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INHIBITION OF INFLUENZA VIRUS HAEMOLYTIC AND HAEMAGGLUTINATION ACTIVITIES BY MONOCLONAL ANTIBODIES TO HAEMAGGLUTININ GLYCOPOLYPEPTIDES HA1 AND HA2

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Summary. — Acid treatment of influenza virus enhanced haemagglutination inhibiting (HI) activity of some anti-HA1 monoclonal antibodies (MoAbs). These changes in the HI-activity could be either due to alteration in the mutual orientation of MoAb (e.g. IC8, IB8) binding epitope to receptor site or to an increase in the number of epitopes accessible to the corresponding MoAbs (e.g. IVA1). HI test with pH 5-virus revealed similar (although not identical) antigenic differences among related virus strains as the HI test with pH 7-virus. Anti-HA2 MoAbs were negative in the HI test with both pH 5- and pH 7-virus. Anti-HA1 MoAbs showed a HI activity with pH 5-treated BHA similar to that with pH 5-treated virus. Surprisingly one out of eight anti-HA2 MoAbs (IIF4) exhibited a relatively high HI activity to pH 5-BHA-mediated haemagglutination. Virus-induced red blood cell haemolysis was efficiently inhibited with several anti-HA1 MoAbs (e.g. IC8, IB8, and IIB4) while other anti-HAl antibodies, including IVA1 and IVG6 with preferential reactivity with pH 5-treated antigens in RIA, gave no inhibition. As a rule, anti-HA2 MoAbs were poor haemolysis inhibitors.

Key words: *influenza A virus*; *haemagglutination*; *haemolysis*; *monoclonal antibody*

Introduction

The low-pH-induced changes in the influenza A virus haemagglutinin (HA), which are essential in the initiation of infection have been extensively through which the fusogenic N-terminal peptide of the HA2 subunit becomes exposed and thus able to attach to and interact with the target cell membranes (for review see Wiley and Skehel, 1987). A number of studies have reported the use of antibodies to detect antigenic alterations that coincides with the acid-induced conformational change in the HA. These studies were made usually with MoAbs specific to major antigenic sites

localized on the HA1 subunit of influenza virus HA (Daniels *et al.*, 1983; Webster *et al.*, 1983; Yewdell *et al.*, 1983; Jackson and Nestorowicz, 1985).

We prepared MoAbs specific to either the HA1 or the HA2 subunit of influenza A virus HA (Russ *et al.*, 1987). Using these MoAbs we were able to examine regions of the molecule not examined in the previous studies. We showed that 1) changes in the antigenicity of the HA molecule at acid pH involve both subunits; 2) bromelain-released HA (BHA) can undergo an antigenic change which is very similar to that in native viral HA; and 3) there are epitopes in the untreated HA preparations to which MoAbs bind with the same affinity as to pH 5-treated HA and which are present in the pH 5-HA in larger quantities (Kostolanský *et al.*, 1988).

In the present study we further investigated the HI activity of these MoAbs against virus and BHA after pH 5 treatment, and their capacity to inhibit virus-induced haemolysis.

Materials and Methods

Viruses. The following influenza A virus strains were used: A/Dunedin/4/73(H3N2),A/Bangkok/1/79(H3N2), A/Hong-Kong/1/68(H3N2), A/England/42/72(H3N2), A/Victoria/3/75(H3N2), A/Texas/1/77(H3N2), A/Belgium/2/81(H3N2), and A/Philippines/2/82(H3N2). The conditions of virus propagation and purification were described elsewhere (Russ *et al.*, 1974).

Haemagglutination and haemagglutination-inhibition titres were determined by a microtechnique as described (Russ *et al.*, 1978). HI titres were expressed per 0.025 ml as reciprocals of the highest initial dilution of serum causing inhibition of 4 to 8 haemagglutination units (HU). Acid-treated haemagglutinating antigens were prepared in the following way: virus or BHA was first dialyzed against 100 vol of phosphate-citrate buffer (McIlvaine pH 5) overnight at 4 °C and thereafter redialyzed against PBS at pH 7.2. Octyglucoside-solubilized haemagglutinin was prepared by incubation of purified virus with 25 mmol/l detergent solution in PBS at room temperature for 30 min followed by removal of octylglucoside by dialysis.

