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MONOCLONAL ANTIBODIES TO GLYCOPOLYPEPTIDES HA_1 AND HA_2 OF INFLUENZA VIRUS HAEMAGGLUTININ

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Summary. — Anti-haemagglutinin monoclonal antibodies were prepared and their HA_1 or HA_2 specificity was determined by solid phase radioimmunoassay (RIA) using purified viral haemagglutinin (HA) and haemagglutinin glycopolypeptides HA_1 and HA_2, by radioimmunoprecipitation followed with SDS-PAGE, by immunoblotting and by inhibition of virus-induced haemagglutination. The capacity of these methods to estimate HA_1 or HA_2 specificity of anti-HA monoclonal antibodies (MoAb) was compared. HA_1 specificity was demonstrated for all hybridomas originating from lymphocytes of mice immunized with complete influenza virus, except IIF4 hybridoma which was HA_2-specific. All hybridomas obtained with lymphocytes from mice immunized with HA glycopolypeptide HA_2 were HA_2-specific. Anti-HA_2 MoAb neither inhibit haemagglutination induced by the virus or by HA subunits nor neutralized viral infectivity, either alone or in mixture. As expected, all anti-HA_1 MoAb were H3 subtype-specific, showing usually good reactivity only with viruses close to the virus strain used for immunization. Two anti-HA_1 MoAb (IVA1 and IVG6) showed unusual cross-reactivity within the H3 subtype. All anti-HA_2 MoAb were broadly cross-reactive within the H3 subtype. Moreover, a half of them showed high cross-reactivity with influenza viruses of the H7 HA subtype. But the same antibodies did not react with HA of H1, H2 and H8 subtypes.

Key words: Influenza virus; haemagglutinin glycopo ypeptides HA_1 and HA_2; monoclonal antibody

Introduction

The haemagglutinin (HA) is the major glycoprotein of influenza virus, it mediates virus adsorption and penetration into host cells and is important for the induction of protective immunity. HA exist in the form of spikes composed of three identical monomers, each consisting of a glycopolypeptide HA_1 and a glycopolypeptide HA_2 joined by a single disulphide bond (for review see Ward, 1981).

Anti-HA antibodies neutralizing virus and inhibiting haemagglutination are directed primarily toward HA_1 (so far, no exception from this rule has been well documented). Four dominant antigenic regions have been defined on the globular head of the molecule formed by this glycopolypeptide (Wiley *et al.*, 1981; Caton *et al.*, 1982).

Studies on the antibody-binding sites of HA have been focused primarily on determinants of the HA_1 glycopolypeptide. Therefore, much less is known about the antigenic properties of the HA_2 subunit of HA which together with a part of HA_1 forms the fibrous stalk region that anchors the HA molecule into the envelope (Wilson *et al.*, 1981).

Early studies suggested that the HA_2 subunit does not contribute significantly to the antigenic structure of HA in the intact virus (Brand and Skehel, 1972; Eckert, 1972; Schild *et al.*, 1976; Kuo *et al.*, 1976). In rabbit sera produced against intact virus or purified undissociated HA (Russ *et al.*, 1978) and, more important, also in human convalescent sera (Styk *et al.*, 1979) we demonstrated antibodies by gel double immunodiffusion and RIA reactive with both influenza HA subunits (HA_1 and HA_2). Moreover, we obtained direct evidence of the immunogenicity of the isolated HA glycopolypeptides HA_1 and HA_2 in rabbits (Styk *et al.*, 1979). It was later confirmed that the animal sera raised to intact influenza A virus preparations or subviral particles exposed to pH 5.0 and lacking the HA_1 subunit contained both HA_1- and HA_2-specific antibodies (Brown *et al.*, 1980; Graves *et al.*, 1983).

Since the HA_2 region of the HA molecule is highly conserved among all influenza A viruses, it was not surprising that, at least in some cases, intersubtype cross-reactivity with HA_2-specific antibodies was demonstrated (Russ *et al.*, 1978; Graves *et al.*, 1983).

