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Monoclonal antibodies to the hemagglutinin Sa antigenic site of A/PR/8/34 influenza virus distinguish biologic mutants of swine influenza virus

(antigenic variation/point mutation/viral genetics/virulence/pleiotropism)

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ABSTRACT **The dimorphic L and H hemagglutinin mutants of A/NJ/11/76(H1N1) (swine) influenza virus differ pleiotropically in their replication and virulence characteristics and in their antigenicity. L mutants replicate less well in chicken embryos and Madin–Darby canine kidney cells and are more infective for swine than are H mutants. L and H mutants are not antigenically distinguishable in cross-neutralization tests with homotypic antisera, but they can be identified with certain heterotypic heterogeneous antisera. The present studies demonstrate that two monoclonal antibodies (Sa-5 and Sa-13) to the Sa antigenic site of the hemagglutinin of A/PR/8/34H1N1 influenza virus react with mutants and viral reassortants containing the H hemagglutinin in radioimmunoassay, neutralization, and hemagglutination-inhibition tests but to a lesser degree or not at all with L mutants and reassortants. Conversely, monoclonal antibody (9C8) to the L mutant does not react with H mutants. L to H and H to L revertants, whether or not selected with monoclonal antibody, demonstrate concomitant change in biological and antigenic phenotype. Reactivity of H mutants with Sa monoclonal antibodies localizes the mutational site to a position on the hemagglutinin near the receptor binding site—a position in which single amino acid changes could readily influence both antigenic and biologic activity.**

Naturally occurring and laboratory-selected hemagglutinin (HA) gene L and H mutants of swine influenza virus (1–3) differ pleiotropically in their capacity to replicate in chicken embryos, Madin–Darby canine kidney (MDCK) cells, and intact swine. These mutants are not distinguishable in reciprocal hemagglutination-inhibition (HI) tests (1). However, L mutants and reassortant viruses that incorporate their HAs are inhibited in HI and neutralization reactions with H mutant-absorbed heterotypic rabbit antiserum to A/SW/Cam/39 (SW/CAM) influenza virus, while H mutants and reassortants are not (Table 1). Certain heterogeneous antisera to A/PR/8/34 influenza virus are reactive with most H mutants or reassortants but not with L mutants (Table 1). Reversion of L to H virus is commonly observed in culture systems that favor the replication of H mutants or when L virus is suppressed with A/SW/Cambridge/39(H1N1) (SW/CAM) antibody. This and other evidence (1, 4) suggests that the complex phenotypes of L and H reflect point mutations in the HA gene. In an exploration for more specific and reactive antibody for characterization of H mutants, it was found that monoclonal antibody to the Sa HA antigenic site of A/PR/8/34(H1N1) (PR8) virus distinguished L and H mutants in radioimmunoassay (RIA) and in biological assay systems (Tables 2 and 3). These observations and others described below were important not only in providing more specific reagents for serologic characterization of the mutants but fortuitously as an indication of the probable mutational site in view of recent correlative studies of HA structure and antigenic sites (5).

MATERIALS AND METHODS

Viruses. Field isolates A/NJ/10/75(H1N1) and A/NJ/11/76(H1N1) (swine influenza viruses) and the reassortant viruses X-53, X-53a, X-53-PR8, and X-53a-PR8 have been described (1). The PR8 strain employed in the production of reassortant viruses and used in present tests is the so-called Mount Sinai strain. Viruses identified as 1–6 in Tables 4 and 5 represent cloned or passage variants of the above reassortants. Their detailed passage history is described elsewhere (6). All virus seeds are allantoic fluids from 12- to 13-day-old White Leghorn chicken embryos.

Antibody Preparations. SW/CAM and PR8/HK antisera were prepared by injection of rabbits at 0 and 40 days with approximately 3,000 hemagglutinating units of A/SW/CAM/39 (1) and PR8/HK reassortant virus (7), with bleedings at 47 days. One-to-ten dilutions of these antisera were absorbed, respectively, with concentrated X-53a-PR8 and X-53-PR8 viruses in final concentrations of 70,000 hemagglutinating units/ml to produce antisera specifically reactive in HI tests with L or H serotype viruses. The monoclonal antibodies to A/PR/8/34 virus have been described (8).