Haemolysis inhibition test. Two antibody dilutions (ascitic fluids) in 0.25 ml of 0.15 mol/l NaCl were mixed with 400 HU of virus and incubated for 30 min. Then 0.25 ml of 2% chicken erythrocytes was added. After 30 min of incubation the pH in the samples was adjusted to 5.0 by adding 1 ml of phosphate-citrate buffer (McIlvaine) pH 5.0. The mixture was then incubated for 60 min at room temperature, centrifuged and released haemoglobulin in the supernatant was determined from the absorbance at 405 nm.

Results

HI activity of some anti-HA1 monoclonal antibodies is increased after treatment of virus at pH 5

A short (60—90 min) exposure of Dunedin or Bangkok viruses to acid environment does not affect their haemagglutinat ng activity. In fact, this activity decreased only slowly within a few days, probably due to virus aggregation (unpublished results). Experiments, therefore, were done to determine whether antibodies to the epitopes more exposed at low pH inhibit haemagglutination and whether the differences between antibodies found in RIA assays (Kostolanský *et al.*, 1988) could be correlated with biological activity.

In the HI test with pH-7 virus anti-HA1 antibodies IB8, IC8, IIB4, IIG3, IIE5, and IIF5 showed anti-haemagglutination activity, but high

Table 1. HI activity of anti-HA1 MoAbs with homologous virus antigenic preparations

MoAb	pH 7 virus	pH 5 virus	pH 5 BHA	OG-virus[1])	Anti-M Ig[2]
IB5	<10	<20	20	<20	<10
IIC5	<10	20	20	<20	<10
IB8	160	1280	1280	4096	2560
IC8	160	640	640	1024	1280
IVA1	<10	640	640	40	<10
IVG6	<10	40	20	80	<10
IVC4	<10	<10	<20	20	<10
IIB4	20 480	20 480	N.D.	N.D.	N.D.
IIG3	160	160	N.D.	N.D.	2560
IIE5	160	160	N.D.	N.D.	2560
IIF5	160	160	N.D.	N.D.	2560

The titres are expressed per 0.025 ml as reciprocals of the highest dilution of antibody causing inhibition of 4—8 HAU of the respective antigenic preparation.
N.D. — not done
[1]) Octylglucoside-treated
[2]) Sheep anti-mouse Ig added to the reaction mixture with pH 7 virus

titres were detected only with IIB4 antibody (Table 1). We found that two of these anti-HA1 antibodies, namely IB8 and IC8 exhibited significant increase (greater than fourfold) in HI titres against pH 5-treated virus compared with untreated virus. No parallel increase was detected in the direct binding assay. MoAbs IB8 and IC8 reacted in RIA equally well with both untreated and pH 5-treated virus (Kostolanský *et al.*, 1988). Other anti-HA1 antibodies (IIB4, IIG3, IIE5, and IIF5) showed the same HI titres with both neutral and pH 5-treated viruses. Anti-HA1 antibodies which were negative in HI test with neutral virus (IB5, IIC5, IVG6, and IVC4) were negative also in the HI test with pH 5-treated virus, with the exception of IVA1 antibody exhibiting a relatively high HI potency to pH 5-treated virus (HI titres about 640). Reactivity of MoAbs IVA1 with pH 7 and pH 5 virus in RIA correlated with the HI data in that IVA1 MoAb exhibited a significantly increased binding to pH 5-treated virus. None of the MoAbs used in the present study showed in the HI test with pH 5-treated virus a lower titre than with neutral virus, or a loss of HI activity. In agreement with this was that no MoAb showed decreased binding in RIA after pH 5 treatment (Kostolanský *et al.*, 1988).

Anti-H2 monoclonal antibodies exert no HI with pH 7 and pH 5-viruses

All 8 anti-HA2 MoAbs described previously (Russ *et al.*, 1987) were negative in HI tests with both neutral and pH 5-treated virus.