The recognition of influenza virus HA by T cells was shown to involve recognition of determinants on both the HA_1 (Lamb *et al.*, 1982; Hurwitz *et al.*, 1984; Katz *et al.*, 1985a) and HA_2 glycopolypeptides (Wabuke-Bunoti and Fan, 1983; Katz *et al.*, 1985b). HA_2 with its conserved regions could be a target antigen for some T cells, particularly for cytotoxic T cells showing an unusually high degree of cross-reactivity (Wabuke-Bunoti and Fan, 1983; Katz *et al.*, 1985b; Kozinowski *et al.*, 1980; Askonas *et al.*, 1980; Anders *et al.*, 1981; Lamb *et al.*, 1982; Braciale, 1984; Fleischer *et al.*, 1985).

Recently, the first panel of anti-HA_2 monoclonal antibodies was used to study the immunogenic properties of HA_2. These monoclonal antibodies were, however, subtype-specific, an no positive results concerning the role of HA_2 in humoral or cell-mediated immunity to influenza have been obtained (Becht *et al.*, 1984).

In the present report we describe the preparation and characterization of MoAb specific to either HA_1 or HA_2 glycopolypeptide.

Materials and Methods

Viruses. The following influenza virus strains and recombinants were used: A/Dunedin/4/73 (H3N2) which will be referred to as strain "Dun"; A/Bangkok/1/79 (H3N2) – "Bang"; A/Hong Kong/1/68 (H3N2) – "HK"; A/Belgium/2/81 (H3N2) – "Belg"; A/Caen/1/84 (H3N2) – "Caen";

A/England/403/80 (H1N1) — "Eng"; A/PR/8/34 (H1N1) — "PR8"; A/Singapore/1/57 (H2N2) — "Sing"; A/Ty/Ont/61/68 (H8N4) — "Ont"; B/Hong Kong/8/73 — "B/HK"; X-42 (H7N2) — "X-42"; X-38 (H7N2) — "X-38"; and X-53 (H1N1) — "X-53". The conditions for infection of embryonated hen's eggs and the mode of purification of the viruses have been described (Styk and Blaškovič, 1973; Russ *et al.*, 1974).

Isolation of bromalain purified HA (BHA), glycopolypeptides BHA_1 and BHA_2, and iodination procedures were described previously (Poláková *et al.*, 1978). Glycopeptides were prepared by pronase digestion of the purified BHA_2.

Preparations of hybridoma cell lines. Hybridoma cell lines were prepared by fusion of mouse myeloma cell line Sp2/0 with spleen cells from immunized BALB/c mice using polyethylene glycol according to established procedures (Gerhard, 1980). BALB/c mice, which had been primed by intraperitoneal (i.p.) injections of purified virus (100 μg), or BHA_2 (100 μg) in incomplete Freund's adjuvant, were challenged intravenously (i.v.) 3 days before fusion. After fusion, the cells were distributed into 96-well microtitre plates on a feeder of spleen cells. The feeder cells (10^5/well) were seeded 24 hr before fusion and on the day of fusion they were irradiated with 20 Gy (^{60}Co, Chisobalt, Chirana, Prague). Cultures producing antibody to the influenza virus were cloned in soft agar. Hybridoma cell cultures were maintained in Dulbeco's modified Eagle's minimal essential medium containing foetal calf serum, 50 μg/ml gentamycine and 2 mmol/l glutamine at 37 °C in humidified atmosphere containing 5% CO_2. Ascitic fluids containing hybridoma antibodies were obtained from paraffine oil-primed BALB/c or DBA mice inoculated i.p. with approximately 5×10^6 hybridoma cells each.

Solid phase radioimmunoassay (RIA). An indirect antibody-binding assay with ^{125}I-labelled rabbit anti-mouse $F(ab')_2$ (Poláková and Russ, 1983) was used for the initial screening of hybridomas as well as for further characterization of monoclonal antibodies.

Determination of Ig class and subclass. The isotypes of the monoclonal antibodies were determined by a double antibody RIA (Poláková and Russ, 1983).

