ANTIBODY RESPONSE TO HIDDEN EPITOPE OF INFLUENZA A HAEMAGGLUTININ ELICITED BY ANTI-IDIOTYPIC ANTIBODIES

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Summary. – Monoclonal antibody (MoAb) IIF4 defines an epitope on the HA2 part of influenza haemagglutinin (HA) (Russ *et al.*, 1987). It was also found this epitope becomes fully accessible after pH 5 treatment of the antigen and is shared by strains of H3 subtype. In this study we found binding of MoAb IIF4 also to some strains belonging to H2, H4, H7, and H10 subtypes. We prepared rabbit polyclonal anti-IIF4 anti-idiotype (anti-Id) antibody. In competitive assays, the inhibition potential of anti-Id was considerably higher than that of native HA. Anti-Id was used for the preparation of mouse Ab3 (anti-anti-IIF4) serum. Reactivity pattern of Ab3 with influenza virus strains differed from Ab1 in (i) appearence of binding to some strains of H2 and H7 subtype and (ii) decreased dependency of Ab3 binding on the pH forms of antigen. The reactivity of Ab1 and Ab3 with two amantadine-resistant virus mutants indicates that IIF4 epitope (and its related region recognized by Ab3) becomes accessible in consequence of destabilization of trimeric arrangement of HA and it also correlates with expulsion of N-terminus of HA2.

Key words: influenza haemagglutinin: HA2 glycopolypeptide: conservative epitope; anti-idiotype; Ab3;

Introduction

Haemagglutinin (HA) is an essential structural protein of influenza virus at early stage of infection of cell and a major target antigen in antibody-mediated immunity. An important feature of this glycoprotein is its extraordinary antigenic variability, associated predominantly with distal HA1 part of HA (Wiley *et al.*, 1981). Proximal HA2 part (light chain) of HA is, in general, antigenically less variable, sterically less accessible (Graves *et al.*, 1983). However, in the course of natural influenza infection of man the production of anti-HA2 antibodies had been proved (Styk *et al.*, 1979). Also immunization of animals with intact virus led to the production of anti-HA2 antibodies (Russ *et al.*, 1978a). HA2-specific MHC class I-restricted cytotoxic T cells (Wabuke-Bunoti *et al.*, 1984; Kuwano *et al.*, 1989; Braciale *et al.*, 1989; Dillon *et al.*, 1992) and HA2-recognizing helper T cells (Katz *et al.*, 1985*a*, 1985*b*; Jackson *et al.*, 1994) were characterized.

Many data prove the role of amino-terminal portion of HA2 at the fusion of influenza virus envelope with endosomal membrane at the initial stage of infection of the cell. The process of fusogenic activation of HA requires mildly acidic environment and is accompanied by irreversible conformational changes (Maeda and Ohnishi, 1980; Huang *et al.*, 1981; Skehel *et al.*, 1982; Doms and Helenius, 1986; Carr and Kim, 1993) resulting in antigenic alterations (Yewdell *et al., 1983;* Webster *et al.*, 1983, Daniels *et al.*, 1983). These antigenic changes occur also in HA2 part of HA (Kostolanský *et al.*, 1989).

From the panel of our anti-HA2 monoclonal antibodies (MoAbs), we focused on the MoAb designated IIF4 which exhibited some unusual properties, mainly broad cross-reactivity within H3 subtype (Russ *et al.*, 1987) and preferential binding to pH 5-treated antigen. The 'pH-dependency' of binding of MoAb IIF4 is manifested by about 100-fold increase of its binding potential after exposing of antigen (isolated HA or whole virus) to pH 5.0 (Kostolanský *et al.*, 1988). Similarly, increased binding of MoAb IIF4 has been detected on the surface of infected MDCK cells after their treatment with acid pH (Varečková *et al.*, 1993). These data suggest that, in native HA, the HA2 epitope defined by MoAb IIF4 is sterically hidden and accessible for interaction with MoAb IIF4 on a minor subpopulation of HA molecules encompassing 1 – 7 % of all HA molecules. The uniqueness of this epitope is stressed by previous observa-

tion that its MoAb IIF4 exhibits haemagglutination-inhibition activity to pH 5-treated BHA but not to the native or acid-treated virus (Kostolanský *et al.*, 1989). However, MoAb IIF4 does not cause an inhibition of haemolytic activity of pH 5-treated virus.

