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LABORATORY CHARACTERIZATION OF INFLUENZA A VIRUSES (H3N2) — PATHOGENS OF LENINGRAD 1983 EPIDEMIC

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Summary. — Seventy strains of influenza A virus (H3N2) isolated in Leningrad during the influenza epidemic in January—March 1983 were studied. The virus isolates appeared to be markedly heterogeneous with respect to antigenic characteristics of surface glycoproteins, biological properties (haemagglutinating and neuraminidase activities, sensitivity to γ-inhibitors, etc.) as well as genome structure. The identity of the virus isolates with the reference influenza A virus was as follows: 10% were similar to A/Texas/1/77 virus, 30% to A/Bangkok/1/79 virus, and 20% to A/Philippines/2/82 virus. A large part of the isolated viruses (27 of 70) were characterized by a certain (mainly unilateral) relatedness with the virus A/Victoria/35/72. The isolates were extremely heterogeneous with respect to genomic characteristics as determined by cRNA-vRNA hybridization test and differed from the corresponding reference strains by the homology of genes 1—6.

Key words: influenza A virus; biological and antigenic properties; inhibitors; RNA-RNA hybridization

Introduction

Influenza A viruses (H3N2) entered into epidemic circulation as early as in 1968. Although it was rather a long time ago and pathogens of subtype A (H1N1) reappeared in human circulation in 1977, the A (H3N2) viruses still remain epidemically active. The influenza epidemic in 1983 was the 8th from the onset of pandemic A (H3N2) cycle (Ivannikov and Ismagulov, 1983). Like the two previous epidemics of this pandemic cycle (1976 and 1980), it was characterized by low morbidity indices in humans older than 15 years (Karpukhin *et al.*, 1983). The bulk of the influenza cases was registered in children and adolescents. Predominant morbidity in these groups can be explained by their immunologically unprotected state with respect to the pathogens of this epidemic (Karpukhin *et al.*, 1983).

The first influenza A (H3N2) viruses from 1983 epidemic were isolated in Leningrad on January 24. The virus isolations were without any serious difficulties, since during the epidemic period were isolated altogether 70 strains of influenza A (H3N2) viruses. The present paper deals with the analysis of isolated virus strains.

Materials and Methods

Viruses. The virus isolation from influenza patients was carried out in chick embryos using a conventional technique. During the epidemic period (24. 1. 1983 – 13. 3. 1983) out of 316 influenza and acute respiratory disease patients examined, 70 strains of influenza A (H3N2) virus were isolated (35 strains from 0 – 14-year-old children and 35 from patients aged 15 or more years). The bulk of virus strains (more than 60 %) was isolated already after primary infection of chick embryos, the rest required 1 or 2 additional passages.

Sera. Primary typing of isolated viruses was performed using diagnostic horse sera produced by Leningrad Research Institute of Vaccines and Sera. For more detailed identification hyperimmune strain-specific sera prepared by four-time intraperitoneal immunization of rats with allantoic virus at 4 – 5 day intervals were employed. The rats were bled 14 to 20 days after last immunization. Rabbit sera to recombinant strains for neuraminidase activity inhibition (NAI) were prepared according to Paniker (1968).

Haemagglutination inhibition (HI) test with the strain-specific sera was made by a conventional technique (one hr incubation of the serum with virus at 20 °C), using sera heated at 58 °C for 30 min and 1 % chick red blood cells. NAI was determined according to Aimard-Henry and Coleman (1974) at the temperature assuring complete inactivation of neuraminidase activity as described elsewhere (Aptekareva *et al.*, 1973).

Determination of ts-phenotype of the viruses was performed by their titration in chick embryos at 34 and 40 °C.

Analysis of the virus genome. The genome of influenza virus isolates was analysed on the basis of the study of double-stranded complexes cRNA/vRNA according to Hay *et al.* (1977); for detailed description see Ghendon *et al.* (1979). In brief: ^{3}H-uridine-labelled (All-Union Society Isotop, USSR, specific activity 1.1 TBq/mM) complementary RNA (cRNA) was isolated from the monolayer of infected chick embryo fibroblasts which were incubated in the presence of 100 μg/ml of cycloheximide (CH), hybridized with an excess of unlabelled virion RNA (vRNA), treated with SI nuclease and the complexes were analyzed in polyacrylamide gel (PAG). In separate experiments, monolayers of canine kidney continuous cell line (MDCK 78/78) have been used without CH treatment.

