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CHARACTERIZATION OF INFLUENZA A-1983 EPIDEMIC STRAINS BY POLYCLONAL AND MONOCLONAL ANTIBODIES AND DETECTION OF TWO CO-CIRCULATING ANTIGENIC VARIANTS

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Summary. — Influenza virus strains isolated during 1985 epidemic in Czechoslovakia proved to be antigenically closely related to A/Bangkok/79, A/Philippines/2/83 and A/Texas/77 (all H3N2) viruses, if examined in haemagglutination inhibition (HI) tests with standard polyclonal antisera. If examined in HI tests with monoclonal antibody (MAb) IIB4, the virus isolates could be separated into two groups: those reacting to high titres (about two thirds of the isolates) and those negative with IIB4 (titre of < 20; rest of the strains). A relationship to MAb IIB4 similar to that of freshly isolated A-H3 influenza virus strains was found with prototype strains A/Belgium/2/81 (highly positive with IIB4, HI titre up to 20 000 per 0.025 ml) and A/Philippines/2/82 (titre < 20). Examination of the isolate labelled A/Prague/2/83, obtained from a single individual, suggested the existence of two stable and passage-independent lines of a single virus strain, namely one HI^+ and the second HI^- (highly positive and negative in HI tests with MAb IIB4, respectively). Solid-phase radioimmunoassay with ^{125}I-labelled MAb IIB4 of the viruses under consideration showed that binding of virus with antibody had occurred in all cases and that, therefore, the negative results of HI tests with HI^- strains were not due to the absence of binding of MAb IIB4 to the respective viral antigen.

Key words: influenza virus A; monoclonal antibody; solid-phase radioimmunoassay

Introduction

A widespread epidemic caused by influenza virus A occurred in Czechoslovakia early in 1983, mainly from the 6th to the 14th calendar week (Walter, 1984; Masár, 1984). The causative viruses were identified as related to or identical with A/Bangkok/1/79 (H3N2) A/Philippines 2/83, and A/Texas/1/77 (H3N2) strains (Tůmová *et al.*, 1985).

We examined a total of 65 positive isolates from this epidemic, obtained in various Czechoslovak localities, mainly in central and western Slovakia and central and eastern Bohemia. We have received either partially characterized strains (mainly from the Czechoslovak Influenza Centre, Institute of Hygiene and Epidemiology, Prague) or, more frequently, allantoic fluid samples following only a few egg passages, immediately after positive haemagglutination had suggested possible presence of influenza virus. The latter materials were obtained mainly from virus laboratories of Regional Hygiene Stations, usually in the 2nd or 3rd egg passage with haemagglutinin titres of 4—16 per ml or less, without any characterization of the presumed virus.

In the present paper we are reporting of investigations on these strains in haemagglutination inhibition (HI) tests and solid-phase radioimmunoassay (SP-RIA) with polyclonal antisera and monoclonal antibodies (MAb). Our results confirmed that all strains isolated from January to March, 1983, were very similar to A/Bangkok/1/79 (H3N2) or A/Texas/1/77 (H3N2) virus and moreover showed that two antigenic variants distinguishable only in HI tests with MAb IIB4 were circulating during the epidemic in question in Czechoslovakia.

Materials and Methods

Virus isolation and associated procedures were done by the standard technique according to Lennette and Schmidt (1969) and Palmer et al. (1975) from throat and/or nasal swab specimens on developing chick embryos. Usually allantoic fluids from the 2nd—4th egg passage, with a titre of 4—16 haemagglutinin units (HAU) per ml, were examined.

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/1/79 using polyethylene glycol according to established procedures (Gerhard, 1980). The MAb obtained will be characterized in detail elsewhere (Russ *et al.*, 1987).

Preparation of polyclonal antisera. With only one exception of a ferret antiserum, rabbit sera were used. The rabbits were immunized with three doses of purified virus or haemagglutinin along with Al-Span-Oil adjuvant (Styk and Blaškovič, 1973).

Haemagglutination inhibition (HI) tests were performed by Takátsy's (1955) microtechnique. HI titres were expressed per 0.025 ml as reciprocals of the highest initial dilution of serum causing inhibition of 4—8 HAU of the respective virus used in the form of infected allantoic fluid.

Polyclonal antisera and MAb-containing ascitic fluids were usually treated with RDE to remove nonspecific inhibitors (Palmer *et al.*, 1975; Styk *et al.*, 1977).