Radioimmunoprecipitation experiments with HA fragments indicate that the target determinant for binding of MoAb IIF4 is localised within a region of amino acids 127–175 from the N-terminus of HA2 (S. Wharton, NIMR, London, personal communication). This sequence is burried deeply into the native HA structure. After acidification, in consequence of conformational changes of HA, IIF4 epitope becomes accessible on those HA molecules where it was burried.

In this work, we produced anti-Id antibodies of beta type specific to MoAb IIF4 with intention to check the possibility of avoiding the inaccessibility of the IIF4 epitope. Subsequently, we characterized this epitope and its related region in different influenza A strains and amantadine-resistant mutants using Ab1 (MoAb IIF4) and Ab3 antibodies.

Materials and Methods

Viruses. The following influenza virus strains and recombinants from the stocks of the Institute of Virology, Bratislava, were used: A/NWS/33, A/PR/8/34, A/FM/1/47, A/Switzerland/74/85, A/Singapore/6/86 (**H1N1**); A/Bratislava/4/57, A/Japan/305/57, A/Singapore/1/57 (**H2N2**); A/Hong Kong/1/68, A/England/42/72, A/Dunedin/4/73, A/Victoria/3/75, A/Texas/1/77, A/Bangkok/1/79, A/Belgium/2/81, A/Philippines/2/82, A/Prague/1/83, A/Prague/2/83, A/Missisippi/1/85 (**H3N2**); A/Duck/England/62, A/Turkey/Alberta/66, A/Duck/CSR/66, A/Duck/Hong Kong/33/76, A/Duck/Slovakia/42/78 (**H4N6**); A/FPV/Rostock/34 (**H7N1**); X-38, X-42, R-4 (**H7N2**); A/Chick/Germany/49 (**H10N7**); A/Quail/Italy/544/66 (**H10N8**); A/Duck/Hong Kong/11/77 (**H10N1**); A/Larus/78 (**H10N4**); B/Lee.

The conditions for infection of embryonated hen's eggs and the mode of purification of the viruses were already described (Russ *et al.*, 1974).

Amantadine-resistant mutants 1a and 4x (Daniels *et al.*, 1985), as well as parental recombinant X-31 (H3N2) were kindly provided by Dr. S.A. Wharton, NIMR, London.

Isolation of HA by bromelain (BHA). Haemagglutinin was released from the influenza virus A/Dunedin/4/73 (H3N2) by treatment with bromelain (Sigma, grade II) and purified by rate zonal centrifugation (Brand and Skehel, 1972; Russ *et al.*, 1978*b*).

MoAbs IIF4 and BB8 specific to HA2 glycopolypeptide of the HA originating from influenza virus A/Dunedin/4/73 (H3N2) were prepared and characterized by Russ *et al.* (1987).

Preparation of anti-Id sera. Anti-HA2 MoAbs IIF4 and BB8 were purified on Protein A-Sepharose (Ey *et al.*, 1978) and administered to rabbits in four doses on day 0, 22, 34, and 56. First dose of 400 μg MoAb in complete Freund's adjuvant was applied subcutaneously into multiple sites, following doses of 100 μg MoAb in PBS intramuscularly. Animals were bled on 8th day after the last dose (modified according to Staudt and Gerhard, 1983).

Immunoaffinity purification of anti-Id Ig from anti-IIF4 serum was carried out by repeated passing through CNBr-Sepharose column coupled with the mouse non-immune Ig (until no binding to mouse Ig was observable), subsequent binding on CNBr-Sepharose column coupled with MoAb IIF4, and final elution using 4.5 mol/l $MgCl_2$.

Preparation of Ab3 sera. BALB/c mice were given four doses of purified anti-IIF4 rabbit Ig, each of 200 μg intraperitoneally, at 2-week intervals. First dosis was emulsified in Freund complete adjuvant, booster inoculations were administered in Freund incomplete adjuvant. At 7th day after the last inoculation the animals were bled.

Iodination procedures. The samples were labelled with Na [^{125}I] (Amersham, 1 mCi/10 μl) using chloramine T (Russ *et al.*, 1978*b*).

Solid-phase RIA (SP-RIA). Purified native viruses were adsorbed onto microplates overnight, 300 ng per well, incubated with either PBS pH 7.2 or with citrate buffer pH 5.0 for 30 mins, then washed with PBS pH 7.2. After saturation with 1% BSA, MoAb IIF4 or Ab3 mouse serum was added for 3 hrs. Binding of antibodies was detected with [^{125}I]-swine anti-mouse Ig.