Results

Characterization of antigenic specificity of influenza A virus (H3N2) haemagglutinins

On the basis of cross-HI with strain-specific rat sera, the influenza A (H3N2) viruses could be classified into 5 rather clearly defined groups (Fig. 1):

1. A relatively small part of the strains A/Leningrad/83 (A/Len/83) (about 10%) was similar to the virus A/Texas/1/77.

2. A large part (~30%) of the isolated viruses was similar to reference strain A/Bangkok/1/79 (A/BK/1/79).

3. About 20% of the isolated strains were related to reference virus A/Philippines/2/82 (A/Phil/2/82). These strains resulting from antigenic drift of the viruses of subtype A (H3N2) lost their relation to reference viruses A/Victoria/35/72 (A/Vic/35/72) and A/Texas/1/77 (A/Tex/1/77), but kept close relations with the chronologically more related virus A/BK/1/79. The

Table 1. Antigenic characterization of neuraminidases of influenza A (H3N2) viruses isolated in Leningrad in 1983 (NAI test)

Influenza virus strain	Titres of the sera to recombinant strains containing neuraminidases of the viruses indicated (reciprocal values)			TIN_{100},* degrees centigrade
	A/Vic/35/72	A/Tex/1/77	A/BK/2/79	
A/Leningrad/83 Groups 1, 2, 3, 5	40—80	160—640	80—640	48—56
A/Leningrad/83 Group 4	640	1280	160	48
A/Vic/35/72	1280	640	640	48
A/Texas/1/77	160	1280	640	52
A/BK/2/79	160	320	1280	56
A/Phil/2/82	60	320	320	48

* Temperature of complete (100%) inactivation of neuraminidase activity of the virus (TIN_{100})

viruses of the 3rd group were characterized by bilateral relations with reference virus A/Phil/2/82. The serum to this strain actively inhibited haemagglutination activity of the isolates and the sera against the isolates interacted with strain A/Phil/2/82.

4. Viruses characterized by a marked relatedness with virus A/Vic/35/72 but dramatically differing from reference viruses A/BK/1/79 and A/Phil/2/82. Although the strains of this group readily interacted with the serum against A/Vic/35/72, they still differed from the virus A/Vic/35/72 with respect to the results of HI with a set of sera to a number of other influenza A (H3N2) strains. This indicates that viruses of this group are both similar to as well as different from the reference virus A/Vic/35/72. The viruses of group 4 were dramatically dissimilar from the strains of Bangkok or Philippine varieties (group 2 and 3). To the group 4 belonged as many as 27 of the 70 isolated virus strains (~40%).

5. Only 6 strains isolated in Leningrad in 1983 interacted with sera to the viruses of all the 4 groups, thus occupying an intermediate position between the viruses of Texas, Bangkok and Philippines varieties on one hand, and "Victoria-like" viruses on the other hand.

Therefore, according to HI findings, influenza A (H3N2) viruses isolated in Leningrad in 1983 were characterized by a marked heterogeneity of haemagglutinin (HA) antigenic pattern.

Characterization of antigenic specificity of influenza A/Leningrad/83 virus neuraminidase

Neuraminidase of the tested epidemic strains differed in the extent of relationship with antibodies to neuraminidase of A/BK/2/79 virus, and

Table 2. Characterization of the heterogeneity of antigenic and biologic properties of A/Leningrad/83 viruses

Antigenic and biological properties of influenza A/Leningrad /83 viruses		Values of the viruses of the groups indicated		
		1, 2 and 3	4	5
Intensity of interaction in HI with sera to the viruses indicated (as related to homologous titre)	A/Victoria/35/72	1/16—1/64	1/4	1/16
	A/Texas/1/77	1/2—1/8	1/16	1/2
	A/Bangkok/1/79	1/2—1/8	1/64	1/4
	A/Philippines/2/82	1—1/4	1/64	1/2
Degree of relationship of neuraminidase of strains investigated to the enzyme of the virus	A/Bangkok/2/79	1/2—1/16	1/8	1/2—1/16
Level of accumulation of the indicated activity				
haemagglutination (in 0.2 ml)		2—64	512—2048	64—128
infectivity (EID_{50}/0.2 ml)		$10^2—10^7$	$10^8—10^{10}$	$10^2—10$
neuraminidase (μg NANA in 1 ml)		15—100	200—600	15—100
Sensitivity to υ-inhibitors in HI with normal horse serum heated at 80 °C for 30 min*		1,280—40,960	10—10	160—640
RTC-marker — degree of inhibition of virus infectivity in the course of reproduction at 40 °C as compared to 34 °C				
(EID/0.2 ml)		$10^5—10^7$	$10^1—10^2$	$10^5—10^7$
TIN_{100}		48—56 °C	48 °C	48—56 °C