Solid-phase radioimmunoassay (SP-RIA). Virus-containing fluids (25 µl per well) were adsorbed on to polystyrene microplates (KOH-I-NOOR, Hardtmuth, Czechoslovakia). After drying the wells with adsorbed antigen were washed with washing buffer (phosphate buffered saline — PBS — pH 7.2, containing 1% foetal calf serum), saturated with 1% bovine serum albumin in PBS, and twofold dilutions in PBS of MAb in the form of ascitic fluid were added. After 2 hr, unbound antibody was washed off by washing buffer and the plates were incubated for 2 hr with ^{125}I-labelled rabbit anti-mouse F(ab')$_2$ Ig (4×10^4 count/min per well). Then followed thorough washing and bound racioactivity was measured on a gamma counter. All SP-RIA steps proceeded at room temperature.

Results

Antigenic analysis of epidemic influenza A-1983 virus strains

Serological studies on freshly isolated strains done by the "standard" technique (HI test with standard RDE-treated polyclonal sera — Palmer

Table 1. Examination of prototype and freshly isolated influenza A (H3N2) virus strains in HI tests with polyclonal antisera and monoclonal antibody IIB4

Virus strain	HI titres with		
	rabbit serum anti A/Bangkok/1/79	ferret serum anti A/Philippines/2/82	Mab IIB4
A/Prague/1/83			
E3	160	160	5 120
E4	640	120	5 120
E5	320	160	10 240
A/Prague/2/83			
E3 (10^{0})	160	160	< 20
E3 (10^{-1})	160	160	< 20
E3 (10^{-3})	320	80	5 120
E4 (10^{-3})	640	320	5 120
A/Ústí/14/83	160	160	< 20
A/Banská Bystrica/65/83	320	160	< 20
A/Bratislava/67/83	160	160	2 560
A/Bratislava/51/83	320	320	10 240
A/Philippines/2/82	160	320	< 20
A/Belgium/2/81	640	1 280	20 480

et al., 1975; Lennette and Schmidt, 1969) yielded results that were in accordance with those obtained in other laboratories (Walter, 1984): strains of influenza virus A, subtype H3, closely related to A/Bangkok/1/79 (H3N2) or A/Texas/1/77 (H3N2) were involved.

But the results of HI tests on the same influenza virus isolates became quite different when polyclonal sera were replaced by MAb IIB4, directed against one of A/Bangkok/1/79 influenza virus haemagglutinin epitopes (the respective hybridoma was obtained following immunization with this virus).

All the fresh isolates and two recent prototype strains proved to be more or less homogeneous or closely antigenically related if examined in HI tests with standard polyclonal antisera (differences in HI titres maximally 4-fold — see Table 1). But the use in HI tests of MAb IIB4 revealed great differences between the virus strains in question. The latter could be separated into two groups: one of completely negative (H^-) strains and the other of highly positive (H^+) strains.

We examined a total of 65 isolates (only typical results were included in Table 1). Irrespective of the place of isolation of the virus in Czechoslovakia, the results of HI tests with MAb IIB4 were either unambiguously negative (in about one third of the isolates, titre of < 20 or 10 per 0.025 ml), or highly positive (in about two thirds of the isolates, titres from 1000- to 10 000-fold higher). Only four of the 65 strains yielded low titres (about 20—80) in these HI tests.

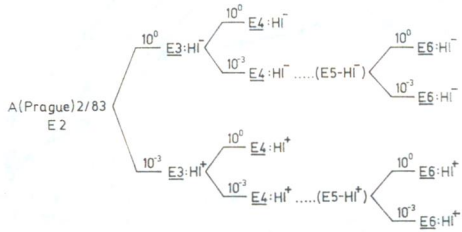

Fig. 1.
Separation of two independent lines from a single virus isolate (A/Prague/2/83)
* HI^- = HI titre < 20
** HI^+ = HI titre $\geq 10\,000$

Evidence of two independent lines of influenza virus from a single isolate

Extreme differences in HI titres established with MAB IIB4 were also recorded in an isolate obtained from a single individual namely strain A/Prague/2/83, depending on the size of inoculum (its dilution) used for further passaging in chick embryos (see Table 1). At a high multiplicity of infection (MOI) — inoculum undiluted or diluted 10^{-1} — the resulting allantoic fluid

Table 2. HI tests with prototype strains of subtype H3 influenza virus and monoclonal antibody IIB4 or polyclonal rabbit antiserum against haemagglutinin of A/Dunedin/73 virus (BHA_{Dun})

