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ANALYSIS OF INFLUENZA A VIRUS NEURAMINIDASE USING LECTIN TEST AND MONOCLONAL ANTIBODIES

E. VAREČKOVÁ, *O. V. LYUBOVTSEVA, G. RUSS, B. STYK, *L. Ya. ZAKSTELSKAYA, *M. A. YAKHNO

Institute of Virology, Slovak Academy of Sciences, 81703 Bratislava, Czechoslovakia, and *D. I. Ivanovsky Institute of Virology, U.S.S.R. Academy of Medical Sciences, 123098 Moscow, U.S.S.R.

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Summary. — Influenza viruses causing epidemics in the U.S.S.R. in 1968—1982 and 1983 were analysed in the lectin test (LT) using polyclonal and monoclonal antibodies with specificity against neuraminidase (NA) of N2 subtype. Heterogeneity of the U.S.S.R. virus isolates in the reaction with monoclonal and polyclonal antibodies was demonstrated, though they were coming from the same year epidemic. The LT turned out to be an appropriate method to detect the antigenic drift in influenza virus NA. The results of LT were in a good agreement with those obtained by colorimetric estimation of the inhibition of enzyme activity and by competitive solid phase radioimmunoassay (SP RIA). In the LT only low steric inhibition of the NA with antihaemagglutinin monoclonal antibodies (MoAb) reacting in haemagglutination inhibition and virus neutralization tests was detected.

Key words: influenza virus neuraminidase; lectin test; monoclonal antibody

Introduction

Neuraminidase (NA), the second influenza virus surface antigen, undergoes changes during the influenza epidemics. Both, antigenic shift and antigenic drift occur in the NA as well as in the haemagglutinin (HA) (Webster *et al.*, 1983). Nine subtypes of influenza A virus NA are known, two of them in the human strains (Assaad *et al.*, 1980). Antigenic shift occurred in 1957, when a new NA subtype emerged designated N2. Since that influenza virus with the N2 subtype NA has been predominant in human population.

Antigenic drift in the NA was studied using enzyme inhibition or RIA methods with polyclonal or monoclonal antibodies (Webster *et al.*, 1983). By colorimetric enzyme-inhibition method (Aymard-Henry *et al.*, 1973) the biologic activity of NA and its inhibition with specific antibody is being detected. Luther has introduced a new method for detection of NA and anti-NA activities — the lectin test (Luther *et al.*, 1979; 1980; 1983; 1984). LT can be easily performed in any biological laboratory and is suitable for screening of many samples. LT was modified and further improved for

antigenic analyses of influenza virus NA with specific polyclonal antisera (Lyubovtseva *et al.*, 1983).

In our work we used LT to compare the NA of the last epidemic strains of N2 subtype (1968—1983) using MoAb. The results of LT were related to those obtained by colorimetric enzyme-inhibition method and by competitive SP RIA. Moreover, steric effects (i.e. inhibition of NA activity by anti-HA monoclonal antibodies) in LT were evaluated.

Materials and Methods

Viruses. The following influenza virus strains were used: A/Singapore/1/57 (H2N2) — which will be reffered to as A/Sing, A/Hong Kong/68 (H3N2) — A/Hong Kong, A/Dunedin/4/73 (H3N2) — A/Dunedin, A/Port Chalmers/1/73 (H3N2) — A/Port Chalmers, A/Victoria/3/75 (H3N2) — A/Vict, A/Texas/1/77 (H3N2) — A/Texas, A/Bangkok/1/79 (H3N2) — A/Bangkok, A/Philippine/2/82 (H3N2) — A/Philippine, A/Bel/42 (H1N1) — A/Bel, A/FM1/47 (H1N1) — A/FM1, B/Hong Kong/73, A/Turkey Ontario/67, Swine/Iowa/46. Viruses were propagated and purified according to Styk and Blaškovič (1973) and Russ *et al.* (1974).

Influenza virus strains from epidemics in the U.S.S.R. in 1983 and other virus strains were used as allantoic fluid. The viruses were grown in 9-day-old chick embryos. The U.S.S.R. virus and the reference strains were obtained from the collection of Influenza Laboratory and Regional Centre of D. I. Ivanovsky Institute of Virology, Academy of Medical Sciences in Moscow.