* Inverse values of serum titres.

reacted with the mentioned serum from 1/16 to 1/2 of the homologous titre (Table 1). Meanwhile, all the strains A/Leningrad/83 except for group 4 virtually lost their relationship with the enzyme of the virus A/Vic/35/72. The strains belonging to group 4 with respect to antigenic characteristics of HA actively interacted in NAI with antibodies to neuraminidase of reference virus A/Vic/35/72.

A/Len/83 viruses also dramatically varied with respect to the indices of temperature of 100% inactivation of neuraminidase activity (TIN): from 48 °C which is characteristic of reference virus A/Vic/35/72 and viruses of group 4 to 56 °C at which the enzyme of A/BK/2/79 was inactivated (Table 1).

The results obtained indicate that the isolated viruses are heterogeneous with respect to antigenic specificity of their neuraminidase and the temperature of enzyme inactivation.

Biological properties of influenza A/Leningrad/83 viruses

Characterization of biological properties of influenza A/Len/83 viruses is represented in Table 2. The virus yield in chick embryos dramatically varied from 1 : 2 to 1 : 2048 when determined by HA test. The viruses with high

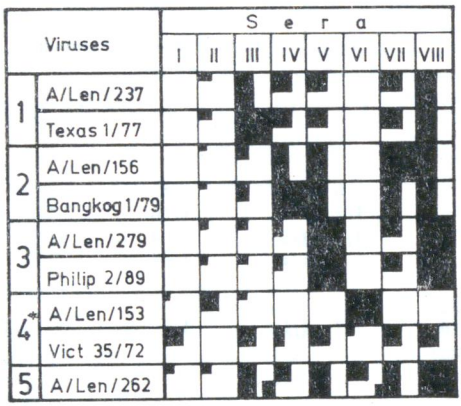

Fig. 1.

Cross-HI of influenza A/Leningrad/83 viruses

In the upper parts of each column the representative strain of each group is shown — (i.e. one of those isolated in Leningrad in 1983); in the lower part — a reference antigen is shown being the most related to the latter.

Sera:
I — to A/Hong Kong/68; II — to A/Vic/35/72; III — to A/Tex/ 1/77; IV — to A/BK/1/79; V — to A/Phil/2/82; VI — to A/Len/153/83; VII — to A/Len/156/83; VIII — to A/Len/279/83. Viruses of groups 1, 2, 3 and 5 were sensitive to γ-inhibitors; group 4 strains (*) were inhibitor-resistant.

yields of infectivity and haemagglutinating activity were resistant to γ-inhibitors of normal heated (80 °C during 1 hr) guinea pig and rabbit sera. Viruses with low and intermediate yields of infectivity and haemagglutinating activity were characterized by different sensitivities to γ-inhibitors (from 1 : 160 to 1 : 640 for the viruses of group 5 and from 1 : 1280 to 1 : 40 960 for the viruses of the first 3 groups, respectively).

Accumulation of neuraminidase activity of the isolates H3N2 of 1983 varied within broad ranges. Antigenic characteristics of the viruses was in a good correlation with their biological features. Thus, for instance, viruses of groups 1, 2 and 3 consisting of Texas, Bangkok, and Philippines varieties were characterized by a low virus yield with respect to infectivity and haemagglutinating activity, were highly sensitive to γ-inhibitors (1 : 2 560 to 1 : 40 000 and higher) and did not essentially reproduce in chick embryos at 40 °C.

The strains of group 4 having an unilateral relationship with the virus A/Vic/37/72 were inhibitor-resistant and had high yields of infectivity and haemagglutinating activity, reproducing intensively at 40 °C.

Viruses of group 5 ("intermediate" strains) had a moderate sensitivity to γ-inhibitors (1 : 160—1 : 1280), intermediate indices of the yields of infectivity and haemagglutinating activity and did not reproduce at 40 °C. Thus, heterogeneity of the isolated viruses was registered not only in the study of antigenic, but also some biological properties.