Immunoprecipitation and SDS-PAGE. S. aureus was incubated first for 15 min with swine anti-mouse Ig serum and then washed 5 times with PBS containing 0.5% Triton-X-100. This pretreatment ensured precipitation of all mouse Ig isotypes. Then *S. aureus* loaded with anti-mouse Ig was added to ^{125}I-labelled BHA which had previously been incubated in the presence of 0.5% Triton-X-100 and 0.1% 2-mercaptoethanol with 5 μl of ascitic fluid. After 1 hr incubation *S. aureus* cells were pelleted and washed 5 times. Adsorbed proteins were eluted from *S. aureus* by boiling in SDS-PAGE sample buffer and the eluates were analysed on 10% polyacrylamide gels (Laemmli, 1970). The gel was dried for autoradiography, and autoradiographs were made with Medix Rapid X-ray film (Czechoslovakia), using intensifying screens at −70 °C.

Immunoblotting. Haemagglutinin glycopolypeptides from SDS-PAGE were electrophoretically transferred to nitrocellulose sheets (Millipore GSWP, 0.22 μm pore size). After incubation of immobilized proteins with MoAb, the immune complexes were detected by using anti-immunoglobulin conjugated to horseradish peroxidase followed by treatment with diaminobenzidine to localize the peroxidase.

Haemagglutination and haemagglutination inhibition (HI) 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 antibody causing inhibition of 4 HA units of the homologous virus. A means for increasing the reactivity of MoAb in HI test was adding sheep anti-mouse Ig into the reaction mixture or detergent treatment of the virus used.

Results

HA_1 and HA_2 specificity of anti-HA monoclonal antibodies

The specificity of anti-HA MoAb to HA_1 or HA_2 glycopolypeptide was first examined in solid phase RIA. The results obtained are shown in Tables 1 and 2. Monoclonal antibodies IB8, IC8, IVA1 and IVG6 prepared against the strain Dun were clearly specific to the HA_1 glycopolypeptide. MoAb IB5 and IIC5 showed significant binding also to BHA_2, but the reactions with BHA_1 were preferential. We can conclude, therefore, that also these MoAb

Table 1. Reactivity of MoAb to HA_1 in solid-phase RIA

MoAb to HA_1 (Dun.)	Parental virus	BHA (Dun.)	BHA_1 (Dun.)	BHA_2 (Dun.)	B/HK
IB5	4 098	4 592	8 610	2 091	287
IIC5	3 102	2 511	4 991	1 302	310
IB8	5 795	522	1 972	**174**	232
IC8	5 480	385	2 310	**220**	247
IVA1	3 398	1 394	3 298	**204**	204
IVG6	4 020	1 292	4 284	**306**	255
MoAb to HA_1 (Bang.)					
IIB4	2 300	1 104	506	*N.D.	299
IIG3	1 502	300	300	N.D.	300
IIE5	3 085	510	390	N.D.	510
IIF5	3 022	390	388	N.D.	390

*N.D. — not done
The figures represent the radioactivity (cpm) bound in solid-phase RIA

were specific to the HA_1 glycopolypeptide. All six MoAb specific to the HA_1 glycopolypeptide mentioned above showed a higher binding to HA_1 than to HA and a lower binding to HA than to the whole virus (particularly MoAb IB8 and IC8). The results did not show fine specificity for the remaining MoAb, since HA from influenza virus A/Bangkok/1/79 (H3N2) was not available. The solid phase RIA with various virus strains and recombinants proved the HA specificity of anti-A/Bangkok MoAb (results not shown). Since all these MoAb were positive in the HI test (Table 3), they are considered as HA_1-specific and listed together with other HA_1-specific MoAb.

Table 2. Reactivity of MoAb to HA_2 in solid-phase RIA

MoAb to HA_2 (Dun.)	Dunedin	BHA (Dun.)	BHA_1 (Dun.)	BHA_2 (Dun.)	*CHO
IIF4	3 400	2 108	2 312	1 496	205
BB8	2 946	3 991	1 119	3 730	596
CF2	1 606	3 062	**125**	2 510	75
EB12	2 361	3 715	**432**	2 880	201
FC12	1 386	2 980	**554**	2 310	346
FE1	530	2 917	**326**	2 040	142
CB8	2 650	4 610	1 016	3 630	363
EF1	662	1 060	770	1 040	391