Solid-phase competitive RIA. A MoAb was adsorbed overnight at a concentration of 1 μg per well. The inhibition of [^{125}I]-BHA binding was examined in the presence of various dilutions of anti-Id sera for 3 hrs at room temperature. Rabbit anti-Id sera were saturated before testing with 2 volumes of mouse non-immune serum overnight at 4 °C, then centrifuged at 15,000 × g for 30 mins.

Liquid-phase competitive RIA. The reaction mixture in a volume of 0.25 ml contained 1% BSA, 0.5% Triton-X-100, 5 mmol/l EDTA, in PBS pH 7.2 (RIA buffer), constant amount of [^{125}I]-BHA or [^{125}I]-anti-Id, respectively, twofold serial dilutions of BHA or anti-Id as a competitor, and appropriate concentration of MoAb IIF4. The concentration of MoAb IIF4 was that precipitating approximately 20% of the binding of label without competitor. After 48 hrs incubation at 4 °C to reach the equilibrium, 25 μl of normal mouse serum and 100 μl of sheep anti-mouse IgG (30 mg/ml) were added for overnight incubation at 4 °C. The reaction mixture was diluted 10 times with RIA-buffer and radioactivity bound in immunoprecipitates was counted.

Affinity estimation by effective affinity evaluation. Both MoAb IIF4 affinity to the antigen and anti-Id effective affinity to the MoAb IIF4 were estimated on the basis of the liquid phase competitive RIA described above. The most simple form of the mass action law for the interaction at equilibrium had been used to evaluate the effective affinity of antibody. The evaluation had been done as follows. The binding potential of the label (Kp), the unitless product of the equilibrium constant of the interaction and the concentration of the label, was evaluated according to the formula

$$Kp\,(1) = \frac{R(1) - R(2)}{(p(2)/\ p(1)) \times X\,(2) - X(1)}$$

where X is a bound-to-total ratio of the BHA or anti-Id, R=X/(1–X) is a bound-to-free ratio of the BHA or anti-Id, K is the equilibrium

constant of the reaction, p is the total input concentration of BHA or anti-Id. The sample without and with competitor was marked by (1) and (2), respectively. Since the concentration p(1) was known the equilibrium constant was computed as $K = Kp(1)/p(1)$. Results represent average values, computed as a geometrical mean, from the samples where 10 – 90% inhibition of binding has been measured. This receipt is principially the same as that used by Rodbard and Feldman (1971), however described in more general form.

Radioimmunoprecipitation. [^{125}I]-labelled BHA (native, or pH 5-treated and redialyzed to neutral pH before iodination) was incubated with MoAb IIF4 (Ab1) and Ab3 mouse antiserum in the presence of 0.5% Triton-X-100 and 0.1% 2-mercaptoethanol and immunoprecipitated using *Staphylococcus aureus* precoated with sheep anti-mouse Ig (see also Russ *et al.*, 1987).

Results

Detection of anti-Id antibodies in rabbit sera

The presence of anti-Id antibodies in rabbit sera saturated with twofold volume of mouse non-immune serum was detected by SP-RIA. As shown in Fig. 1, binding of [^{125}I]-BHA to MoAb IIF4 adsorbed to solid phase was inhibited only by anti-IIF4 sera No. 3 and 4, but not by anti-BB8 serum No. 1. Symmetrically, binding of [^{125}I]-BHA to MoAb BB8 was inhibited by serum No. 1, but not by anti-IIF4 sera. In no case decrease of binding of labelled antigen was observed in the presence of corresponding preimmune serum. Nearly 100% inhibition of antigen binding indicates that immunization of rabbits with MoAb IIF4 as well as MoAb BB8 led to the production of anti-Id antibodies which bind to the paratope or in close vicinity to the paratope.

It can be concluded from inhibition curves that anti-Id sera were closely specific to the immunizing MoAb, directed against unique idiotopes. This also shows that removal of balast antibodies to mouse Ig from anti-Id sera by mouse non-immune serum was efficient.