Molecular-biologic analysis of the A/Leningrad/83 viruses

The genomes of A/Leningrad/83 isolates belonging to different antigenic groups characterized in Fig. 1 were compared in the cRNA-vRNA hybridization test.

Figs. 2—4 present the results of the data concerning the genomes of the following strains: A/Len/237/83 (group 1), A/Len/156/83 (group 2), A/Len/153/83, A/Len/171/83 (group 4), and A/Len/262/83 (group 5). cRNAs of the above listed strains were hybridized with vRNA of the reference virus A/BK/1/79 (see Fig. 2), vRNA of the virus A/Vic/35/72 (see Fig. 3), and vRNA of the isolate A/Len/153/83 (see Fig. 4). The results obtained allowed the following conclusions:

1. On the basis of all the 3 tests of RNA—RNA hybridization, viruses of group 4 (A/Len/153/83 and A/Len/171/83) appeared to be virtually identical with respect to all the genes, and similar to the virus A/Vic/35/72, except for gene 7(M), which in the viruses tested was different from the mentioned reference virus (see Figs. 3 and 4). In both viruses all the genes differed from those of A/BK/1/79 (see Fig. 2).
2. Viruses A/Len/156/83 and A/Len/263/83 belonging to groups 2 and 5, respectively, based on their virological analysis, were similar according to the results of molecular hybridization, but appeared nonidentical with the reference virus A/BK/1/79 in several genes, including the gene coding for HA (see Fig. 2).
3. Virus A/Len/237/83 (group 1, see Fig. 2) was different from viruses A/Len/153/83 (group 4) and A/Len/262/83 (group 5) with respect to mobility of several cRNA-vRNA duplexes. Meanwhile, it was not completely identical with the reference virus A/BK/1/79. Yet, based on this analysis, isolates 237, 153 and 262 can be conventionally defined as "Bangkok-like".

In the experiments presented in Figs. 2—4 the viruses of antigenic groups 1, 2 and 4 were compared. This comparison is continued in Fig. 5 with addition of the reference virus A/Phil/2/82 and "Philippines-like" strain A/Len/257/83 (group 3). As shown in Fig. 3, there were significant differences between A/Phil/2/82 virus and A/Len/257/83 isolate as evident from the comparison of the mobility of the duplexes produced during hybridization of A/Len/257/83 vRNA and cRNA of the virus A/Phil/2/82 in 7.5% PAG. Strain A/Len/257/83 differed from the A/Phil/2/82 virus in the genes 1, 2, 3, 4, 5, 6 and in the same genes from the reference virus A/BK/1/79.

At the next stage of molecular-biological analysis, the comparison of the genomes of isolates A/Len/153/83 and reference strain A/Victoria/35/72, possessing a similar antigenic HA specificity, was continued (Fig. 1). As already mentioned above, these viruses were found to have different mobilities of gene 7 in 4% PAG (Figs. 3 and 4). For their further comparison 7.5% PAG containing 6 M urea has been used, i.e. a system capable of detection minor differences in the genome homology. As follows from Fig. 6, the genomes of the viruses under comparison turned out to be nonidentical not only with respect to gene 7, but also in some others (1, 2, 4, 5). It should be emphasized that in spite of a certain similarity of the viruses mentioned with respect to the antigenic specificity of surface glycoproteins, molecular hybridization allowed us to reveal differences between these strains, in particular, in gene 4, which codes for HA.

Discussion

The evidence for heterogeneity of the strains of influenza A (H3N2) viruses isolated during the same epidemic with respect to specificities of HA and neuraminidase and some biological features was obtained in the second half of the 70s (Luzyanina *et al.*, 1975, 1978). This was further supported by the data on heterogeneity of such isolates with respect to their gene composition, applying not only to the genes which code for external glycoproteins, but also to those coding for unglycosylated proteins. Thus, for instance, the strains isolated in the U.S.S.R. and G.D.R. during the influenza A (H3N2) epidemic in 1979—1980 were classified into 3 groups on the basis of gene composition: 1) strains similar to the virus A/Texas/1/77; 2) strains similar to the reference virus A/Bangkok/1/79 with respect to all the genes or differing from it in separate genes; 3) recombinant strains of subtype A (H3N2). The strains isolated in the G.D.R. during the 1981—1982 epidemic, though similar to the virus A/Bangkok/1/79, could be also classified into 3 independent groups based on the gene composition and HA antigenic structure (Ghendon *et al.*, 1981; Klimov *et al.*, 1984).