*CHO — oligosaccharides obtained by pronase digestion of the HA_2 glycopolypeptide

Table 3. HI activity of MoAb to HA_1: standard conditions and potentiation with sheep anti-mouse Ig

MoAb to HA_1	Parental virus	
	standard conditions	sheep anti-mouse Ig added
Dunedin		
IB5	<10	<10
IIC5	<10	<10
IB8	80–160	2 560
IC8	80–160	1 280
IVA1	<10	<10
IVG6	<10	<10
IVC4	<10	<10
Bangkok		
IIB4	> 2 560	> 2 560
IIG3	80–160	2 560
IIE5	80–160	2 560
IIF5	80–160	2 560

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

HA_1-specific MoAb were produced by hybridomas obtained by fusion of myeloma cells with splenocytes from mice immunized with influenza viruses A/Dunedin/4/73 (H3N2) (i.e. IB5, IIC5, IB8, IC8, IVA1, and IVG6) or A/Bangkok/1/79 (H3N2) (i.e. IIB4, IIG3, IIE5, and IIF5).

The results of solid phase RIA concerning MoAb specific to the HA_2 glycopolypeptide are summarized in Table 2. These MoAb showed preferential binding to BHA_2. The HA_2-specificity was evident particularly for the four MoAb CF2, EB12, FC12 and FE1. Nevertheless, the less clear reactivity pattern for the remaining MoAb of this group is also in agreement with their HA_2-specificity. These results revealed no glycopolypeptide specificity for IIF4 MoAb. Because HA_2-specificity of this MoAb was unambigously proved by radioimmunoprecipitation and immunoblotting, we included the IIF4 MoAb into Table 2. Rather high binding of all anti HA_2 MoAb to HA indicates that either corresponding epitopes are accessible or they became accessible due to changes in BHA by adsorption to polystyrene microplate (see FE1, Table 2). It is noteworthy that for all MoAb listed in Table 2 their carbohydrate specificity was excluded not only by lack of reactivity with influenza virus B (results not shown) but also with glycopeptides obtained from purified BHA_2 by exhaustive pronase digestion. All anti-HA_2 specific MoAb, with the exception of the IIF4 MoAb, were produced by hybridomas obtained by fusion of myeloma cells with splenocytes from two mice immunized with purified BHA_2. MoAb IIF4 was produced by

Table 4. "Cross-reactivity" of MoAb to HA_1 in solid-phase RIA

MoAb to HA_1	HK 68	Dun. 73	Bang. 79	Belg. 81	Caen. 85	B/Hk
IB5	1 681	4 100	205	*N.D.	N.D.	287
IIC5	1 085	3 120	310	N.D.	N.D.	310
IB8	1 102	5 795	174	N.D.	N.D.	232
IC8	1 105	5 492	220	N.D.	N.D.	247
IVA1	**1 961**	**3 925**	**3 720**	**3 896**	**3 404**	182
IVG6	**2 503**	**5 045**	**4 396**	**4 442**	**4 085**	256
IVC4	N.D.	**5 322**	**3 402**	**3 762**	**2 946**	342
IIB4	2 166	2 778	4 613	4 704	1 778	174
IIG3	N.D.	236	4 648	4 189	3 517	258
IIE5	N.D.	168	6 213	5 633	4 919	218
IIF5	N.D.	384	6 551	5 948	5 627	294

*N.D. — not done
The figures represent the radioactivity (cpm) bound in solid-phase RIA.
None of the MoAb given in this table reacted with the following influenza virus strains and recombinants: Eng. (H1), PR8 (H1), Sing. (H2), X-42, X-38, X-53, Ont.

a hybridoma obtained after fusion of myeloma cells with splenocytes from mice immunized with influenza virus A/Dunedin/4/73 (H3N2).

