species-specific antibodies conjugated with horseradish peroxidase (Dako, USA), followed by the identification of positive components by chemiluminescence (ECL) with a super-substrate (Pierce, USA), as previously described [13].

## RESULTS

### Variability of the *NSP* gene in human influenza H3N2 viruses.

At first, we estimated the variability of the *NSP* gene and the corresponding protein over the course of

**Table 1.** Primers used for the detection of mRNA of IFNγ and ribosomal mouse 28S RNA

| Primer assignment | Primer structure |
|---|---|
| 1. Primer for the synthesis of 28S RNA fragment (F) | *5'-GCTCATTAAATCAGTTATGGTTC-3'* |
| 2. Primer for the synthesis of 28S RNA fragment (R) | *5'-GAGGTTATCTAGAGTCACCA-3'* |
| 3. Probe for the detection of 28S RNA | *P [R6G]-CGCTCGCTCCTCTCCTACTTGG-[BHQ2]* |
| 4. Primer for the synthesis of IFNγ fragment (F) | *5'-GCCTAGAAAGTCTGAATA-3'* |
| 5. Primer for the synthesis of IFNγ fragment (R) | *5'- CCAGATATCCAAGAAGAG -3'* |
| 6. Probe for the detection of IFNγ | *P [R6G]-TCTTCCACATCTATGCCACTTGA-[BHQ2]* |

natural evolution of influenza virus. For this purpose, the primary *NSP* gene structure of influenza viruses of H3N2 subtype circulating in humans from 1968 (when this viral subtype emerged) to the present time was analyzed. The dynamics of mutation occurrence in the NSP protein is shown in Fig. 2. It is evident that the primary structure of the NSP protein has changed significantly, and mainly the N- and C-terminal regions of the protein were affected, whereas in the central part only minor changes occurred. To assess the rate of variability, the coefficient of variation (CV) of the protein was calculated. The CV shows the number of mutations per 100 amino acids in the protein per year (Table 2). The CV of NSP protein was 0.34, which is similar to the CV of hemagglutinin (HA) and neuraminidase (NA) – 0.4 – that are the most variable surface proteins of influenza virus. The obtained results also suggest that the significant variability of the N-terminal NSP region (Fig. 2) was determined by the changes in the *NSP* gene sequence leading to multiple mutations in the NSP protein but not in the NEP and NS1 proteins since both of them have much lower variability – CV 0.08 and 0.26, respectively (Table 2). The presented data show that the NSP protein is exposed to significant evolutionary variability probably due to its adaptation to tissue factors, and/or viral proteins with which it might interact and


**Fig. 2.** The mutations accumulation dynamics in the NSP protein of the human influenza H3N2 viruses in the course of the natural evolution from 1968 to 2018.

The chart was composed using the influenza virus *NS* gene sequences of viruses isolated in the period from 1968 to 2018 and presented in the GenBank database.

**Table 2.** The evolutionary variability of proteins of A/H3N2 influenza viruses isolated in the period 1968-2018

| Viral protein | Molecular weight (Da) | The number of analyzed sequences over 50 years | Number of detected mutant positions | Coefficient of variation (CV)[a] |
|---|---|---|---|---|
| **PB1** | 86,000 | 13,215 | 23 | 0.05 |
| **PB2** | 87,000 | 13,201 | 34 | 0.08 |
| **PA** | 85,000 | 13,183 | 47 | 0.1 |
| **HA** | 75,000 | 19,639 | 139 | 0.4 |
| **NP** | 56,000 | 13,505 | 58 | 0.2 |
| **NA** | 56,000 | 17,147 | 114 | 0.4 |
| **M1** | 27,000 | 16,145 | 12 | 0.09 |
| **NS1** | 27,000 | 13,847 | 34 | 0.26 |
| **M2** | 14,000 | 15,438 | 7 | 0.14 |
| **NEP** | 14,000 | 13,739 | 5 | 0.08 |
| **NSP** | 25,000 | 14,331 | 37 | **0.34** |

[a] The evolutionary variability of the protein was assessed by the CV, which was determined by the number of amino acid positions in a separate viral protein that underwent mutations in the human influenza A/H3N2 virus strains from 1968 to 2018. CV was calculated as the total number of mutant amino acid positions in each protein in one year per 100 amino acids. The sequence data were obtained from GenBank.

change concurrently or as a result of escape from the host immune factors.