Determination of NA and anti-NA activities.

I. *Lectin test.* NA and anti-NA activities were estimated in modified LT with guinea pig (Lyubovtseva *et al.*, 1983) or human erythrocytes (Luther *et al.*, 1983). The LT was done in 96-well polystyrene plates (KOH-I-NOOR, Czechoslovakia) using spiral loops according Takátsy (1955). The lectin was isolated from *Arachis hypogaea* according to Terao *et al.* (1975). The reaction mixture for NA activity titration contained: 25 μl purified virus in two-fold dilutions in saline containing 5 mmol/l $CaCl_2$ and 0.1 % NaN_3; 25 μl 3 % human erythrocytes in the same incubation fluid. After 18 hr incubation at 37 °C, the lectin was added in 25 μl volume (0.3 $mg.ml^{-1}$). One hr later, the NA titre was read and expressed as reciprocal of the highest virus dilution, which agglutinated the erythrocytes after lectin addition. For determination of the anti-NA activity the antisera or ascitic fluid (25 μl) containing anti-NA monoclonal antibody (MoAb) in two-fold dilutions and influenza virus (25 μl) with LT NA titre 16 were incubated for 1 hr at 25 °C. Thereafter, 3 % human erythrocytes and lectin were added as desccibed above, the anti-NA titre was read after addition of lectin and expressed as reciprocals of the highest antibody dilution (serum or ascitic fluid) which completely inhibited the agglutination.

II. Colorimetric enzyme-inhibition assay: Anti-NA activity of antiserum or of MoAb was determined in NA-inhibition test using fetuin as substrate (Aymard-Henry *et al.*, 1973). The NA-inhibition titre was determined as the dilution of antiserum or ascitic fluid reducing NA activity to 50 % of the control.

SP RIA. Purified monoclonal antibody IIIE 11 was labelled with radioactive iodine Na ^{125}I by the chloramine T procedure (Russ *et al.*, 1978). The homologous antigen (purified virus A/Bangkok) was adsorbed on the surface of the microtitration plate (KOH-I-NOOR, Czechoslovakia, 96 wells) for two hr at 25 °C (900 ng of virus protein in 50 μl per well). Plates were saturated with 1 % bovine serum albumin in phosphate buffered saline (PBS), pH = 7.2 for 1 hr at 25 °C and washed with 1% foetal calf serum (FCS) in PBS. The competing purified virus in two-fold dilutions (25 μl per well) and 25 μl of ^{125}I-labelled IIIE 11 were added to the reaction mixture (45 000 cpm per well). The labelled MoAb was titrated with the homologous Bangkok virus adsorbed on the plate. The amount of labelled MoAb giving 50 % of the radioactivity bound was used in the assay. After 24 hr competition at +4 °C, the reaction mixture was washed with FCS solution and radioactivity was measured in a γ-counter.

Antisera. Rabbits and rats were immunized as described (Yakhno *et al.*, 1978).

Preparation of hybridoma cell lines. Hybridomas were prepared by fusion of mouse myeloma cell line SP2/0 with spleen cells from BALB/c mice immunized with influenza virus A/Bangkok/79 using polyethylene glycol according to established procedures (Gerhard, 1980). Monoclonal antibodies will be characterized in details elswhere (manuscript in preparation). Purification of MoAb was done as described previously (Poláková *et al.*, 1983).