The specificity of the MoAb was further characterized in a radioimmunoprecipitation test employing ^{125}I-labelled BHA or virus. The precipitation was performed after incubation of BHA with MoAb under reducing conditions, which allowed dissociation of HA_1 and HA_2, either directly with *S. aureus* or after loading *S. aureus* with swine anti-mouse Ig. As expected, anti-HA_2 MoAb, with the exception of EF1 MoAb, precipitated preferentially the HA_2 chain (Figs. 1 and 2). But some anti-HA_1 MoAb (IB5, IIC5, IVA1, and IVG6) precipitated equally both chains — HA_1 and HA_2 (Fig. 1). The remaining anti-HA_1 MoAb IIB4, IB8, and IC8 and anti-HA_2 MoAb EF1 did not precipitate any labelled antigen under the experimental conditions employed.

Table 5. "Cross-reactivity" of MoAb to HA_2 in solid-phase RIA

MoAb to HA_2	HK	Dun.	Bang.	Belg.	X-42	Caen	B/HK
IIF4	3 100	4 579	3 682	3 376	204	2 942	488
BB8	4 902	5 029	4 402	4 466	**2 761**	4 687	969
CF2	2 019	2 584	2 215	2 345	**1 430**	2 223	112
EB12	3 899	4 441	4 309	4 108	**2 131**	4 019	714
FC12	2 916	3 431	3 318	3 199	415	2 154	540
FE1	1 574	2 059	1 329	1 257	286	783	342
CB8	3 326	3 794	3 295	3 252	**1 960**	3 274	608
EF1	1 066	1 184	1 146	1 096	610	1 101	628

The figures represent the radioactivity (cpm) bound in solid-phase RIA.

Table 6. Data summarizing the fine specificity of MoAb to HA

MoAb	SP-RIA	RIP	Blot	HI-VN	Isotype
IB5	HA_1 (?)	$HA_1 + HA_2$	HA_1	—	IgG2a
IIC5	HA1 (?)	$HA_1 + HA_2$	HA_1	—	IgG3
IB8	HA_1	neg.	neg.	+	IgG2a
IC8	HA_1	neg.	neg.	+	IgG2a
IVA1	HA_1	$HA_1 + HA_2$	HA_1	—	IgG2a
IVG6	HA_1	$HA_1 + HA_2$	HA_1	—	IgG1
IVC4	N.D.	N.D.	HA_1	—	
IIB4	N.D.	neg.	neg.	+	IgG2a
IIG3	N.D.	N.D.	neg.	+	IgG1
IIE5	N.D.	N.D.	neg.	+	IgG2a
IIF5	N.D.	N.D.	neg.	+	IgG2a
IIF4	HA_2 (?)	HA_2	HA_2	—	IgG1
BB8	HA_2 (?)	HA_2	HA_2	—	IgG1
CF2	HA_2	HA_2	neg.	—	IgG1
EB12	HA_2	HA_2	HA_2	—	IgG1
FC12	HA_2	HA_2	HA_2	—	IgG1
FE1	HA_2	HA_2	HA_2	—	IgG2a
CB8	HA_2 (?)	HA_2	HA_2	—	IgG1
EF1	HA_2 (?)	neg.	(HA_2)	—	IgM

N.D. — not done
parentheses indicate low reactivity
+ and — means positive or negative in HI and VN tests respectively
? means that the results of SP RIA were ambiguous

Finally, the specificity of the MoAb was analysed by immunoblotting. Figs. 3 and 4 shows the results obtained. Immunoblotting thus confirmed the HA_2-specificity of the IIF4, BB8, EB12, FC12, FE1, CB8, and EF1 MoAb. The remaining six anti-HA_1 MoAb (IB8, IC8, IIB4, IIG3, IIE5, IIF5) and one anti-HA_2 MoAb (CF2) did not react with immobilized proteins. The same MoAb, with the exception of CF2 MoAb, were negative in both radioimmunoprecipitation and immunoblotting.

The capacity of anti-HA_1 and anti-HA_2 monoclonal antibodies to inhibit haemagglutination and to neutralize virus

In HI tests, anti-HA_1 MoAb IB8, IC8, IIB4, IIG3, IIE5, and IIF5 showed anti-haemagglutination activity, but high anti-HA titres were detected only with IIB4 MoAb (Table 3). We found that low anti-HA titres increased either by adding sheep anti-mouse Ig into the HI test system (Table 3) or by using this test with HA subunits instead of intact virus (results not shown). These modifications, however, failed to potentiate the eventual low HI activity of those MoAb, which were negative under standard conditions.