### *In silico* analysis of the immunological domains in the NSP protein structure

The results of the computational analysis of the predicted T- and B-cell humoral epitopes based on the primary structure of influenza A virus NSP protein are shown in Fig. 3. T-cell epitopes were identified using software for predicting the binding of peptide domains to the receptors of the major histocompatibility complex-1 (MHC-I) [14] and MHC-II [15]. B-cell humoral epitopes were evaluated based on the antigenic index algorithm according to Jameson and Wolf [16]. As shown in Fig. 3, three T-epitope regions are predicted in the NSP protein – in amino acid positions 10-25, 40-50, and 85-110 (Fig. 3f, g). B-cell epitope regions are predicted by the software in three sites – amino acid positions: 70-80, 85-95, and 110-120 (Fig. 3e) – which are characterized by low hydrophobicity (Fig. 3d) and partially overlap with one MHC-II epitope (Fig. 3g). These results indicate that the $NSP_{82-119}$ peptide used in this study includes two neighboring epitopes recognized by both MHC-I (Fig. 3f) and MHC-II (Fig. 3g) receptors. The presence of such epitopes in the $NSP_{82-119}$ molecule could provide the immune response of both B- and T-cell types in virus-infected mice.

### The recombinant NSP protein

The identity of the recombinant NSP protein expressed in *E. coli* BL 21(DE3)pLysS cells was analyzed by the

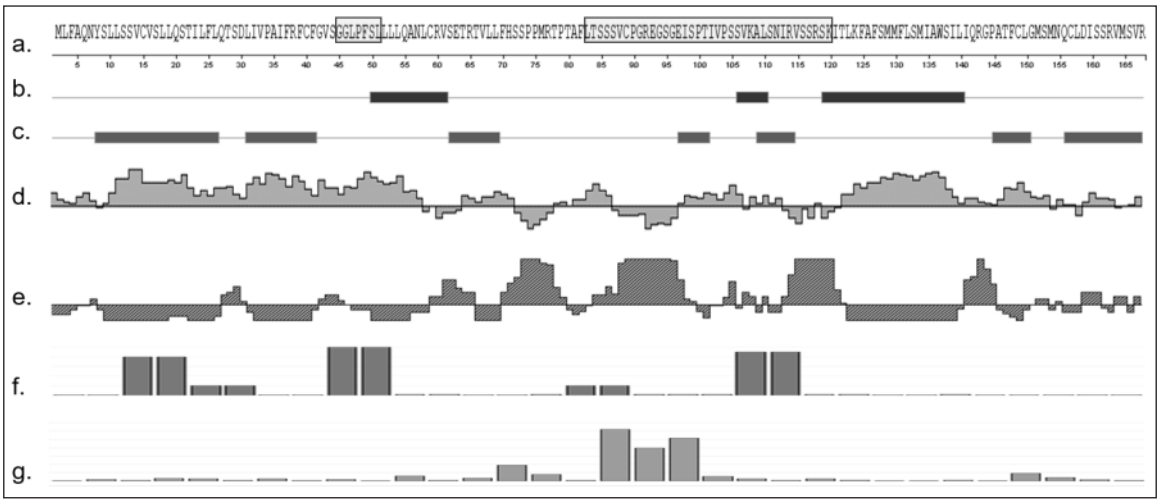


**Fig. 3.** The domain structure of influenza A/WSN/33 (H1N1) virus NSP protein.

The primary structure of the NSP protein (MW 19 kDa), compiled on the basis of nucleotide sequence of influenza A/WSN/33 virus (H1N1) *NSP* gene: (a) amino acid sequence of the NSP protein is presented in a single letter code; (b) hydrophobicity profile as determined by the Kyte-Doolittle method; (c, d) the areas of α-helices and β-sheets, respectively, identified by the Chou-Fasman method; (e) B-cell humoral epitopes predicted by the Jameson-Wolf antigenicity index method; (f, g) T-cell epitopes predicted by the algorithm of interaction with MHC-I and MHC-II receptors. The ordinate axis shows the number of MHC-I (f) alleles capable of recognizing each of these epitopes, and the affinity index of the interaction of 12 amino acids epitopes with MHC-II in the NSP molecule (g). The frames show the amino acids 44-51 and 82-119 positions, corresponding to the peptides studied in the paper published by Zhong *et al*. [17] and in the present study, respectively.