Characterization of anti-IIF4 idiotypes

The characterization of anti-IIF4 antibodies comprised affinity evaluation of anti-Id to MoAb IIF4 and type determination of anti-Ids in competitive-inhibition tests. In the following experiments affinity-purified anti-Id immunoglobulin obtained from rabbit serum No. 3 (anti-IIF4) was used.

Affinity was determined in liquid-phase competitive inhibition RIA experiments with homologous competitor. Effective affinity of anti-Id to MoAb IIF4 was found 1.2×10^9 mol^{-1} (Fig. 2, curve B) whereas the affinity of MAb IIF4 to BHA at the native pH 7 from was 1.7×10^8 mol^{-1} (Fig. 2, curve A).

In competitive test with heterologous competitor where BHA inhibited binding of [^{125}I]-anti-Id to MoAb IIF4 (Fig. 2, curve C), BHA could inhibit binding of only those

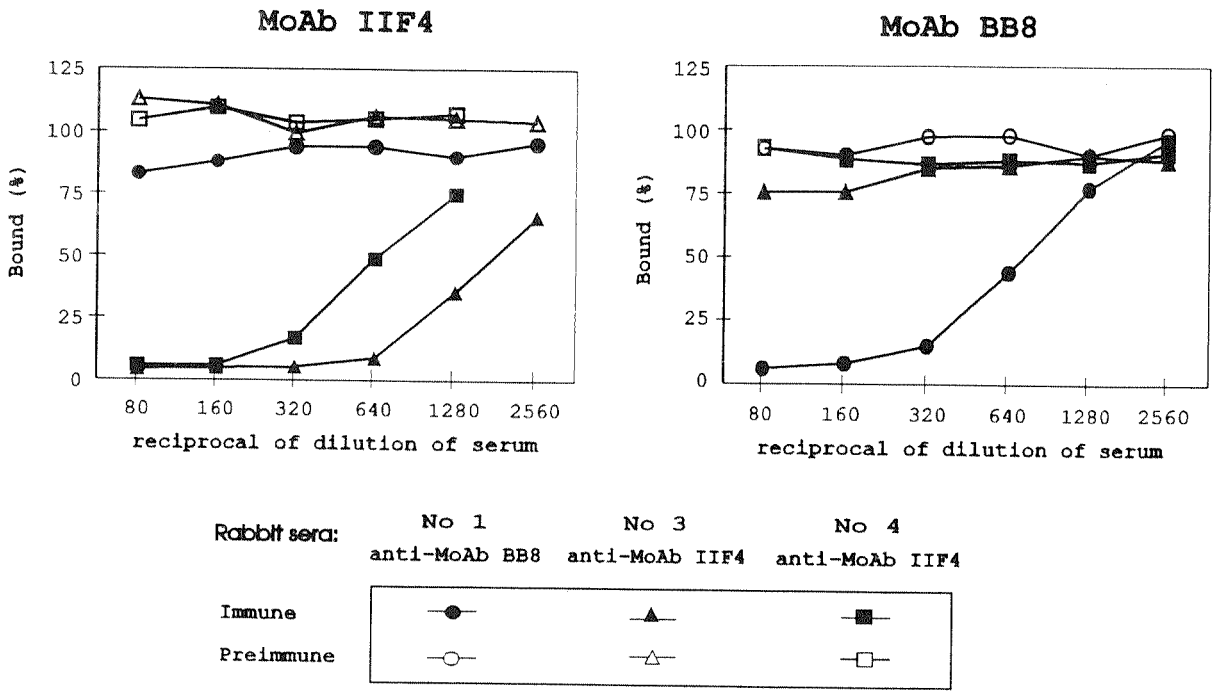

Fig. 1

Binding of [^{125}I]-BHA to MoAb IIF4 or BB8 in the presence of different concentrations of anti-Id serum in competitive SP-RIA

100% represents binding of [^{125}I]-BHA in the absence of competitor.

