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Relationship between Mitogenic Activity of Influenza Viruses and the Receptor-Binding Specificity of Their Hemagglutinin Molecules

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The relationship between the mitogenic activity of influenza type A viruses for murine B lymphocytes and the receptor-binding specificity of their hemagglutinin was examined. Receptor-binding specificity was determined by the ability of the virus to agglutinate erythrocytes that had been sialidase treated and then enzymatically resialylated to contain sialyloligosaccharides with defined sequences. Distinct differences in receptor-binding specificity were observed between strongly and weakly mitogenic viruses of the H3 subtype, with strong mitogenic activity correlating with the ability of the virus to recognize the sequence *N*-glycolylneuraminic acid α2,6 galactose (NeuGcα2,6Gal). Viruses isolated early in the evolution of the H3 subtype (from 1968 to 1971) are relatively weak mitogens and recognize the sequence *N*-acetylneuraminic acid α2,6 galactose (NeuAcα2,6Gal) but not NeuGcα2,6Gal. H3 viruses isolated since 1972 are strongly mitogenic, and these viruses recognize both NeuGcα2,6Gal and NeuAcα2,6Gal. The amino acid substitution of Tyr for Thr at residue 155 of HA_1 may be critical to this change in receptor-binding specificity and mitogenic activity of the later H3 viruses. Horse serum-resistant variants of H3 viruses, which bind preferentially to the sequence NeuAcα2,3Gal, are poorly mitogenic. Differences were also observed between the receptor-binding specificity of the strongly mitogenic H3 viruses and viruses of the H2 and H6 subtypes, the mitogenic activity of which is limited to strains of mice that express the class II major histocompatibility complex glycoprotein I-E. The results indicate that the receptor-binding specificity of the hemagglutinin plays a critical role in determining the mitogenic activity of influenza viruses.

Many strains of influenza A virus behave as T-cell-independent B-cell mitogens for murine lymphocytes in vitro (2). Two distinct mechanisms of mitogenesis appear to be involved. Viruses of the H2 and H6 subtypes are strong mitogens, but only for strains of mice that express the cell-surface class II major histocompatibility complex (MHC) glycoprotein I-E (16). Evidence from competition studies with monoclonal antibody (MAb) against I-E suggests that these viruses bind directly to I-E molecules on the B-cell surface (17). Viruses of the H3 subtype are also mitogenic, but mitogenesis is independent of I-E antigen expression and is presumed to involve binding of these viruses to a different receptor on the B cell. Within the H3 subtype, however, certain strains of virus are much more mitogenic than others.

We have recently shown (P. Poumbourios, E. M. Anders, A. A. Scalzo, D. O. White, A. W. Hampson, and D. C. Jackson, manuscript in preparation) that, like intact virus, purified hemagglutinin (HA) prepared from detergent-disrupted virus is a B-cell mitogen and that the response to HA of the H2, but not H3, subtype is dependent on I-E antigen expression. It seemed likely, therefore, that distinctions in mitogenic specificity and activity between different influenza virus strains may reflect differences in the fine specificity of the receptor-binding site of the HA molecule. Such differences in receptor-binding specificity of HA are well established and include the ability to distinguish different naturally occurring sialic acids (SAs) as well as different anomeric linkages of the terminal SA to the proximal sugar in the oligosaccharide side chains (3, 8, 12, 14).

In this report we examine the role of receptor-binding specificity of the HA molecule in influencing the mitogenic activity of influenza viruses.

MATERIALS AND METHODS

Mice. Mouse strains BALB/c (*H-2*^{d}), C57BL/10 (referred to as B10) (*H-2*^{b}), and B10.D2 (*H-2*^{d}) were bred and maintained in the animal facility of the Department of Microbiology, University of Melbourne. All of the mice were females and were used at 6 to 10 weeks of age.

Viruses. Influenza A virus strains used in this study were Port Chalmers = A/Port Chalmers/1/73 (H3N2), Victoria = A/Victoria/3/75 (H3N2), Texas = A/Texas/1/77 (H3N2), Bangkok = A/Bangkok/1/79 (H3N2), Eng69 = A/England/878/69 (H3N2), and Eng72 = A/England/42/72 (H3N2) and the recombinant viruses Jap_H-Bel_N = A/Japan/305/57 × A/Bel/42 (H2N1), $Shear_H$-Bel_N = A/Shearwater/E. Aust/1/72 × A/Bel/42 (H6N1), $Mem71_H$-Bel_N = A/Memphis/1/71 × A/Bel/42(H3N1), and PNG_H-Bel_N = A/Papua New Guinea/75 × A/Bel/42 (H3N1). Horse serum-resistant (HS^r) variants $Mem71_H$-Bel_N/HS^r, Port Chalmers/HS^r, Victoria/HS^r, PNG_H-Bel_N/HS^r, and Texas/HS^r were selected by growing the parent virus in hens eggs in the presence of nonimmune horse serum (HS) and subsequently cloning by growth at limiting dilution in eggs. Other HS-sensitive (HS^s) and -resistant (HS^r) pairs of influenza A viruses used included the substrains $RI/5^+$ = A/$RI/5^+$/57 (H2N2) (HS^s) and $RI/5^-$ = A/$RI/5^-$/57 (H2N2) (HS^r) isolated by Choppin and Tamm (4); the cloned variants of Mem72 = A/Memphis/102/72 (H3N2), M1/5 (HS^s) and