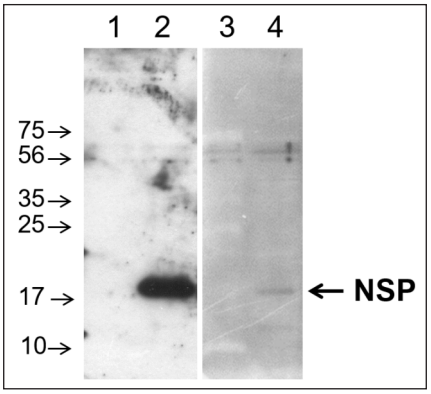


**Fig. 4.** Analysis of the recombinant NSP protein by the Western blot.

The recombinant influenza virus A/WSN/33 (H1N1) NSP protein, flanked on the N and C termini by 6xHis peptides, was expressed in competent BL21 (DE3) pLysS *E. coli* cells. After purification on the Ni-NTA agarose, it was analyzed by electrophoresis in PAAG with subsequent Western blot analysis. Lanes 1, 3 – control preparation of non-transformed BL21 (DE3) pLysS cells; lanes 2, 4 – preparation of NSP protein purified from BL21(DE3) pLysS cells transformed with pET30a-NSP(-26)-his+. Tetra-His antibodies (lanes 1, 2) and antibodies to $NSP_{82-119}$ peptide (lanes 3, 4) were used for primary staining. The positions of the marker proteins are shown on the left, with indicated MW (kDa).

Western blot analysis with specific antibodies obtained for $NSP_{82-119}$ peptide and for the 6xHis oligopeptide. The NSP protein of A/WSN/33 virus containing two 6xHis peptides on the N- and C-termini was synthesized according to the method described in the Materials and Methods. As shown in Fig. 4, the NSP protein synthesized in bacterial cells reacted with both anti-$NSP_{82-119}$ and anti-6xHis antibodies (Fig. 4, lane 2 and 4). These results confirm that the recombinant protein expressed in *E. coli* cells corresponds to the NSP protein of influenza A/WSN/33 virus.

## Sequential infection of mice with two influenza viruses of H1N1 and H3N2 subtypes

The preliminary attempts to measure the distinct cellular immune response to the NSP protein after a single influenza infection of mice were unsuccessful. In order to enhance the expected immune response, the mice were infected twice with two influenza strains causing the disease with an interval of 30 days. For this purpose, the animals were infected first with influenza A/WSN/33 (H1N1) virus carrying *NS* gene encoding NSP protein (18 kDa) [4]. After the recovery from the primary infection, the animals were reinfected with heterologous reassortant A/Aichi/2/68-WSN (H3N2) virus, in which the *NS* genomic segment was replaced by that of A/WSN/33 virus.

The level of the disease after primary infection with A/WSN/33 virus was assessed by measuring the loss of body weight, a parameter that is known to reflect the morbidity. As shown in Fig. 5, the slowdown in weight gain was observed in mice from the 3rd to 8th day after infection by aerosol influenza virus compared to the uninfected mice. The increase of animal weight was recovered by the 15th day post infection. In the animals' sera, obtained on the 21st day post immunization, the anti-HA antibodies against A/WSN/33 virus were detected with titers that ranged from 1/40 to 1/160 (the data are not shown) as measured by HAI assay. These data confirm that all of the animals were properly infected with the virus.

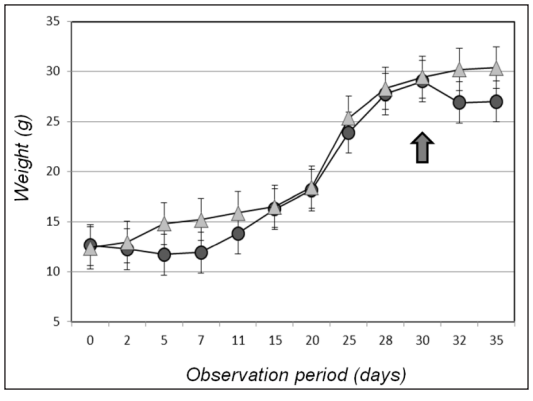


**Fig. 5.** The dynamics of the mouse weight changes upon infection with the influenza viruses.

BALB/c mice were infected (day 0) with A/WSN/33 (H1N1) virus. On the 30th day, the mice were reinfected with the reassortant A/Aichi/2/68-WSN (H3N2) virus carrying the *NS* gene of A/WSN/33 (H1N1). The arrow shows the day of reinfection. Each mouse was weighed during the observation period. The body weight curves for non-infected (▲) and infected (●) groups of mice are shown. The data are presented as M±SD.