Table 2. Reactivity of H3N2 virus strains exposured to pH 7 or pH 5 in HI test

Ab	HK 68 pH 7	HK 68 pH 5	Eng 72 pH 7	Eng 72 pH 5	Dun 73 pH 7	Dun 73 pH 5	Vict 75 pH 7	Vict 75 pH 5	Tex 77 pH 7	Tex 77 pH 5	Bang 79 pH 7	Bang 79 pH 5	Belg 81 pH 7	Belg 81 pH 5	Phil 82 pH 7	Phil 82 pH 5
IB8	40	80	80	5120	40	1280	20	<20	<20	<20	<20	<20	<20	<20	<20	<20
IC8	40	80	80	2560	80	640	20	<20	<20	<20	<20	<20	<20	<20	<20	<20
IVA1	<20	<20	<20	1280	<20	320	<20	<20	<20	40	<20	80	<20	80	<20	40
IIB4	<20	<20	20	40	160	128	1280	2560	5120	5120	20480	20480	10240	10240	20	40
332*	160	320	640	2560	1280	1280	640	320	160	80	80	80	40	40	40	320
223*	2560	5120	2560	5120	2560	5120	1280	1280	1280	80	160	80	160	40	80	160

The titres are expressed per 0.025 ml as reciprocals of the highest dilution of antibody causing inhibition of 4—8 HAU of the respective virus.
* Rabbit polyclonal antiserum

HI-activity of anti-HA1 monoclonal antibodies with pH 5-BHA is similar to that with pH 5-treated virus

After pH 5-treatment BHA acquired the ability to agglutinate chicken erythrocytes (Skehel *et al.*, 1982). Anti-HA1 antibodies IB8, IC8, and, the most important, also IVA1 showed with pH 5-treated BHA a HI activity similar to that with pH 5-treated virus (Table 1).

One anti-HA2 monoclonal antibody (IIF4) inhibits pH 5-BHA mediated haemagglutination

Surprisingly, the IIF4 anti-HA2 antibody exhibited a relatively high HI activity (> 640) to pH BHA-mediated haemagglutination, although the HI titres varied extremely (640—20 000). Other anti-HA2 MoAbs failed to inhibit pH 5-BHA-mediated haemagglutination.

HI activity with pH 7-virus treated with octylglucoside

The capacity of IIF4 antibody to inhibit pH 5 BHA-mediated HA cannot be explained by the conformational change at acidic pH itself, because this antibody has no capacity to inhibit haemagglutination of pH 5-treated virus.

To examine whether this IIF4 HI activity can be explained simply by the solubilization of HA, we performed a HI test with pH 7-virus disrupted with octylglucoside (pH 7-BHA has no ability to agglutinate chicken erythrocytes, therefore a HI-test allowing a direct comparison cannot be done). We found an extremely low HI activity of IVA1 antibody (as expected) and no HI activity of IIF4 antibody under these conditions.

Table 1 supplemented with the results of HI tests presented previously (Russ *et al.*, 1987) shows that low HI titres of IB8, IC8, IIG3, IIE5, and IIF5 increased by adding sheep anti-mouse Ig into the HI test with pH 7-virus, whereas the HI activity (or its absence) of other antibodies was not influenced under these conditions.

Analysis of influenza A(H3N2) strains in the HI test with pH 5-virus revealed similar antigenic differences to a test with pH 7-virus

Acid treatment of the virus enhanced HI activity of some anti-HA1 MoAbs. Moreover, MoAb IVA1, negative in the HI test with pH 7-virus manifested a HI activity against pH 5-virus. We were interested to know which antigenic differences can be detected with the use of these MoAbs in HI tests with pH 5-viruses. As a control we used MoAb IIB4 with a high HI activity and reacting in RIA equally well with pH 7 and pH 5-virus (Kostolanský *et al.*, 1988). As summarized in Table 2 the antigenic relationships among various influenza A virus strains estimated by HI tests with these MoAbs were similar (although not identical) when using both pH 7 and pH 5-viruses. Analogous results were obtained with the rabbit polyclonal sera against virus A/Dunedin (No. 223) or its BHA (No. 332). MoAb IVA1 gave positive results only with pH 5-treated homologous or closely related virus strains. By contrast, this MoAb showed in RIA an unusually high cross-reactivity (Kostolanský *et al.*, 1988).