Production of Monoclonal Antibody Against Swine Influenza Virus L HA. Female BALB/c mice were inoculated intraperitoneally with 10 μg of viral protein of swine influenza virus reassortant X-53(CL)-PR8(2)P4T (recloned). A boosting dose of 10 μg was given intravenously 10 weeks after the initial injection. Three days later splenocytes from a boosted animal were fused with SP2/0 mouse myeloma cells by adapting the methods of Köhler and Milstein (9) with modifications by Koprowski *et al.* (10). Fused cells were initially seeded in 96-well Linbro plates and subsequently transferred to 24-well Corning plates. The hybridoma culture fluids were tested for anti-influenza activity by RIA and enzyme-linked immunosorbent assay (ELISA). Hybridoma 9C8 was found to produce antibody specific for the L hemagglutinin variant in RIA, ELISA, and HI tests. This hybridoma was further cloned by limit dilution. Ascitic fluids containing the monoclonal antibody were produced by injection of 9C8 hyridoma cells into the peritoneal cavity of pristane-primed BALB/c mice (10, 11).

Assays. Viral HA and HI titrations were carried out in microtiter plates (12). In HI tests 16–32 HA units were employed

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Abbreviations: HA, hemagglutinin; HI, hemagglutination-inhibition; SW/CAM, A/SW/Cambridge/39(H1N1); PR8, A/PR/8/34(H1N1); MDCK cells, Madin–Darby canine kidney cells; RIA, radioimmunoassay.

Table 1. Biologic and antigenic phenotype of swine influenza virus HA mutants in relation to genotype defined by RNA gel migration

Virus	RNA gel genotype*								HI†		Chicken embryo viral yield‡	Plaque size in MDCK cells, mm	Swine ID_{50}§
	1	2	3	4	5	6	7	8	SW/CAM antiserum	PR8/HK antiserum			
A/NJ/10/76(L)	S	S	S	S	S	S	S	S	320	<10	16	1–2.5	
A/NJ/11/76(L)	S	S	S	S	S	S	S	S	320	<10	16	1–2.5	10^2
X-53	—	—	—	S	—	S	—	—	320	<10	512	1–3.0	
X-53-PR8	—	—	—	S	—	—	—	—	80	20	1,024	1–3.0	$10^{4.4}$
A/NJ/10/76(H)	S	S	S	S	S	S	S	S	<10	20	128	2–4	
A/NJ/11/76(H)	S	S	S	S	S	S	S	S	20	<10	128	2–4	10^4
X-53a	—	—	—	S	—	S	—	—	<10	40	4,096	2–5	
X-53a-PR8	—	—	—	S	—	—	—	—	<10	40	8,192	2–5	$>10^{5.6}$

*S, migration of RNA on polyacrylamide gel characteristic of A/NJ/11/76 (prototype) virus (1, 6); —, migration of RNA on polyacrylamide gel characteristic of A/PR/8/34 virus (1, 6).
†HI titer, expressed as reciprocal of serum dilution at endpoint.
‡Hemagglutination titer in allantoic fluid, expressed as reciprocal of virus dilution at endpoint.
§Minimal egg infectious dose required to infect 50% of inoculated swine (2).

and 0.5% human type O erythrocytes were added after incubation of virus–antibody mixtures at room temperature for 30 min. Tests were read after further incubation for 75 min. Sera or murine ascites fluids were treated with receptor-destroying enzyme prior to use.

RIA. The RIA was performed as described (13), using 20 HA units of partially purified virus as solid-phase immunoadsorbents and iodinated $F(ab')_2$ fragments of affinity-purified rabbit antibodies to mouse-immunoglobulin to quantitate bound anti-viral hybridoma antibodies.

Virus Plaquing. Plaquing and plaque neutralization of viruses were carried out in MDCK cells with trypsin-containing media (14) by methods described previously (15).