Four weeks after the first infection, the animals were reinfected by the aerosol administration of a sublethal dose of A/Aichi/2/68-WSN (H3N2) reassortant. As in the case of primary infection, the animals showed a slowdown of weight gain confirming the infection process. The virus titers measured in the bronchopulmonary washes on the 3rd day post reinfection, varied in the range of $10^2$–$10^4$ FFU/ml (data not shown). These results confirmed the development of productive virus infection after the second infection.

On the 5th day after boost immunization, mice were sacrificed, the spleen was removed and processed to isolate the leukocyte fraction, in which the level of sensitization to $NSP_{82-119}$ peptide or to NSP protein was tested.

## The NSP-dependent immune reactivity of leukocytes obtained from the infected animals

One of the methods for assessing the cellular immune response after viral infection in animals is the determination of immune leukocytes that are measured by the level of IFNγ induced by the leucocytes of infected animals exposed *in vitro* to the viral proteins or their peptides. The level of IFNγ can be assessed by the measurement of the corresponding mRNA by quantitative RT-PCR [18]. The leucocytes of infected mice were sensitized with $NSP_{82-119}$ peptide and the level of IFNγ mRNA was determined by RT-PCR. In parallel, leukocytes were sensitized with two preparations of purified NSP protein and with the whole virus. As shown in Fig. 6, the level of IFNγ induction in response to the stimulation of leukocytes by $NSP_{82-119}$ peptide – 2.3 AU – was significantly higher ($p<0.05$) than the values obtained for the control leukocytes stimulated with BSA (0.7 AU). The amount of IFNγ induced in response to the stimulation of leukocytes by both recombinant protein NSP preparations was even higher (12.0

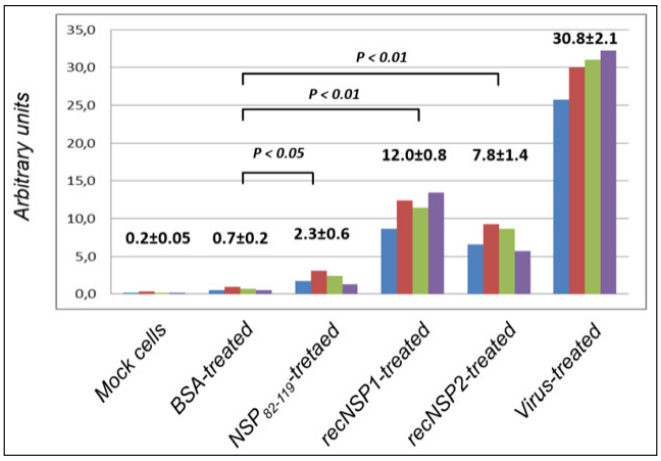


**Fig. 6.** The levels of IFNγ mRNA in leucocytes of the infected mice stimulated by the target antigens.

Leukocytes were isolated from the spleens of infected mice and sensitized with: (1) BSA; (2) $NSP_{82-119}$ oligopeptide; (3, 4) recombinant NSP protein from two different batches of isolation, respectively; (5) influenza A/Aichi/2/68-WSN. The bars show the values of mRNA in AU for each of 4 mice. The mean values for the group are shown above the columns (M±SD). Mock cells – leucocytes obtained from uninfected mice. The significance of the differences between the groups was evaluated by the Student's t-test.

and 7.8 AU, p<0.01). The highest levels of expression of IFNγ (30.8 AU) were found in leukocytes sensitized by the virus reflecting the cumulative effect of epitopes present in the target antigens on peptide, recombinant protein and whole virus.

The control testing of IFNγ induction by the leukocytes in non-infected animals sensitized with $NSP_{82-119}$ peptide did not show the reliable induction of IFNγ (0.06 AU, p>0.05) (Table 3). A slight (0.78 AU) increase in IFNγ induction after stimulation of leukocytes of uninfected mice with recombinant NSP protein was comparable to the response to the unspecific stimulation of leucocytes with BSA (0.7 AU) that is most likely due to the residual impurity of bacterial proteins in the NSP protein preparation. A significant induction of IFNγ upon stimulation of leukocytes by the virus quite logically reflects the primary immune response of the splenocytes to virus infection.