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M1/HS8 (HS^r) (14); and the recombinant virus X-31 = A/Aichi/2/68 × A/PR/8/34 (H3N2) and its HS^r variant X-31/HS (13).

Jap_H-Bel_N, $Shear_H$-Bel_N, and PNG_H-Bel_N viruses, grown as described previously (1) and purified by rate-zonal centrifugation, were kindly provided by Alan Hampson, Commonwealth Serum Laboratories, Melbourne. All other viruses were used as unpurified allantoic fluid stocks or as semipurified concentrates, obtained from allantoic fluid preparations by adsorption to and elution from chicken erythrocytes. Purified viruses and virus concentrates were stored at 4°C in the presence of sodium azide (0.1% [wt/vol]). Before use in lymphocyte cultures, viruses were dialyzed to remove azide and then inactivated by exposure to UV irradiation (2 min, 14 cm from a 15-W germicidal lamp) which reduced their infectivity for eggs to undetectable levels (<10 50% egg infective doses per ml).

Preparation of derivatized erythrocytes. Methods for the enzymatic modification of human erythrocyte oligosaccharides have been described previously (12). CMP-SA donor substrates were prepared as described previously (7) with either *N*-acetylneuraminic acid (NeuAc) or *N*-glycolylneuraminic acid (NeuGc) as the SA. Sialidase-treated human erythrocytes were resialylated with either the galactose β1,3 *N*-acetylgalactosaminide α2,3-sialyltransferase (EC 2.4.99.4) or the galactose β1,4 *N*-acetylglucosamide α2, 6-sialyltransferase (E.C. 2.4.99.3) to form the sequences SAα2,3Galβ1,3GalNAc (SAα2,3Gal) and SAα2,6Galβ1, 4GlcNAc (SAα2,6Gal), respectively, on glycoprotein oligosaccharides (15, 19). The amount of NeuAc and NeuGc incorporated was 111 and 110 nmol/ml of packed erythrocytes, respectively, for preparations containing the SAα2,3Gal linkage and 40 and 35 nmol/ml of packed erythrocytes, respectively, for preparations containing the SAα2,6Gal linkage.

Hemagglutination and hemagglutination inhibition assays. Routine hemagglutination and hemagglutination inhibition (HI) titers were determined as described by Fazekas de St. Groth and Webster (6). Virus doses are expressed in hemagglutinating units (HAU). For determination of virus receptor-binding specificity, hemagglutination assays with 1.5% (vol/vol) suspensions of native, asialo, or resialylated human erythrocytes were performed in the presence of the neuraminidase inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DDN; 1 mM) as described previously (12).

Lipopolysaccharide. *Escherichia coli* (serotype O111:B4) lipopolysaccharide (LPS) W was purchased from Difco Laboratories (Detroit, Mich.).

MAb. Cells of the hybridoma cell line 14-4-4 (anti-I-$E^{k,d}$, specificity Ia.7) (11) were kindly provided by I. F. C. McKenzie, Department of Pathology, University of Melbourne. Purified immunoglobulin G (IgG) of MAb 14-4-4 was prepared from ascitic fluid by affinity chromatography on protein A-Sepharose (5).