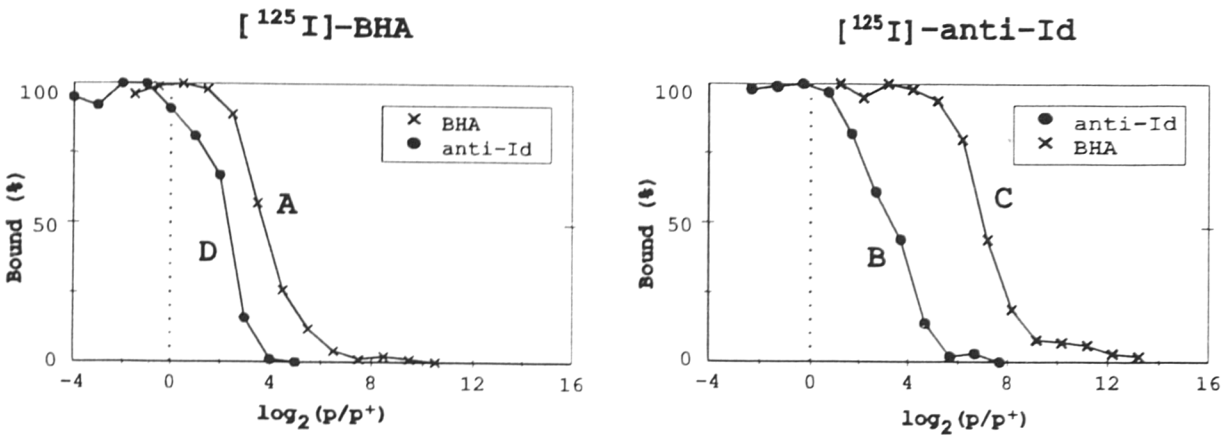

Fig. 2
Inhibition of binding of [^{125}I]-BHA or [^{125}I]-anti-Id to MoAb IIF4 by BHA and anti-Id in liquid phase competitive RIA
p/p^+ is concentration of competitor per concentration of label. 100% represents binding of label in the absence of competitor.

anti-Id antibodies which bind to the IIF4 paratope or its nearmost surroundings. The lower tail of the curve C indicates the presence of a minor part – less than 10% of anti-Id antibodies directed to the MoAb IIF4 idiotopes which did not interfere with its antibody-combining site (i.e., anti-Id of alpha type). Supposing that this minor subpopulation of alpha type anti-idiotypes could significantly affect neither the effective affinity estimation of anti-Id to MoAb IIF4 nor the position of inhibition curve B, we can conclude that the inhibition potential of anti-Id was higher then that of BHA in accordance with the two effective affinities estimated in competitive tests with homologous competitors (curves A, B). In reciprocal competitive test with heterologous competitor (i.e. anti-Id as an inhibitor of [^{125}I]-BHA binding to MoAb IIF4 (Fig. 2, curve D)), higher inhibition potential of anti-Id was confirmed too.

Specificity of Ab3 sera

Two sera from mice immunized with purified anti-IIF4 rabbit immunoglobulin were tested for the presence of virus-specific antibodies in SP-RIA using purified native virus A/Dunedin. As preliminary screening showed, both sera had the same titer 1:1000 (data not shown), and were pooled for subsequent experiments. Anti-HA specificity of Ab3 antibodies was confirmed in radioimmunoprecipitation, where native (pH-neutral) and fusogenic (pH 5-treated) BHAs labelled with [^{125}I] were used. As shown in Fig. 3, [^{125}I]-BHA at native conformation reacted with MoAb IIF4, but reaction with Ab3 serum was not visible. Fusogenic form of [^{125}I]-BHA yielded very strong reactivity with MoAb IIF4 and also significant reactivity with Ab3 serum. The bands represented HA2 glycopolypeptide of HA to which the MoAb IIF4 specificity has been proved previously (Russ *et al.*, 1987). The reactivity patterns of MoAb

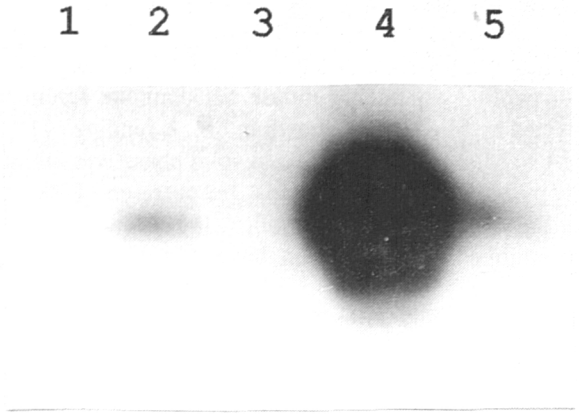

Fig. 3
SDS-PAGE of immunoprecipitates of [^{125}I]-BHA and MoAb IIF4 or Ab3
Approximately 10^5 cpm of [^{125}I]-labelled BHA from A/Dunedin/4/73 virus (corresponding to about 10 ng of protein) was incubated with MoAb IIF4 or Ab3 serum. Immunoprecipitates were analysed in 10% SDS-PAGE under reducing conditions with subsequent autoradiography. Lanes: 1, mouse preimmune serum + [^{125}I]-BHA (pH 7); 2, Ab3 serum + [^{125}I]-BHA (pH 5); 3, Ab3 serum + [^{125}I]-BHA (pH 7); 4, MoAb IIF4 + [^{125}I]-BHA (pH 5); 5, MoAb IIF4 + [^{125}I]-BHA (pH 7).