Table 1. Reaction of influenza viruses with IIIE 11-MoAb detected in LT and NA-inhibition colorimetric test

Virus strain	MoAb IIIE 11 titre	
	LT	NA inhibition test
A/Singapore/1/57	< 100+	< 50
A/Hong Kong/1/68	< 100+	< 50
A/Port Chalmers/1/73	6 500+	1 000
A/Victoria/3/75	100+	50
A/Texas/1/77	8 100	10 000
A/Bangkok/1/79	24 300+	50 000
A/Philippine/2/82	17 000	50 000
A/USSR/2/83	5 400	3 000
A/USSR/3/83	8 100	5 000
A/USSR/4/83	10 500	2 000
A/USSR/5/83	8 100	4 000
A/USSR/6/83	16 200	50 000
A/USSR/7/83	< 100	< 50
A/USSR/9/83	9 000	10 000
A/Bel/42	< 4++	—
A/FM1/47	< 4++	—
B/Hong Kong/73	16++	—
Swine/Iowa/46	0++	—
A/Turkey Ontario/67	0++	—

IIIE 11-ascitic fluid-containing MoAb against A/Bangkok/79 was obtained as described in Materials and Methods. Antibody titres in LT are expressed as reciprocal of the highest dilution of ascitic fluid providing complete inhibition of erythrocyte agglutination after adding peanut lectin

+ — obtained with guinea pig as well as with human erythrocytes

++ — obtained with human erythrocytes; the rest of LT values was obtained with guinea pig erythrocytes

Antibody titres in NA inhibition test are expressed as reciprocal of ascitic fluid dilution causing 50% inhibition of the enzyme activity

Results

Reactivity of the influenza A virus NA with monoclonal and polyclonal antibodies in LT

The reactivity of N2 NA was determined in LT with MoAb IIIE 11 (in the form of ascitic fluid). The N2 NA of the "old" virus strains (isolated before Bangkok virus circulated) and the "new" epidemic viruses causing epidemics 1979—1983 were compared (Table 1). MoAb IIIE 11 reacted to a high titre with Bangkok virus NA. The "old" virus strain N2 NA could be divided into three groups according to their reactivity with IIIE 11 MoAb: 1. A/Sing and A/Hong Kong showing no reaction (<100), 2. A/Vict showing weak reaction (100—5 000), 3. A/Port Chalmers, A/Dunedin, A/Texas showing strong reaction (> 5 000). The "recent" strains (A/Philippine and influenza viruses from the U.S.S.R. epidemic in 1983) reacted up to titres

Table 2. Antigenic specificity of influenza A virus NA (N2 subtype) in lectin test with polyclonal antibodies

Virus strain	Serum titre to N2 NA of			
	Victoria+ 1	Texas++ 2	Bangkok+ 3	Philippine++ 4
A/Hong Kong/68	240	120	480	60
A/Port Chalmers/1/73	1 920	480	1 920	480
A/Victoria/75	2 840	960	960	960
A/Texas/1/77	960	3 840	1 920	3 840
A/Bangkok/79	1 920	960	7 680	3 840
A/Philippine/82	3 840	480	1 920	7 680
A/USSR/2/83	240	240	3 840	7 680
A/USSR/3/83	240	1 920	1 920	7 680
A/USSR/4/83	120	480	3 840	3 840
A/USSR/5/83	480	960	1 920	3 840
A/USSR/6/83	240	480	1 920	7 680
A/USSR/7/83	480	240	480	3 840
A/USSR/9/83	960	120	1 920	7 680

Titres are expressed as in Table 1; values were obtained with guinea pig erythrocytes according Lyubovtseva *et al.*, 1983

The following strains and reassortants were used for immunization:

1 — A/Equi/Prague/1/56 — A/Victoria/3/75 (H7N2)
2 — A/Equi/Prague/1/56 — A/Texas/1/77 (H7N2)
3 — A/Equi/Prague/1/56 — A/Bangkok/1/79 (H7N2)
4 — strain A/Philippine/2/82 (H3N2)

+ rabbit serum
++ rat serum

5 400—16 000 except of the isolate A/USSR/7/83 whose reaction was lower than 100. No reaction (< 16) was detected with viruses possessing NA of another subtype and with the influenza B viruses.

Polyclonal rabbit or rat antisera with the specificity against N2 NA subtypes A/Vict/75, A/Bangkok/79, A/Texas/77, A/Philippine/82 were used in lectin test to compare the analysed virus strains. Antigenic relationships among various influenza virus strains estimated by LT were similar when using both rabbit antiserum against Bangkok virus NA and IIIE 11 MoAb (Table 2).