The study of the strains A (H3N2) isolated during 1983 influenza epidemic in Leningrad revealed even higher heterogeneity. Based on the antigenic pattern of HA and neuraminidase, these strains could be classified into five groups related to basic reference viruses of this subtype known from the epidemic events of 1972—1982: A/Victoria/35/72, A/Texas/1/77, A/Bangkok/2/82 (see Fig. 1 and Table 1). However, in addition to the relationship with the reference viruses listed, virtually all the isolates were characterized by a marked heterogeneity not only of antigenic, but also some biological properties (Table 2).

The analysis of gene composition of these viruses also indicated their marked heterogeneity, although it was inconsistent with the data on their antigenic pattern. Thus, for instance, "Victoria-like" viruses A/Leningrad/153/83 identical to each other differed from the reference virus A/Victoria/35/72 in the analysis of the mobility of RNA-RNA duplexes in 4% PAG with respect to 1 gene (7) and in 7.5% PAG with respect to 5 genes (gene 4 coding for HA including). Viruses A/Leningrad/156/83 and A/Leingrad/262/83 belonging to different groups, as indicated by the analysis of antigenic properties of their HAs (see Fig. 1), appeared similar in their gene composition, and together with virus A/Leningrad/237/83 (see Fig. 1, group 1) were grouped with "Bangkok-like" viruses on the basis of genome structure. "Philippines-like" virus A/Leningrad/257/83 was different from reference viruses A/Bangkok/1/79 and A/Philippines/2/82 in a number of genes (see Fig. 5). Thus, on the basis of cRNA-vRNA hybridization it can be concluded that most of the isolated viruses are not quite identical with but only similar to the corresponding reference viruses. This applies to all the virus groups tested and allows one to suggest that during the influenza epidemic in 1983 "Victoria-like" (1972—1973), "Texas-like" (1977), "Bangkok-like" (1979) and "Philippines-like" (1982) strains had been circulating.

The data obtained confirm the suggestion that during drift changes of influenza viruses the evolution of genes coding for unglycosylated proteins can be independent of the variability of the gene coding for HA (Palese and Young, 1982). Hence, the highly marked heterogeneity of gene composition of the isolates is not necessarily consistent with their classification on the basis of the characteristics of HA and neuraminidase antigenic properties. The causes of inconsistency between HI results during identification of antigenic pattern of viruses and the data on the mobility of RNA-RNA duplexes of gene 4 coding for HA need special discussion. These facts support the suggestion that under the conditions of natural circulation of influenza viruses, mutations may also arise in nonantigenic regions of HA. These mutations without affecting the antigenic properties of HA can still affect electrophoretic mobility of RNA-RNA duplexes (Klimov and Ghendon, 1985).

Summing up, the results of the present study indicate that influenza virus strains isolated in Leningrad during the epidemic in 1983 are charaterized by a marked heterogeneity with respect to the antigenic specificity of HA and neuraminidase by a complex of biological properties and by the homology of a number of genes.

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Explanation of Figures (Plates XXX—XXXIII):

Fig. 2. Electrophoresis in 4 % PAG of double-stranded RNAs obtained by hybridization of vRNA of A/Bangkok/1/79 strain with cRNA of some A/Leningrad/83 strains. Here and hereinafter: B — A/Bangkok/1/79, V — A/Victoria/35/72.
237 — A/Len/237/83, 262 — A/Len/262/83, 156 — A/Len/156/83, 153 — A/Len/153/83, 171 — A/Len/171/83. Dots and figures indicate the positions and numbers of homologous double-stranded RNA segments.

Fig. 3. Electrophoresis in 4 % PAG of double-stranded RNAs obtained by hybridization of vRNA of A/Victoria/35/72 with cRNA of some A/Leningrad/83 isolates.

Fig. 4. Electrophoresis in 4 % PAG of double-stranded RNAs produced by hybridization of vRNA of A/Len/153/83 isolate with cRNAs of different influenza virus isolates.

Fig. 5. Electrophoresis in 7 % PAG containing urea (6 M) of double-stranded RNAs produced by hybridization of vRNA of strain A/Len/257/83 with cRNAs of different influenza virus strains. Ph — A/Philippines/2/82.

Fig. 6. Electrophoresis in urea-containing (6 M) 7.5 % PAG of double-stranded RNAs produced by hybridization of vRNA of A/Victoria/35/72 virus with cRNA of homologous virus and strain A/Len/153/83.
Notations as in Fig. 2.