IIF4 and Ab3 serum demonstrated strong dependence of binding on the pH form of BHA, i.e. preferential binding of both MoAb IIF4 and Ab3 to conformationally altered haemagglutinin. Ab3 specificity furnished a definite proof that there are anti-Ids of beta type among Ab2 antibodies.

Cross-reactivity patterns of MoAb IIF4 and Ab3

The reactivity of MoAbIIF4 and Ab3 antibodies with a panel of influenza strains belonging to H1, H2, H3, H4, H7, and H10 subtypes, both native and acid-treated viruses, was

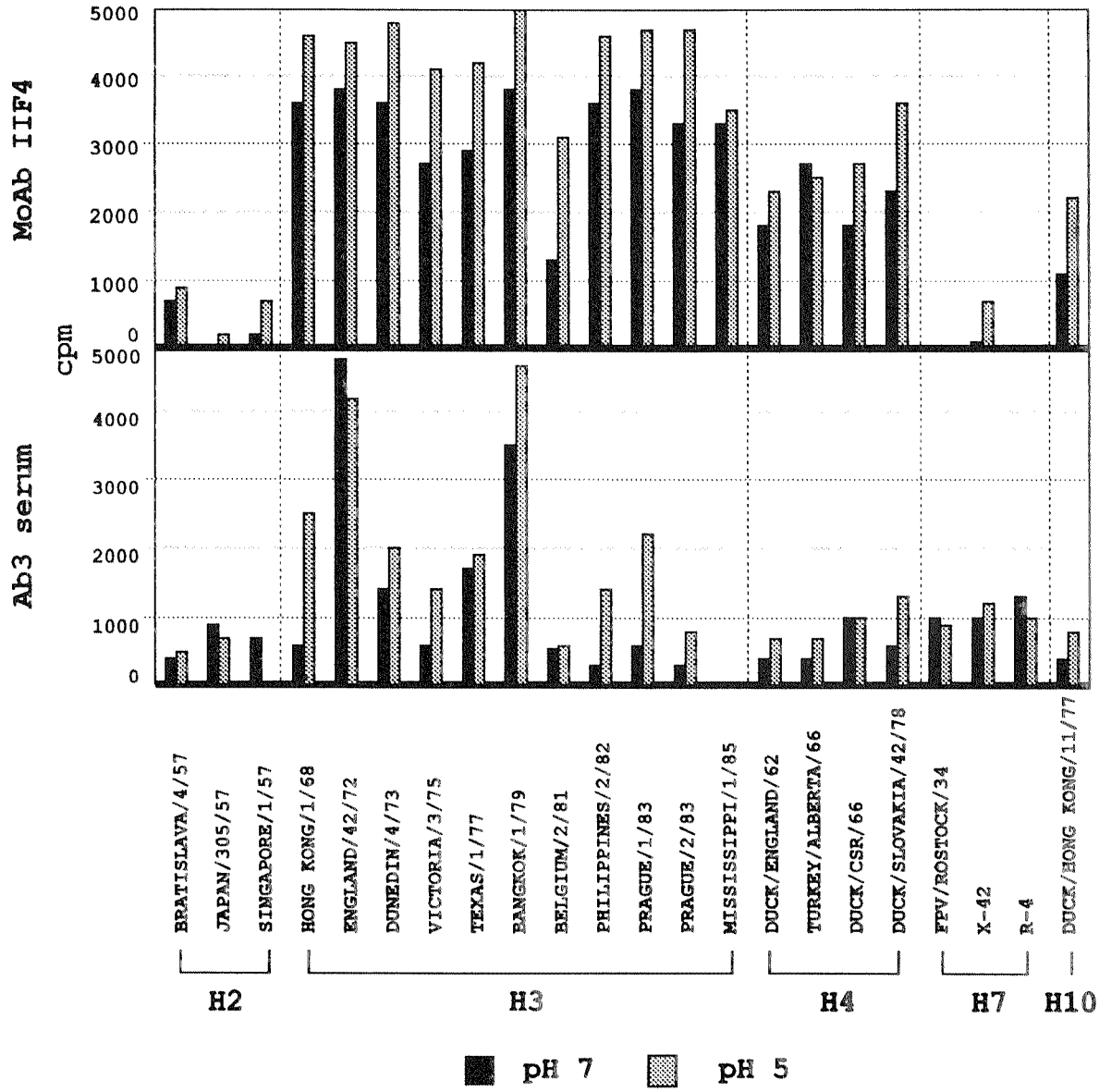

Fig. 4
SP-RIA reactivity pattern of MoAb IIF4 and Ab3 serum with different influenza A virus strains, native (pH 7) or acid-treated (pH 5)

investigated in SP-RIA. The concentration of antibody (Ab1 or Ab3) providing 75% value of the maximal binding (plateau) with native A/Dunedin virus was used. MoAb IIF4 revealed high level of cross-reactivity not only with all tested strains of H3 subtype within a period of years 1968–1985, but bound also to some strains belonging to subtypes H2, H4, H7, H10 (Fig. 4). As the data show, the reaction of MoAb IIF4 was higher with acid-treated viruses than with the native ones in most of strains tested and therefore, limited reactivity of the respective epitope in native virions seems to be its general property.

Binding of Ab3 antibodies was generally at lower level than that of MoAb IIF4, with exception of the reactions of Ab3 with strains A/England and A/Bangkok. Despite this, in some cases Ab3 binding appeared to those strains with which MoAb IIF4 didn't react (A/FPV/Rostock; R-4) or did at very low level and only after pH 5.0 treatment of virus (A/Japan). Conversely, the reaction of Ab3 with strain A/Missisippi disappeared. Enhanced binding of Ab3 to acid-treated viruses was also observed although at restricted number of strains as compared to MoAb IIF4. Especially