Haemolytic activity of virus is inhibited by some anti-HA1 monoclonal antibodies

Influenza virus A/Dunedin caused extensive haemolysis at pH 5.0. MoAbs were examined in haemolysis inhibition assays at dilutions of 1/20 1/80. Anti-HA1 MoAbs IB8, and IIB4 efficiently inhibited haemolysis (10% of total haemolysis at 1/80 antibody dilutions). Other anti-HA1 antibodies (including IVA1 and IVG6 with preferential binding to pH 5-treated antigens) did not reduce haemolysis (>90% of total haemolysis at 1/20 antibody dilutions).

Anti-HA2 monoclonal antibodies are poor inhibitors of haemolysis

Anti-HA2 MoAbs were poor inhibitors of haemolysis (60—100% of total haemolysis at 1/20 antibody dilutions). Using various MoAbs as control we found that anti-neuraminidase antibody IIIE11 very efficiently inhibited haemolysis mediated by influenza virus Dunedin (20 % of haemolysis at 1/80 antibody dilution).

Discussion

A number of studies have reported the use of MoAbs to detect acid-induced changes in influenza virus HA by HI tests. These studies were usually done with MoAbs specific to major antigenic sites; both elevations and decreases in HI titre were observed after pH 5 treatment (Daniels *et al.*, 1983; Webster *et al.*, 1983; Yewdell *et al.*, 1983; Nestorowicz *et al.*, 1985). In the present study we carried out further investigations on the effect of acid pH on antigenic properties of influenza virus haemagglutinin. First of all, we examined in detail HI-activity of MoAbs specific to either the HA1 or HA2 subunit (Russ *et al.*, 1987). Using these MoAbs we have been able to examine regions of the HA molecule not analysed in previous studies.

We showed that low HI activity of MoAbs IC8 and IB8 significantly increased after treatment of virus at pH 5. This enhancement in HI activity cannot be explained simply by increased binding to acidified virus, because MoAbs IC8 and IB8 reacted (in RIA) to the same extent at the same affinity with untreated and pH 5-treated virus (Kostolanský *et al.*, 1988). We assume that, due to conformational change at low pH, MoAbs IC8 and IB8 get closer to the receptor binding site and therefore the binding to red blood cells is then more efficiently inhibited. At present we cannot exclude also the possibility that binding of IB8 and IC8 to acidified (but not to neutral) HA induces a change in the receptor site (enhances affinity of receptor site). We consider such explanation as very unlikely.

MoAbs IIG3, IIE5, and IIF5 proved to be similar. They also have a low HI activity and the same reactivity in RIA with untreated and pH 5-treated virus. As distinct from MoAbs IC8 and IB8, they also gave the same HI titre with both neutral and pH 5-treated virus. Enhancement of HI activity due to alteration in the mutual orientation of epitope to receptor binding site thus apparently takes place only with epitopes properly localized in the tertiary HA structure.

Anti-HA1 MoAb IVA1 shows no detectable HI activity with pH 7-virus. This MoAb, however, exhibited a relatively high HI-activity against pH 5-virus. We detected a parallel increase also in RIA (Kostolanský *et al.*, 1988).

Previously (Kostolanský *et al.*, 1988) we showed that MoAb IVA1 binds with the same affinity to pH 7 and to pH 5-HA and we postulated that at pH 7 the HA preparations contain two subpopulations of HA molecules. The minor subpopulation of the HA molecules contains the IVA1 epitope accessible to MoAb, whereas the major subpopulation of HA molecules contains the IVA1 epitope not accessible to MoAbs. After pH 5 treatment the IVA1 epitope became accessible in a larger extent of HA molecules, therefore, this MoAb showed a greatly enhanced reactivity with acid-treated HA. This assumption is also supported by the finding that HI activity of IVA1 at pH 7 cannot be potentiated by adding anti-mouse Ig into the HI test. Anti-mouse Ig cannot potentiate HI activity, because MoAb IVA1 is not bound to most of the HA molecules in HA preparations at pH 7. By con-

trast, low HI activity of all MoAbs with an equal reactivity with untreated and pH 5-treated virus (e.g. IC8, IIG3, etc) is potentiated by anti-mouse Ig.