Virus strain	HI titre per 0.025 ml with	
	MAb IIB4	Polyclonal serum BHA_{Dun}
A/equine/Miami/1/63	< 10	< 10
A/duck/Ukraine/1/63	< 10	< 10
A/Hong-Kong/1/68	< 10	80
A/England/42/72	10	320
A/Dunedin/4/73	80	2 560
A/Victoria/3/75	5 120	640
A/Texas/1/77	10 240	80
A/Bangkok/1/79	10 240	< 10
A/Belgium/2/81	20 480	< 10
A/Philippines/2/82	< 10	< 10
A/Bratislava/1/83	< 10	< 10
A/Prague/1/83	2 560	< 10
A/Prague/2/83		
HI^- line	< 10	< 10
HI^+ line	10 240	< 10

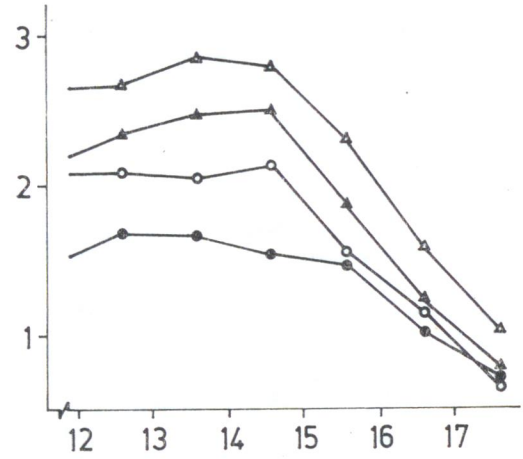

Fig. 2.

Reactivity of anti-haemagglutinin monoclonal antibody IIB4 in SP-RIA with influenza A (H3N2) viruses A/Prague/2/83, 10^{-1} (●); A/Prague/2/83, 10^{-3} (○); A/Belgium/2/81 (▲); and A/Philippines/2/82 (△)

Abscissa: antibody dilution reciprocal ($\log_2$); ordinate: counts/min $\times 10^3$ of ^{125}I-labelled rabbit anti-mouse F(ab')$_2$ Ig bound

(E3) was negative in the HI test with IIB4. With higher inoculum dilutions (at lower MOI), the resulting allantoic fluids reacted to a high titre with MAb IIB4.

Since a mixture of two virus populations could have been involved, we attempted to obtain pure HI$^+$ and HI$^-$ lines by simple passaging (i.e. without adding IIB4 or other antibody) at low and high MOI. The procedure and results are shown in Fig. 1. We obtained both, i.e. HI$^+$ and HI$^-$, lines of A/Prague/2/83 virus and maintained them up to the 7th egg passage.

Different sensitivity to MAb IIB4 of various prototype strains of the influenza A/H3 subtype

It soon became evident (see Table 1) that extreme differences in HI titres with MAb IIB4 occurred not only among the freshly isolated virus strains but also with two recent A/H3 prototype strains, namely A/Belgium/81 and A/Philippines/82. We examined, therefore, several available A/H3 prototype strains, including the oldest ones. The results summarized in Table 2 are supplemented with those obtained in HI tests done with rabbit polyclonal antiserum prepared against purified haemagglutinin of A/Dunedin/4/73 virus.

It is evident that the activity in HI tests of MAb IIB4 with prototype strains showed a time-dependence. The reaction first appeared in strains isolated in 1972—73, reaching a peak in strains from the period 1975—81. Strains isolated later fell into two diametrally different groups — HI$^+$ and HI$^-$.

Binding of MAb IIB4 with HI$^+$ and HI$^-$ influenza virus strains in SP-RIA

The extremely different behaviour in the HI test of HI$^+$ and HI$^-$ strains (e.g., the two lines of the strain A/Prague/2/83 or the prototype strains A/Belgium/81 and A/Philippines/82) was documented above. It was of

interest to check the reactivity of these virus strains (and of other A/H3 prototype strains) with MAb IIB4 in another test system that would offer objective and quantitative information about the viruses binding with the given antibody. For this purpose we used SP-RIA, employed by Kostolanský *et al.* (1986) for the characterization of some influenza A virus isolates from the 1983 epidemic.

SP-RIA carried out under standard conditions failed to reveal any differences between freshly isolated or prototype strains that gave distinct reactions in HI tests with MAb IIB4. The titration curves were practically identical — see Fig. 2.