**Table 3.** Level of IFNγ induction in the splenocytes of uninfected mice

| Antigens used for stimulation | The level of the induced IFNγ (AU) | Differences significance |
|---|---|---|
| 1. mock | 0.04 ± 0.02 | — |
| 2. $NSP_{82-119}$ | 0.06 ± 0.02 | 2 versus 1, p> 0.05 |
| 3. NSP | 0.78 ± 0.26 | 3 versus 1, p<0.05 |
| 4. A/Aichi/2/68-WSN | 14.50 ± 5.1 | 4 versus 1, p<0.01 |

The levels of IFNγ mRNA expressed in AU determined in leucocyte samples obtained from uninfected mice (4 per group), sensitized with: (1) intact splenocytes (mock); (2) $NSP_{82-119}$ peptide, (3) recombinant NSP protein; (4) A/Aichi/2/68-WSN virus. The data are presented as M±SD. The statistical significance of the differences is estimated by the Student's t-test.

The obtained results show that the development of influenza infection in mice stimulated the generation of sensitized white blood cell clones, which recognize the epitopes of viral proteins, including the NSP protein, and induce the production of IFNγ.

## DISCUSSION

An extended ORF with ATG start and TAG stop codons of a full gene, and structural elements for recognition by ribosomes was revealed in the smallest *NS* segment of the influenza A virus genome besides the *NS1* and *NEP* genes [8]. The main feature of the ORF of *NSP* gene is that it is located in the negative-sense RNA segment, in contrast to the ORF of *NS1* and *NEP*, which are known to be of positive polarity, complementary to the vRNA (Fig. 1). The special polarity of the *NSP* gene suggests a specific mechanism of its expression, which should be different from that of other viral proteins. It can be assumed that there is a direct translation of a free negative-sense *NS* vRNA in infected cells prior to its encapsidation by the nucleoprotein (NP). NP protein forms viral ribonucleoprotein (RNP) complexes (nucleocapsids) with vRNA and, probably, acts as a negative translational regulator, closing the access of ribosomes to vRNA. The mechanism of positive regulation through the viral NS1 protein, which can enhance the translation of genomic RNA in infected cells, cannot be ruled out [19-21]. It is possible that cellular factors play a decisive role in the NSP expression and determine the tissue tropism of this protein synthesis in an infected organism [22]. Finally, an alternative specific mechanism of a subgenomic mRNA/NSP synthesis on the viral cRNA template in flu virus-infected cells also cannot be excluded.

The assumption of direct *NS* vRNA translation is consistent with recent observations that showed the ability of the virion full-length *NS* RNA of influenza A/Aichi/2/68 (H3N2) virus to be effectively translated *in vitro* by mammalian cell ribosomes to form a 23 kDa and a 13 kDa polypeptides that specifically reacted with antibodies to the $NSP_{82-119}$ peptide in immune precipitation reaction [8]. This result suggests the possibility of a specific translation of the *NS* negative polarity RNA of influenza virus in infected host cells.

The data obtained in the present study most likely indicate the generation of a specific cellular immune response to the viral NSP protein in mice infected with influenza A virus. This conclusion implies the formation of NSP protein (or its components) during viral replication in the body of infected animals. Indirect proof of the expression of the *NSP* gene was recently obtained in experiments with the recombinant influenza A/Puerto Rico/8/1934 (A/PR/8) virus containing a chimeric *NSP* gene with the expression reporter in mice [23]. The results of our study are also consistent with the observations of Zhong *et al*. [17], who examined the immune profile of mouse leukocytes after infection with A/PR/8 (H1N1) virus. The authors found leukocyte clones reacting with GGLPFSLL peptide, denoted by the authors as a "hypothetical peptide". It is important to mention that both the $NSP_{82-119}$ and the GGLPFSLL peptide, identical to the 44-51 amino acid positions of the A/WSN/33 NSP, correspond to the T-cell epitopes predicted in the NSP protein structure (Fig. 3g, f). Therefore, these results confirm the expression of the *NSP* gene in an infected organism, and the presence of T-cell epitope in the central part of the encoded NSP protein.