All anti-HA_2 MoAb were negative in the HI test under standard conditions and with sheep anti-mouse Ig. Moreover, they were all negative also in the

HI test with HA subunits solubilized from virus with either Triton-X-100 or deoxycholate, sarcosyl, or octylglucoside.

The VN capacity of anti-HA_1 MoAb correlated exactly with the results of the HI test, i.e. only MoAb positive in the HI test had a virus neutralizing capacity. Anti-HA_2 MoAb, either alone or in complete mixture, did not neutralize the virus infectivity.

Cross-reactivity of anti-HA_1 and anti-HA_2 monoclonal antibodies

Variation within the HA_1 subunit is higher than in the HA_2 subunit. Accordingly, we found that anti-HA_2 MoAb were more cross-reactive than anti-HA_1-specific antibodies. All anti-HA_2 antibodies were fully cross-reactive within the H3 subtype; moreover, a half of them cross-reacted with the H7 subtype (Table 5). No cross-reactivity was detected with HA of H1, H2, and H8 subtypes (results not shown); further HA subtypes were not tested.

By contrast, all anti-HA_1 antibodies were H3 subtype-specific and they usually showed a good reactivity only with strains close to the parental virus (i.e. virus used for immunization and selection of hybridomas). Nevertheless, MoAb IVA1 and IVG6, specific to HA_1, showed a remarkable cross-reactivity within the H3 subtype. These MoAb reacted with influenza viruses isolated in the period from 1968 to 1985 (Table 4).

Discussion

The purpose of our work was to prepare anti-haemagglutinin MoAb and to determine their specificity to HA glycopolypeptides HA_1 and HA_2. We have analysed this fine specificity of anti-HA MoAb by solid phase RIA (using purified HA and HA glycopolypeptides HA_1 and HA_2), radioimmunoprecipitation with SDS-PAGE and immunoblotting. Furthermore we considered the ability of MoAb to inhibit virus induced haemagglutination as a good evidence for its HA_1 specificity. This assumption inferred logically from the knowledge of the threedimensional structure of influenza virus HA and from the failure of all anti-HA_2 MoAb, either those presented by us or those described by Becht *et al.* (1984), to inhibit virus-mediated haemagglutination.

The data on fine specificity of anti-HA MoAb obtained by different analyses are compared in Table 6. Solid phase RIA used in the present report should have provided a rapid, sensitive technique for distinguishing between antibodies directed against the HA_1 or HA_2 subunit. The results obtained, however, often did not clearly reveal the fine specificity of anti-HA MoAb (e.g. IIF4, CB8). A disadvantage of this otherwise simple method is that highly purified HA and HA glycopolypeptides HA_1 and HA_2 are required. In spite of that solid phase RIA proved to be very useful, because, when the HA and HA glycopolypeptides HA_1 and HA_2 were available, it revealed the HA_1 specificity of MoAb capable to inhibit virus-induced haemagglutination (i.e. IB8, IC8).

All anti-HA MoAb negative in the HI test gave immunoprecipitation and

yielded two different radioimmunoprecipitation patterns: (1) equal precipitation of both HA subunits, HA_1 and HA_2; and (2) preferential precipitation of the HA_2 chain. Coprecipitation of HA_1 and HA_2 due to the disulphide bond (joining HA_1 and HA_2 in the HA) could be excluded, because radioimmunoprecipitation was carried out under reducing conditions which ensured dissociation of HA_1 and HA_2 glycopolypeptides. We, therefore, explain these results by high hydrophobicity of the glycopeptide HA_2 (Poláková *et al.*, 1978). Most of the HA_2 molecules are present in the immunoprecipitation mixture as large aggregates containing few entrapped HA_1 molecules. Monoclonal antibody directed against an epitope on HA_1 almost completely precipitates also HA_2 molecules (precipitation pattern 1). In contrast, anti HA_2 MoAb precipitated preferentially the HA_2 subunit, because most of the HA_1 molecules exist in the reaction mixture in a monomeric form and thus cannot contaminate the HA_2 precipitate. This explanation was verified by immunoblotting, i.e. under conditions, under which such aggregates cannot be formed. With the exception of MoAb CF2, monoclonal antibodies negative in the HI test showed good reactivity also in immunoblotting. Anti-HA MoAb bound either to immobilized HA_1 (anti-HA_1) or HA_2 (anti-HA_2). No anti-HA MoAb was found which would bind to both immobilized HA glycopolypeptides, HA_1 and HA_2.