outside of H3 subtype, preferential Ab3 binding to acid-treated antigens became rare and not so apparent.

Despite high degree of cross-reactivity of both MoAb IIF4 and Ab3, none of them reacted either with any tested strain of H1 subtype (A/NWS/33, A/PR/8/34, A/FM/1/47, A/Switzerland/74/85, A/Singapore/6/86) or with some of H4 (A/Duck/Hong Kong/33/76) and H10 (A/Chick/Germany/49, A/Quail/Italy/544/66, A/Laruss/78) strains (data not included in Fig. 4).

Reactivity with amantadine-resistant mutants in SP-RIA

Binding of MoAb IIF4 and Ab3 serum was tested with two amantadine-resistant mutants '1a' and '4x' with conformationally altered haemagglutinins in consequence of defined amino acid substitutions (Daniels *et al.*, 1985), derived from the parental recombinant virus X-31 (H3N2). For standardization of conditions and for sensitive detection of 'pH-dependence' of binding, the concentrations of MoAb IIF4 and Ab3 serum giving equal binding and less than 50% of maximal binding to the native A/Dunedin virus were used in SP-RIA.

MoAb IIF4 in the reaction with both A/Dunedin and X-31 showed preferential binding to acid-treated virus (Table 1). However, this dependence was negligible with mutants 1a and 4x, where the binding to pH 7 form of antigen reached the level of binding to pH 5-treated antigen – apparently as a consequence of structural changes in the mutant HA.

Table 1. Reactivity of MoAb IIF4 (Ab1) and Ab3 serum with amantadine-resistant mutants of influenza virus

	MoAb IIF4 (Ab1) (cpm)		Ab3 serum (cpm)	
	pH 7	pH 5	pH 7	pH 5
A/Dunedin/4/73	1654	3906	1658	1924
X-31	2090	4028	1028	1172
1a	3862	4066	1552	1614
4x	2898	3240	964	1052

Reactivity detected in SP-RIA.

Ab3 serum did not reveal significant difference in binding to native and acid-treated viruses A/Dunedin or X-31, as well as it gave equal binding to both pH forms of mutants 1a and 4x. Mutant 1a (but not 4x) yielded markedly higher binding of Ab3 when compared to parental X-31.