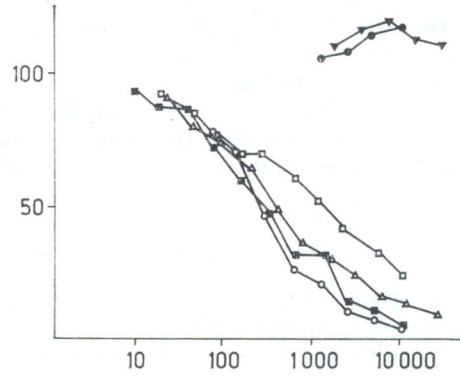
Colorimetric enzyme-inhibition assay

The colorimetric estimation of NA activity inhibition was used for the determination of antigenic relationships between influenza virus NA according to the method recomended by W.H.O. (Aymard-Henry *et al.*, 1973). The titres estimated with IIIE 11 MoAb for various influenza virus strains in comparison with the results of LT are shown in Table 1. It follows from this table that both methods gave comparable data.

Fig. 1.
Competition of influenza virus neuraminidase in SP RIA

—■—■— — A/Bangkok/1/79 (H3N2)
—○—○— — A/Philippine/2/82 (H3N2)
—△—△— — A/Dunedin/4/73(H3N2)
—□—□— — A/Victoria/3/75 (H3N2)
—▼—▼— — A/Hong Kong/1/68 (H3N2)
—●—●— — A/Bel /42(H1N1)

Abscissa: competing purified influenza virus protein in ng (log_{10}); ordinate: proportion of radioactivity of ^{125}I-MoAb IIIE 11 bound to immobilized virus in the presence of soluble competing virus related to the radioactivity bound to the immobilized virus in the absence of the competing virus (in per cent)



Competitive SP RIA

The antigenic relationship of influenza A virus NA can be evaluated in competitive SP RIA (or ELISA) irrespectively on the capacity of examined antibodies to inhibit the enzymatic activity of NA. For this purpose we used ^{125}I-labelled IIIE 11 MoAb. The unbound purified virus was allowed to compete with the immobilized virus for binding of a limited amount of ^{125}I-MoAb IIIE 11. Influenza viruses, whose NA activity was inhibited with IIIE 11 MoAb in LT as well as in NA inhibition test, competed with the immobilized virus for binding of the ^{125}I-labelled antibody (Fig. 1). Viruses, whose NA activity was inhibited neither in LT nor in NA inhibition test with the MoAb IIIE 11 (i.e. A/Hong Kong, A/Bel) did not compete with immobilized Bangkok virus.

Inhibition of neuraminidase activity in LT by antihaemagglutinin MoAb

It is well known that the NA activity can be inhibited nonspecifically by the polyclonal antibodies against relevant haemagglutinin in colorimetric

Table 3. Inhibition of NA activity in LT by anti-haemagglutinin MoAb

Virus	Titre of MoAb			
	IIG 3	IIB 4	IIE 5	IIF 5
A/Bangkok	<40	100	40	40
A/Port Chalmers	<40	40	<40	<40
Equi/56-Bangkok/79+	<40	<40	<40	<40

+ — recombinant A/Equi/Prague/1/56 — Bangkok/1/79 (H7N2)
Titres LT expressed as in Table 1

test and LT (Russ *et al.*, 1974; Lyubovtseva *et al.*, 1983). In our experiments we examined the effect of anti-haemagglutinin MoAb on NA activity in the LT. Using four MoAb against different antigenic sites of haemmagglutinin, we found, that only IIB4 MoAb (i.e. efficient in inhibition of haemagglutinin activity) caused significant inhibition of NA activity under given conditions. The remaining three anti-haemagglutinin MoAb (with low or no haemagglutination inhibition capacity) showed no detectable steric inhibition (Table 3).