All examined anti-HA MoAb with the capacity to inhibit virus-induced haemagglutination were negative in both radioimmunoprecipitation and immunoblotting. These MoAb are aparently directed against discontinuous (conformational) epitopes, which were destroyed during radioimmunoprecipitation and immunoblotting under the experimental conditions used.

HA_1 specificity was demonstrated for all hybridomas originating from lymphocytes of mice immunized with complete virus, except IIF4 hybridoma, which was proved to be HA_2-specific. There is no problem to obtain anti-HA_2 polyclonal antibodies after immunization with complete virus or purified undissociated HA (Russ *et al.*, 1978). Such antibodies are present also in human and avian convalescent sera (Styk *et al.*, 1979; Becht *et al.*, 1984). Nevertheless, we confirmed here previous experience (Russ and Gerhard, unpublished results) that hybridomas with HA_2 specificity are rare or absent at all after using splenocytes from mouse immunized with complete virus for construction of hybridomas. The appearance of the IIF4 hybridoma might be accidental and not related to the immunization procedure at all. At present, we are unable to explain the phenomenon described above. Further anti-HA hybridomas were prepared by fusion of mouse myeloma cells with lymphocytes from mice immunized with purified HA glycopolypeptide HA_2. As expected, HA_2 specificity was demonstrated for all these hybridomas.

Anti-HA_1 monoclonal antibodies revealed high, low or no capacity to inhibit haemagglutination and to neutralize virus. We were able to increase low antihaemagglutinin titres either by adding sheep anti-mouse Ig into the HI test or by using HA subunits instead of intact virus in this test. A similar approach for increasing the reactivity of antibodies in the HI test has been described for many influenza viruses (Lu *et al.*, 1982; Kendal and Cate, 1983). But none of the modifications tested did potentiate an eventual low HI

activity of those MoAb which were negative under standard conditions. All anti-HA_2 MoAb were negative in HI tests under standard conditions, with sheep anti-mouse Ig and with HA subunits. We cannot explain why anti-HA_2 MoAb prepared by Becht *et al.* (1984) did inhibit haemagglutination of isolated HA and our MoAb did not. Anti-HA_2 MoAb, either each alone or in a mixture, did not neutralize virus infectivity.

When HA sequences for different HA subtypes were compared, little sequence homology was found within the HA_1 portion of the genes, while there was a significant homology between the HA_2 subunits (Krystal *et al.*, 1982). Since the region is highly conserved among all influenza A and B virus strains, it could provide a basis for eliciting inter-subtype cross-reactive antibodies. We described that rabbit antisera produced against intact virus of purified undissociated HA contained antibodies reactive with both HA glycopolypeptides HA_1 and HA_2, and that antibodies to the later showed inter-subtype cross-reactivity (Russ *et al.*, 1978). Graves *et al.* (1983) confirmed that anti-HA_2 specific antisera were cross-reactive with the HA_2 of an influenza A virus of a different subtype. Others found only subtype-specific anti-HA_2 antibodies (Brown *et al.*, 1980; Becht *et al.*, 1984). We found that HA_2 MoAb were more cross-reactive than HA_1-specific MoAb. All anti-HA_2 MoAb showed good reactivity with all examined strains of the H3 subtype, isolated from 1968 to 1985. Moreover, a half of them showed also inter-subtype cross-reactivity with the H7 subtype. This is in good agreement with the fact that H3 is even more related to the avian H7 than to other human HA subtypes (Air *et al.*, 1981; Saitov and Nei, 1986). Three out of four MoAb that cross-reacted with H7 subtype are directed against a single antigenic site (Russ *et al.*, 1987). It will be important to determine whether these MoAb are cross-reactive with other related subtypes, as H4 and H10. (Anti-HA_2 MoAb described by Becht *et al.* (1984) were subtype-specific.)