Based on the available data, it remains a challenge to determine the level of synthesis and tissue localization of NSP protein (or its products) in the infected animals. Moreover, the earlier attempts to detect mature NSP protein *in vitro* in cell culture infected with influenza A virus were unsuccessful [23], (Arikainen A. et al., Does segment 8 of influenza A virus encode a negative-strand polypeptide? 4th Influenza ESWI Conference, Malta 2011, abstract B305P, 179). The difficulties of the NSP protein detection may be due to its rapid proteolysis with the formation of short functional peptides of the kinin type. It is possible that the expression of the *NSP* gene may be limited to certain types of cells that depend on specific factors present only in certain tissues of the host organism.

The important question is how the new ambipolar *NSP* gene could emerge in the genome of influenza A virus. The appearance of ambipolar gene suggests the possibility of the regulation of expression of the ambisense genes in RNA molecule by the rules that are currently unknown (or by the reverse determination rule). The

presence of such a region in the center of the *NSP* gene is marked with grey field (Fig. 1). The other two sites of the *NSP* gene overlap with either the *NEP* gene (at 5' end) or with *NS1* (at 3' end). It is quite possible that a certain gene in the virus genome can predetermine the mechanism of the ambipolar gene emergence and its properties. It is assumed that in the absence of determination mechanism, random mutations are accumulated, leading to the emergence of a new functionally semantic gene, followed by selection. The probability of such an event is low, given the ambipolar overlapping of several genes, when changes in one gene lead to changes in the linked ambipolar genes. In this case, the variability and selection of mutations should be linked together in three viral genes: *NS1, NEP*, and *NSP*.

The conclusion based on the obtained results may be important for the classification of influenza viruses. It raises the question of the existence of an additional genus in the *Orthomyxoviridae* family, with the ambisense expression of the viral genome in the process of its life cycle, such as the representatives of known viral bipolar genera of phlebo-, tospo-, arena-, and tenuiviruses [24]. While the direct product of the *NSP* gene is not identified in infected cell cultures or human/animal organisms, the question of classification remains open so far. At the same time, the preservation of a functional *NSP* gene in a population of human influenza viruses for more than 100 years indicates the importance of this gene for the hypothetical group of ambisense influenza viruses [6]. It is noteworthy that the pronounced evolutionary variability of the NSP protein, similar to that of the most variable viral proteins HA and NA, did not cause nonsense mutations forming stop codons that would destroy the *NSP* gene supporting the idea of the biological determinism of this gene.

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## CONFLICT OF INTEREST

The authors do not pursue commercial or financial interests.

## CITATION

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## REFERENCES

1. Pflug A, Lukarska M, Resa-Infante P, Reich S, Cusack S. Structural insights into RNA synthesis by the influenza virus transcription-replication machine. Virus Res 2017; 234, 103-17. doi: 10.1016/j.virusres.2017.01.013.
2. Dou D, Revol R, Ostbye H, Wang H, Daniels R. Influenza A Virus Cell Entry, Replication, Virion Assembly and Movement. Front Immunol 2018; 9, 1581. doi: 10.3389/fimmu.2018.01581.
3. Baez M, Zazra JJ, Elliott RM, Young JF, Palese P. Nucleotide sequence of the influenza A/duck/Alberta/60/76 virus NS RNA: conservation of the NS1/NS2 overlapping gene structure in a divergent influenza virus RNA segment. Virology 1981; 113(1), 397-402. PubMed PMID: 6927848.
4. Zhirnov OP, Poyarkov SV, Vorob'eva IV, Safonova OA, Malyshev NA, Klenk HD. Segment NS of influenza A virus contains an additional gene NSP in positive-sense orientation. Dokl Biochem Biophys 2007; 414, 127-33. PubMed PMID: 17695319.
5. Beklemishev AB, Blinov VM, Vasilenko SK, Golovin S, Karginov VA. Synthesis, cloning and determination of the primary structure of a full-size DNA copy of fragment 8 from the influenza virus type A genome. Bioorg Khim 1985; 11(5), 641-5. PubMed PMID: 4038350.
6. Zhirnov OP, Vorobjeva IV, Saphonova OA, Poyarkov SV, Ovcharenko AV, Anhlan D, et al. Structural and evolutionary characteristics of HA, NA, NS and M genes of clinical influenza A/H3N2 viruses passaged in human and canine cells. J Clin Virol 2009; 45(4), 322-33. doi: 10.1016/j.jcv.2009.05.030.
7. Zhirnov OP, Klenk HD. Integration of influenza A virus NSP gene into baculovirus genome and its expression in insect cells. Vopr Virusol 2010; 55(2), 4-8. PubMed PMID: 20455463.