Discussion

For further study of the HA region associated with IIF4 epitope we developed polyclonal Ab3 antibodies by immunization with anti-Id antibodies (Ab2) substituting IIF4 epitope. Paratope-related specificity of Ab2 was proved in competitive-inhibition tests where anti-Id antibodies inhibited binding of antigen to MoAb IIF4. The presence of anti-Id of beta type was confirmed by the HA2 specificity of Ab3. A remarkable property of our anti-Id antibodies is their binding potential, higher than that of BHA, as revealed in competitive inhibition experiments. Lower inhibition potential of BHA reflects (i) the higher (approx. 7-fold) affinity of anti-Id to MoAb IIF4 than that of MoAb IIF4 to BHA (at the native pH 7 form) and (ii) the heterogeneity of native BHA molecules, where the majority of them does not take part in the competition.

We suppose that our findings may be generalized into a conclusion that the specific response to poorly accessible antigenic determinants can be reached via anti-Id antibodies of beta type. This approach could be valuable especially for those antigenic determinants which are conformationally dependent and cannot be substituted by synthetic peptides.

Immunization with Ab2 antibodies of beta-type usually leads to the production of a spectrum of Ab3 antibodies among which there are those of the same or similar specificity as Ab1 (Bona and Kohler, 1984). The degree of this similarity in terms of specificity to original and related antigens varies, being dependent on many factors where important role play regulatory idiotope interactions and the fidelity of antigenic mimicry of Ab2. To a limited degree, similar relations in reactivities of Ab1 and Ab3 with various influenza strains have been observed in our experiments. However, Ab3 recognized those viruses to which Ab1 did not bind (A/FPV/Rostock/34 (H7N1), R-4 (H7N2)), or did at limited level after exposure of virus to pH 5 (A/Japan/305/57 (H2N2)). This observation could be associated with the fact that anti-Id antibodies may not always provide exact image of external antigens (Bentley *et al.*, 1990). As polyclonal Ab3 consists of many Ig populations of drifted specificity, it is possible that some of them exhibit the ability to recognize IIF4 epitope modified either in the primary structure or conformationally. This concerns the evolutionary more distant strains of H2 and H7 subtypes mentioned above (see Fig. 4).

MoAb IIF4 gave homogeneous reactivity pattern with virus strains of H3 subtype characterized by relatively high level of binding and well pronounced 'pH-dependency'. Therefore, a close structural similarity of IIF4 epitope within the strains of H3 subtypes could be expected. However, Ab3 exhibited surprisingly heterogeneous pattern of reactions with particular H3 strains where the binding to one of them (A/Mississippi/1/85) disapeared, and on the other hand, the reaction with some strains markedly rose (A/England/42/72, A/Bangkok/1/79). However, Anders *et al.* (1989) described very similar paradoxical observation of Ab3 specificity on the model of influenza.

The dependence of MoAb IIF4 binding to HA on conformational state of the antigen is a characteristic property of majority of virus strains tested. This dependence was conserved in the reactivities of Ab3 with some strains, mainly with those belonging to H3 subtype, that indicates that a subpopulation of Ab3 antibodies bears the fine specificity of MoAb IIF4. Another subpopulation of Ab3 antibodies of altered fine specificity appears to be involved in equal binding to both pH forms of viruses. The participation of these Ab3 antibody subpopulations seems to be different in different virus strains. pH-dependent conformational changes on HA trimer were studied by selection of amantadine-resistant influenza A virus mutants. The substitution of the amino acid 112 Asp to Gly on HA2 (mutant 1a) leads to destabilization of amino terminal peptide of HA2 at pH-neutral conditions, and the substitution of the amino acid 81 Glu to Gly on HA2 (mutant 4x) causes destabilization of trimeric arrangement of HA (Daniels *et al.*, 1985). We examined the impact of the described conformational changes of HA of these mutants on the IIF4 epitope. Analysis of the reactions of 1a and 4x mutants with Ab1 and Ab3 shows that more relevant changes in relation to IIF4 epitope reveals mutant 1a. With respect to structural changes of mutant HAs proposed by Daniels *et al.* (1985) we can conclude that uncovering of IIF4 epitope correlates in more extent with exposition of N-terminus of HA2 than with destabilization of trimeric arrangement of HA. Biological aspect of the importance of MoAb IIF4-binding region of HA2 glycopolypeptide will be taken into account in future studies.

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