All anti-HA_1 MoAb were subtype-specific and usually showed good reactivity only with viruses close to the virus used for immunization and selection of hybridomas. MoAb IVA1 and IVG6 are in this respect of particular interest. As anti-HA_1-specific antibodies they showed unusual cross-reactivity within the H3 subtype (see Both *et al.*, 1983).

The present, anti-HA_2 MoAb are of particular interest. Their availability made it possible to determine the four independent antibody binding sites on the HA_2 glycopolypeptide (Russ *et al.*, 1988), and to show that changes in the antigenicity of the HA molecule at acidic pH involve both HA glycopolypeptides, HA_1 and HA_2 (Kostolanský *et al.*, 1988). Experiments are in progress to show whether epitopes corresponding to these MoAb are present on the surface of infected cells and to find out whether these MoAb recognize amantadine resistant mutants (Daniels *et al.*, 1985).

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Explanation of Figures (Plates LV–LVIII)

Fig. 1. SDS-PAGE of immunoprecipitates of ^{125}I-BHA and anti-HA MoAb
Approx. 2×10^5 cpm of ^{125}I-labelled BHA from A/Dunedin/4/73 (H3N2) virus (corresponding to about 20 ng of protein) was incubated with MoAb separately. Immunoprecipitates were analysed in SDS-PAGE (10 %) under reducing conditions with subsequent autoradiography. Lanes: 1. ^{125}I-BHA (total); 2. IVG6; 3. IIF4; 4. IB8; 5. IC8; 6. IB5; 7. IIC5; 8. IVA1; 9. IIB4; 10. IVE8 (anti-NP); 11. mouse anti-A/Dun. serum; 12. mouse preimmune serum. (Lanes 2.–10.: MoAb.)

Fig. 2. SDS-PAGE of immunoprecipitates of ^{125}I-BHA and anti-HA MoAb
Experimental conditions were the same as described in Fig. 1.

Lanes: 1. ^{125}I-BHA (total); 2. IIIE11 (anti-NA); 3. FC12; 4. BB8; 5. EB12; 6. IIB4; 7. FE1; 8. IB8; 9. IIF4; 10. IVG6; 11. mouse anti-A/Dun. serum; 12. mouse preimmune serum. (Lanes 2.—10.: MoAb.)

Fig. 3. Immunoblotting analysis of fine specificity of anti-HA MoAb.
Purified BHA originating from A/Dun. virus was separated to its HA_1 and HA_2 glycopolypeptide in SDS-PAGE under reducing conditions and glycopolypeptides were blotted electrophoretically onto nitrocellulose sheet. After incubation with MoAb separately, immunocomplexes were detected using anti-mouse Ig, conjugated to horseradish peroxidase.
Lanes: 1. mouse anti-A/Dun. serum; 2. Sp2/0 cells supernatant; 3. IB5; 4. IIC5; 5. IB8; 6. IC8; 7. IVA1; 8. IVG6; 9. IIB4; 10. IIF4; 11. BB8; 12. CB8; 13. CF2; 14. EF1; 15. EB12; 16. FC12; 17. FE1. (Lanes 3.—17.: MoAb.)

Fig. 4. Immunoblotting analysis of fine specificity of anti-HA MoAb.
Purified virus A/Bangkok/1/79 (H3N2) was separated to structural subunits in SDS-PAGE under reducing conditions and proteins were blotted electrophoretically onto nitrocellulose sheet. After incubation with monoclonal antibodies separately, immunocomplexes were detected using anti-mouse Ig conjugated to horseradish peroxidase.
Lanes: 1. mouse anti-MRC 11 serum; 2. mouse preimmune serum; 3. IVA1; 4. IVG6; 5. IVC4; 6. IIB4; 7. IIG3; 8. IIE5; 9. IIF5; 10. IIF4; 11. BB8; 12. CF2; 13. EB12; 14. FC12; 15. FE1; 16. CB8; 17. EF1. (Lanes 3.—17.: MoAb.)