The eventual increase in HI activity by alterations in accessibility of epitopes also depends on the nature of the epitope, because MoAb IVG6 with a similarly enhanced binding to pH 5-treated virus like MoAb IVA1 showed only an extremely low HI activity with pH 5-virus. Anti-HA2 MoAb, like with pH 7-virus, were also negative with pH 5-virus.

HI activity of anti-HA1 MoAbs with pH 5-BHA was similar to that with pH 5-virus. The most important was the finding that anti-HA2 MoAb IIF4 exhibited a relatively high HI activity against pH 5-BHA-mediated haemagglutination. The acid-induced conformational change in the intact viral HA and isolated BHA seems to be similar if not identical (Daniels *et al.*, 1983; Doms *et al.*, 1985). Therefore, as expected, HI activity of anti-HA1 with pH 5-BHA was similar to that with pH 5-treated virus. The pH 5 BHA-mediated haemagglutination was inhibited also with anti-HA2 MoAb IIF4, although the latter together with other anti-HA2 MoAbs showed no HI activity against neutral, pH 5 and octylglucoside-treated virus. To complete the characterization of this MoAb it is important to remember that after treatment of virus at pH 5, its binding in RIA significantly increased (Kostolanský *et al.*, 1988). All these results suggest that acid-induced conformational change in the intact viral HA, or solubilization of neutral virus with octylglucoside does not produce preparations whose haemagglutination activity could be inhibited with MoAb IIF4. We can speculate that the potency of MoAb IIF4 to inhibit pH 5 BHA-mediated haemagglutination is probably caused by both bromelain solubilization of HA and pH 5-induced conformational change. We believe, however, that in fact the peculiar IIF4 HI activity reflects differences in conformational changes in intact viral HA and BHA, reported recently (Doms and Helenius, 1986; Boulay *et al.*, 1987).

Analysis of influenza A(H3N2) strains in the HI test with pH 5 virus revealed similar antigenic differences to the test with pH 7 virus. This confirms previous conclusion (Kostolanský *et al.*, 1988) that our MoAbs react by the same affinity with pH 5 and pH 7 viruses, i.e. that essentially the same epitopes (although at pH 5 and pH 7 at different quantities) are involved.

At last we analysed the capacity of anti-HA1 and anti-HA2 MoAbs to inhibit virus-mediated haemolysis. The latter was efficiently inhibited (with anti-HA1 MoAb which were able to inhibit also pH 7 virus haemagglutination (i.e. IC8, IB8, and IIB4). These MoAbs obviously inhibit haemolysis by preventing the adsorption of virus onto erythrocytes. Other MoAbs including IVA1 and IVG6 with preferential reactivity with pH 5-treated antigens, gave no inhibition at all. Similarly, all anti-HA2 MoAbs were not able significantly inhibit haemolysis. We assume that these MoAbs are directed against regions of the HA molecule which are not directly involved in the fusion. This explanation is consistent with previous findings that at least 3 out of 4 (but probably all) independent antigenic sites defined on the HA2 subunit by our MoAb (Russ *et al.*, in preparation) are not localized in the N-terminal region of HA2 which is responsible for the fusion

activity (Kostolanský *et al.*, 1988). Becht *et al.* (1984) also found that anti-HA2 antibodies do not inhibit virus-induced haemolysis.

At present we cannot explain why anti-NA MoAb IIIE11 inhibits haemolytic activity. Kida *et al.* (1983) described inhibition of haemolytic activity of influenza virus with a single anti-NA MoAb. Nevertheless, Webster *et al.* (1984) later showed with a large panel of anti-NA MoAbs that anti-NA MoAbs do not inhibit haemolytic activity of influenza virus. Experiments with cells that express HA from a cloned copy of HA gene inserted into a recombinant vector demonstrated that the HA molecule displays fusion activity in the absence of any other influenza virus encoded components (White *et al.*, 1982).

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