Discussion

Examination by MAb of influenza A virus isolates from a 1983 epidemic disclosed differences of a previously unreported type between the isolates. Routine HI tests with the use of polyclonal (e.g., rabbit, ferret or other animal) sera revealed no substantial antigenic differences between the isolates. But when one of our anti-haemagglutinin MAb (Russ *et al.*, in press), namely the labelled IIB4, was employed, extreme differences in HI titres (greater than 1000-fold) between the strains were established (titres of < 10 versus $\geq 10\,000$).

A sensitivity to MAb IIB4 like that of epidemic A-1983 strains was also observed in fresh prototype strains of the H3 subtype (A/Belgium/2/81 and A/Philippines/2/82). We, therefore, analysed the whole group of the H3 subtype that had originally started in 1968 (A/Hong-Kong/1/68) and which at present, after reclassification of the viruses A/duck/Ukraine/1/63 (Hav7 → H3) and A/equine/Miami/1/63 (Heq1 → H3), persists for a period of over 20 years. Among the prototype strains examined, the epitope IIB4 was fully manifested "chronologically in retrospect" up to 1975 (A/Victoria/3/75, see Table 2). Since only prototype strains from the previous periods were examined, we cannot say definitely when the antigenic variants distinguishable in HI tests with MAb IIB4 did actually appear. It is interesting that also influenza virus strains isolated in 1985 in the U.S.S.R. proved to be either highly positive or negative in HI tests with MAb IIB4 (S. F. Shenderovich and M. A. Yakhno, Ivanovsky Institute of Virology, Moscow — personal communication). Two distinct antigenic variants, distinguishable in HI tests with MAb IIB4, thus co-circulate even at present and outside of Czechoslovak territory.

In any case, after supplementing the just mentioned results with those obtained with polyclonal sera (illustrated in Table 2 by an antiserum against the haemagglutinin of A/Dunedin/73 virus from the chronological centre of the H3 subgroup), our approach shed new light on the great subgroup of A-H3 influenza virus strains. This does not mean that we consider strains classified as the H3 subtype so distant and heterogeneous that their rearrangement would be required. Based on the occurrence of a single epitope or any other marker such a conclusion would not be warranted. But recently, in view of new findings obtained with MAb, the role of the latter

in evaluating relationships between virus strains is becoming increasingly attractive. This applies, e.g., to relationships within a subtype, as concerns new "Reconsiderations" on H3 strains and subtypes. Finally, the epitope determined by MAb IIB4 represents an antigenic component of at least a part of strains (viruses) belonging to the H3 subtype, and its detection could be one of the many antigenic-immunological aspects which — along with any others that might be detected in the future — should be taken into account in the preparation of an eventual new "Reconsideration" of H3 strains and subtypes (see "A WHO Memorandum" — Bull. Wld. Hlth Org., 1980).

MAb IIB4 directed against a certain epitope on the haemagglutinin of A/Bangkok/79 virus made it possible to differentiate two lines (H^+ and H^-) of a single virus isolate obtained from a single individual (strain A/Prague/ /2/83). As shown in Fig. 1 and Table 2, we carried out a number of passages at various MOI (only some of them are presented in Fig. 1). The strain in question probably was separated already after a single passage in our laboratory into two more or less stable lines that no more were dependent on the the MOI: a) line HI^- (from an inoculum originally undiluted or diluted 10^{-1}, i.e. obtained at a high MOI) and b) line HI^+ (from inoculum originally diluted 10^{-3}, i.e. at a low MOI). (The use of a term like "low, limiting MOI" would be inappropriate since the infectivity titre of the inoculum was not determined before each passage). Both lines of A/Prague/2/83 virus were carried through 7 passages (Fig. 1) and they appeared to be genetically stable.

The comparison of the reactions of individual virus strains with MAb IIB4 in HI tests and SP-RIA was interesting. It became evident that both HI^+ and HI^- strains bound with IIB4 antibody in SP-RIA, practically in the same way under standard conditions. An enhanced reaction of HI^+ strains as compared with HI^- strains was only detected in SP-RIA after direct binding of ^{125}I-labelled IIB4 to firmly adsorbed viruses (unpublished results). This applies to both isolates from the 1983 epidemic and recent prototype strains. Consequently, the negative results of HI tests with HI^- strains are not due to an absence of binding of MAb BII4 to these virus strains. This paracoxical situation may be explained as follows:

1) MAb IIB4 is bound to a site distant from the erythrocyte receptor and inhibits haemagglutination indirectly (e.g., by inducing a conformation change);
2) the quantitative ratios in SP-RIA under standard conditions make it impossible to detect even rather great differences in binding of various viruses with MAb IIB4; or
3) in any other way.

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