Discussion

In this paper we present the results of analysis of influenza virus NA (N2 subtype) obtained with LT in comparison with the results obtained by standard colorimetric method (recomended by W.H.O.) and by competitive SP RIA. We confirmed the conclusion of Luther *et al.* (1983) and Lyubovtseva *et al.* (1983) that LT is suitable for sensitive determination of NA activity, for specific detection of anti-NA antibodies and for identification of NA variability. Both LT and the colorimetric method (generally applied so far) showed results which were in good agreement. LT can be easily performed in any biological laboratory (i.e. there is no need for colorimetric measurements) and is particularly suitable for screening of large sample collections. According to our experience, however, LT is less reproducible as compared with the colorimetric procedure. This can be explained by the need of red blood cells which cannot be completely standardized.

We showed that MoAb against influenza virus NA can be tested and characterized in the LT. We used anti-haemagglutinin MoAb to show whether some steric hindrance of NA activity could be detected. The hybridoma producing MoAb IIIE 11 was prepared by fusion of mouse myeloma cell line SP2/0 with splenocytes from mice immunized with A/Bangkok virus.

The antigenic drift in N2 NA of influenza viruses isolated in 1957—1983 was demonstrated by three methods. The epitope determined by MoAb IIIE 11 appeared to be identical in viruses A/Philippine/82, A/Bangkok/79, A/USSR/4/83, A/USSR/6/83 based on the high titres of MoAb in LT and NA-inhibition test. This was supported by SP RIA as well. The variability of IIIE 11 MoAb titres with other analysed reference virus strains was probably caused by a particular change in that epitope. MoAb reacted with A/Vict virus to a low titre and there was no reaction with the strains A/ /Sing and A/Hong Kong. It can be proposed from our results that the epitope recognized by MoAb IIIE 11 was not present in the NA of "old" virus strains A/Sing and A/Hong Kong but was present in the NA of the isolates from the 1973—1983 epidemic. The variability in the titres of MoAb with the U.S.S.R. epidemic strains indicate the heterogeneity of virus population isolated during the 1983 epidemic in the U.S.S.R. The USSR/7/83 virus isolate differed from the others, because it did not react with MoAb IIIE 11.

The same reactivity patterns were obtained in LT and NA-inhibition test with polyclonal sera prepared by immunization with recombinant virus A/Equi/Prague/1/56 — Bangkok/1/79 (H7N2). Surprisingly, IIIE 11 MoAb

revealed similar antigenic relationships among various examined influenza virus isolates as the rabbit polyclonal serum against this recombinant virus.

A/Viet virus NA reaction with IIIE 11 MoAb and polyclonal anti-NA of A/Bangkok virus was low. This indicate its special position in the evolution of NA; it could be proposed by analogy to the evolution of HA (Both *et al.*, 1983) that the direct progenitors of A/Bangkok were A/Port Chalmers/73 and A/Texas/77, and that A/Victoria/75 strain was a terminal bypass from the main evolution tree.

It was shown that polyclonal sera containing antibodies to relevant haemagglutinin cause nonspecific steric inhibition in colorimetric enzyme inhibition (Russ *et al.*, 1974) and in LT (Lyubovtseva *et al.*, 1983). In the present work we examined whether the monoclonal antibody to the relevant HA can nonspecifically inhibit the NA activity in LT. We detected low steric inhibition of NA activity (to the titre 40). The only anti-haemagglutinin MoAb IIB4 which inhibited the NA activity of A/Bangkok virus in LT was positive also in haemagglutinin-inhibition test (in preparation). Similar results we obtained with MoAb against the haemagglutinin of A/Dunedin virus with A/Dunedin virus (results not shown). The other anti-haemagglitinin MoAb used, did not cause steric hindrance in LT. It follows from our results presented here (and from unpublished results with influenza A/Dunedin) that nonspecific inhibition of the NA with anti-haemagglutinin MoAb depends on spatial localization of the corresponding epitope and is probably related to the capacity of haemagglutinin inhibition (i.e. the higher was the efficiency of MoAb to inhibit haemagglutination, the higher was the nonspecific inhibition of NA).

In summary, LT can be used for detection of specific polyclonal and monoclonal antibodies against influenza A virus NA and for characterization of the antigenic variability of NA. It seems to be particularly suitable for initial screening of human sera and influenza virus isolates during influenza epidemics when many samples have to be tested. LT is also suitable for screening of MoAb with the capacity to inhibit the enzymatic activity of NA.

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