RESULTS

The PR8 monoclonal antibodies Sa-5, Sa-10, and Sa-13 reacted significantly in RIA with the high-yielding field strain A/NJ/10/76(H) and the vaccine reassortant virus X-53a of similar biologic and serologic phenotype (Tables 1 and 2). However, these antibodies did not react to a significant degree with the L mutant or reassortant. PR8 monoclonal antibodies defining other antigenic sites either did not react with or did not distinguish the mutants (Table 2).

Reactivity of Sa-5 and Sa-13 antibody with L and H mutants in plaque neutralization and HI tests was concordant with RIA (Table 3). A mutant previously identified as non-L and non-H in serologic phenotype did not react to a significant degree with either Sa site antibody.

In parallel studies, nucleotide sequence analysis of the HA gene of several L and H reassortants has been undertaken to identify the mutated site responsible for the pleiotropic differences between the mutants. These viruses are listed in Table 4. It will be seen that reassortants of L phenotype are not inhibited or are inhibited only by low dilutions of Sa-13 antibody, whereas H phenotype viruses 4 and 5 are inhibited by high antibody dilutions. X-53-PR8 (virus 2), the only L phenotype virus significantly inhibited by Sa-13 antibody in HI, is clearly distinguishable from H phenotype viruses in plaque neutralization tests. Of special significance are viruses 5 and 6, which are L to H revertants of quadruply plaque-cloned viruses, which emerged as large-plaque high-yielding variants without selective pressure of anti-L (SW/CAM) antibody. Coincident with change in biologic phenotype, they have become highly reac-

Table 2. Crossreaction in RIA of monoclonal anti-PR8 antibodies with swine influenza virus HA mutants

Antibody	Antigenic site	cpm in RIA						
		A/PR/8/34	A/SW/31	A/NJ/10/76 (L)	A/NJ/10/76 (H)	A/NJ/10/76 (non-L, non-H)	X-53	X-53a
Y8-2C6(Sa-5)	Sa	3,101	2,260	177	1,899	119	139	1,728
H28-C1		7,086	4,458	84	1,140	84	67	1,256
Y8-3B3(Sa-13)		2,985	2,130	158	1,372	106	77	1,440
H2-5B6	Ca	2,034	785	67	9	18	46	14
Y8-2D1		1,747	1,951	97	66	14	60	7
H18-S210		6,021	2,387	144	55	83	104	82
H33-23		6,143	250	762	968	885	945	693
H17-L10		5,864	5,260	4,274	5,120	4,382	3,873	4,432
H17-L2		4,022	4,085	3,143	3,673	3,548	3,405	3,370
H18-S413		6,543	3,913	3,235	3,988	3,795	3,858	3,569
H18-S28		4,825	2,764	2,260	2,483	2,624	2,676	2,362
H33-48		4,714	4,544	2,009	2,675	2,466	2,256	2,004
H18-S13	Cb	3,004	1,233	892	1,325	1,380	1,042	1,249
H18-S121		6,286	2,927	1,874	2,921	2,878	2,341	2,734

Hybridoma culture fluids were used in RIA at 1:20 dilution. Results are given as mean cpm, of duplicate samples, above assay background (= cpm against influenza virus B/Lee/40).

Table 3. Inhibition by PR8 monoclonal antibodies* of swine influenza virus L and H variants

Virus†	Titer‡			
	Sa-5 antibody		Sa-13 antibody	
	PI	HI	PI	HI
1. A/NJ/10/76(L)	<20 (800)	<10	40 (1,600)	<10
2. X-53	<20	<10	<40	<10
3. A/NJ/10/76(H)	>1,280 (6,400)	320	1,280 (12,800)	640
4. X-53a	1,280	80	1,280	320
5. A/NJ/10/76 (non-L, non-H)	<20	<10	20	20
6. A/PR/8/34	1,280 (>6,400)	640	>1,280 (>6,400)	1,280

* Ascites fluid. All antibody preparations were treated with receptor-destroying enzyme.
† Viruses 1 and 2 are L phenotype—i.e., reactive with heterogeneous antiserum to A/SW/CAM/39. Viruses 3 and 4 are H phenotype—i.e., reactive with heterogeneous antiserum to A/PR/8/34 virus.
‡ PI, plaque inhibition in MDCK cells (preinoculation neutralization). Parenthetical titers are postinoculation (antibody in agar) neutralization. Titers are reciprocals of arithmetic dilution at the end point.