8. Zhirnov OP, Akulich KA, Lipatova AV, Usachev EV. Negative-sense virion RNA of segment 8 (NS) of influenza a virus is able to translate in vitro a new viral protein. Dokl Biochem Biophys 2017; 473(1), 122-7. doi: 10.1134/S1607672917020090.
9. Clifford M, Twigg J, Upton C. Evidence for a novel gene associated with human influenza A viruses. Virol J 2009; 6, 198. doi: 10.1186/1743-422X-6-198.
10. Gong YN, Chen GW, Chen CJ, Kuo RL, Shih SR. Computational analysis and mapping of novel open reading frames in influenza A viruses. PLoS One 2014; 9(12), e115016. doi: 10.1371/journal.pone.0115016.
11. Yang CW, Chen MF. Uncovering the Potential Pan Proteomes Encoded by Genomic Strand RNAs of Influenza A Viruses. PLoS One 2016; 11(1), e0146936. doi: 10.1371/journal.pone.0146936.
12. Sabath N, Morris JS, Graur D. Is there a twelfth protein-coding gene in the genome of influenza A? A selection-based approach to the detection of overlapping genes in closely related sequences. J Mol Evol 2011; 73(5-6), 305-15. doi: 10.1007/s00239-011-9477-9.
13. Zhirnov OP, Klenk HD. Alterations in caspase cleavage motifs of NP and M2 proteins attenuate virulence of a highly pathogenic avian influenza virus. Virology 2009; 394(1), 57-63. doi: 10.1016/j.virol.2009.08.013.
14. Bhasin M, Raghava GP. A hybrid approach for predicting promiscuous MHC class I restricted T cell epitopes. J Biosci 2007; 32(1), 31-42. PubMed PMID: 17426378.
15. Jensen KK, Andreatta M, Marcatili P, Buus S, Greenbaum JA, Yan Z, et al. Improved methods for predicting peptide binding affinity to MHC class II molecules. Immunology 2018; 154(3), 394-406. doi: 10.1111/imm.12889.
16. Jameson BA, Wolf H. The antigenic index: a novel algorithm for predicting antigenic determinants. Comput Appl Biosci 1988; 4(1), 181-6. PubMed PMID: 2454713.
17. Zhong W, Reche PA, Lai CC, Reinhold B, Reinherz EL. Genome-wide characterization of a viral cytotoxic T lymphocyte epitope repertoire. J Biol Chem 2003; 278(46), 45135-44. doi: 10.1074/jbc.M307417200.
18. Hempel DM, Smith KA, Claussen KA, Perricone MA. Analysis of cellular immune responses in the peripheral blood of mice using real-time RT-PCR. J Immunol Methods 2002; 259(1-2), 129-38. PubMed PMID: 11730848.
19. Liu Y, Chia ZH, Liew J, Or SM, Phua KKL. Modulation of mRNA Translation and Cell Viability by Influenza A Virus Derived Nonstructural Protein 1. Nucleic Acid Ther 2018; 28(3), 200-8. doi: 10.1089/nat.2017.0712.
20. Panthu B, Terrier O, Carron C, Traversier A, Corbin A, Balvay L, et al. The NS1 Protein from Influenza Virus Stimulates Translation Initiation by Enhancing Ribosome Recruitment to mRNAs. J Mol Biol 2017; 429(21), 3334-52. doi: 10.1016/j.jmb.2017.04.007.
21. Nordholm J, Petitou J, Ostbye H, da Silva DV, Dou D, Wang H, et al. Translational regulation of viral secretory proteins by the 5' coding regions and a viral RNA-binding protein. J Cell Biol 2017; 216(8), 2283-93. doi: 10.1083/jcb.201702102.
22. Jan E, Mohr I, Walsh D. A Cap-to-Tail Guide to mRNA Translation Strategies in Virus-Infected Cells. Annu Rev Virol 2016; 3(1), 283-307. doi: 10.1146/annurev-virology-100114-055014.
23. Hickman HD, Mays JW, Gibbs J, Kosik I, Magadan JG, Takeda K, et al. Influenza A Virus Negative Strand RNA Is Translated for CD8(+) T Cell Immunosurveillance. J Immunol 2018; 201(4), 1222-8. doi: 10.4049/jimmunol.1800586.
24. Nguyen M, Haenni AL. Expression strategies of ambisense viruses. Virus Res 2003; 93(2), 141-50. PubMed PMID: 12782362.