Table 4. Correlation of phenotype of various swine influenza virus mutants and reassortants with inhibition by monoclonal antibodies

Virus	HA/NA serotype	Phenotype		HI titer*	
		Biologic†	Serologic‡	Sa-13 antibody	9C8 antibody
1. X-53-LP	SW/SW	L§	L	10	160
2. X-53-PR8	SW/PR8	L	L	80	320
3. X-53-PR8 p4t	SW/PR8	L	L	<10	640
4. X-53a	SW/SW	H	H	640	10
5. A/NJ/11/76 L-P(MH)	SW/SW	H	H	160	<10
6. X-53-PR8 p4t H^2 (L to H revertant)	SW/PR8	H	H	640	10
7. X-53a-PR8(H) (non-L, non-H mutant)	SW/PR8	H	Non-L, non-H	<10	10

* Titers are reciprocals of arithmetic dilutions at the endpoint.
† L indicates low yield in chicken embryos, ≤ 1:2,048 HA titer, and small plaques (1–3 mm) in MDCK cells. H phenotype is reverse.
‡ Inhibited (L) or not inhibited (H) by SW/CAM heterogeneous antibody (1:40 dilution) in HI.
§ Large plaque L variant but low yield in eggs.

tive with Sa-13 antibody and have lost reactivity with swine influenza virus monoclonal antibody 9C8, which is maximally reactive with L mutants.

Passage of L mutant viruses (Table 5, viruses 8 and 9) in chicken embryos with monoclonal antibody 9C8 led to the isolation of revertants of H biologic and antigenic phenotype. An L phenotype revertant of virus 10 [X-53-(CL)-PR8(2) p4t H^2 p^4], itself an L to H revertant, emerged at neutralization endpoint as a "breakthrough" plaque after inoculation of MDCK cell plates with $10^{6.5}$ plaque-forming units of virus 10 and a 1:40 dilution of Sa-5 antibody.

DISCUSSION

Swine and PR8 viruses are both members of the influenza A H1N1 subtype, so the extensive crossreactions of PR8 monoclonal antibodies with the swine influenza virus mutants were not unexpected. The differential reaction of antibodies to the region defined by Gerhard *et al.* (8) as the Sa region of PR8 with the swine influenza virus mutants points to the nucleotides encoding that region as the probable site of nucleotide changes responsible for the differences in the complex phenotypes of L and H viruses. Indeed, parallel studies of nucleotide sequence of the HA1 portion of the L and H HAs have defined differences (glycine to glutamic acid) at residue 155 (4). The structurally equivalent residue of the PR8 HA is residue 154, which lies within the Sa antigenic site (5). Although several previous studies have correlated structure and antigenic sites on the HA molecule (5, 16–19), none has correlated these sites with identifiable differences in viral biologic activity, as is the case in the present studies. The Sa site, which occupies a position relatively near the receptor binding site of the adjacent HA monomer when the trimeric protein is assembled, is at a location in which single amino acid changes could readily influence both antigenic and biological activity. Intensive preliminary studies of comparative receptor binding and absorption kinetics of L and H mutants have not yet demonstrated significant differences between them that could explain HA-determined replication differences in these viruses.

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Table 5. Covariation of antigenic and biologic phenotype of revertant viruses selected with L- and H-specific monoclonal antibodies

Virus	HI titer*		Viral yield*	Mean plaque size, mm	Phenotype†	Selection	Host system‡
	Sa-5 antibody	9C8 antibody					
8. A/NJ/11/76(L)	<10	160	16	2.5	L	9C8 antibody	Chicken embryo
Revertant	2,560	<10	128	4.0	H		
9. X-53(CL)	20	320	256	4.0	L	9C8 antibody	Chicken embryo
Revertant	160	<10	4,096	5.0	H		
10. X-53(CL)-PR8(2) p4t H^2 p4	80	<10	8,192	5.0	H	Sa-5 antibody	MDCK cells
Revertant	10	640	2,048	4.0	L		

* See footnotes to Table 1.
† See text.
‡ Chicken embryo refers to the allantoic sac.

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