Lymphocyte proliferation assay. The culture medium used was RPMI 1640 (Flow Laboratories, Victoria, Australia) supplemented with 5% heat-inactivated (56°C, 30 min) fetal calf serum (FCS; GIBCO Laboratories, Grand Island, N.Y.)–2 mM glutamine–2 mM sodium pyruvate–10^{-4} M 2-mercaptoethanol–100 IU of penicillin per ml–100 μg of streptomycin per ml. In some experiments, 2% goat serum or 2% sheep serum was used instead of FCS to obtain lower levels of background proliferation in unstimulated cultures (2). Spleen cell suspensions were prepared as described previously (1) and were cultured in 96-well, flat-bottomed microtiter trays (2×10^5 to 4×10^5 cells per well) in 0.25 ml of culture medium containing various doses of UV-inactivated influenza virus, 20 μg of LPS, or medium alone at 37°C in a humidified atmosphere of 5% CO_2 in air. In one experiment MAb 14-4-4 that had been dialyzed against culture medium was added to spleen cells 1 h before the addition of virus. Proliferative responses were determined by the incorporation of [^{3}H]thymidine (0.5 μCi per well; specific activity, 5 Ci/mmol; Amersham Corp., Sydney, Australia) for the period 24 to 42 h after initiation of culture, as described previously (2). Data are expressed as mean counts per minute incorporated, or as a stimulation index (*T/C*), where *T* is the mean counts per minute incorporated in triplicate cultures containing virus or LPS, and *C* is the mean counts per minute incorporated in sextuplicate cultures containing medium alone. The standard error of the mean of the counts per minute in replicate cultures was always less than 10%.

TABLE 1. Mitogenic activity of different influenza A virus strains of the H3 subtype for BALB/c spleen cells

Virus	Year of isolation	HAU[c]/culture	Stimulation index in medium containing[a]:	
			5% FCS	2% goat serum
X-31 (H3N2)	1968[b]	400	2.4	7.9
		100	2.2	6.2
		10	1.7	2.8
$Mem71_H$-Bel_N (H3N1)	1971[b]	400	2.2	4.6
		100	1.8	3.2
		10	1.5	2.7
Mem72 (M1/5) (H3N2)	1972	100	15.4	55.9
		10	2.6	5.8
Port Chalmers (H3N2)	1973	100	26.5	86.7
		10	26.2	96.2
Victoria (H3N2)	1975	100	14.7	35.6
		10	9.9	28.1
PNG_H-Bel_N (H3N1)	1975[b]	100	14.7	20.6
		10	8.1	17.8
Texas (H3N2)	1977	100	9.9	26.9
		10	14.5	57.5
Bangkok (H3N2)	1979	100	9.5	35.2
		10	10.3	25.0

[a] Background responses in the absence of virus were 3,315 cpm for medium containing 5% FCS and 261 cpm for medium containing 2% goat serum.

[b] In the case of strains derived in the laboratory by genetic reassortment, the year of isolation given is that of the naturally occurring strain donating the HA.

[c] Virus doses are expressed in HAU (6).

RESULTS

Mitogenic activity of different influenza viruses of the H3 subtype. We have previously shown (2, 16) that strains of influenza A virus of the H3 subtype are mitogenic for murine B lymphocytes and that mitogenic activity is not restricted to mice of a particular MHC haplotype. Study of a larger number of H3 virus strains has revealed that they fall into two groups according to the strength of the proliferative response that they induce (Table 1). X-31 (1968) and $Mem71_H$-Bel_N (1971) viruses are only weakly mitogenic, whereas viruses that have arisen subsequently, Mem72 (1972), Port Chalmers (1973), Victoria (1975), PNG_H-Bel_N (1975), Texas (1977), and Bangkok (1979), are strongly mitogenic.

Receptor-binding specificity differences between H3 virus strains. To examine whether the lower mitogenicity of X-31

TABLE 2. Agglutination of erythrocytes from different animal species by influenza A viruses of the H3 subtype

Virus	Hemagglutination titer[a]		
	Chicken	Bovine	Goat
X-31 (H3N2)	830	<4	<4
Mem71$_H$-Bel$_N$ (H3N1)	1,180	<4	<4
Port Chalmers (H3N2)	830	830	128
PNG$_H$-Bel$_N$ (H3N1)	1,024	724	170

[a] Hemagglutination titer with erythrocytes from the species of animal indicated; assays were performed as described by Fazekas de St. Groth and Webster (6).

and Mem71$_H$-Bel$_N$ viruses, compared with other H3 strains, reflects a difference in specificity of the receptor-binding site on the HA molecule, the viruses were tested for their ability to agglutinate erythrocytes from different animal species. Whereas all strains tested agglutinated chicken erythrocytes, a clear distinction was observed with bovine and goat erythrocytes, which were agglutinated by the strongly mitogenic viruses Port Chalmers (1973) and PNG$_H$-Bel$_N$ (1975) but not at all by X-31 (1968) or Mem71$_H$-Bel$_N$ (1971) viruses (Table 2).

The receptor-binding characteristics of the viruses were further compared by testing their ability to agglutinate human erythrocytes specifically derivatized to contain sialyloligosaccharides with defined sequences. After treatment with sialidase to destroy virus receptors, the erythrocytes were enzymatically resialylated to contain one of two naturally occurring SAs (NeuAc or NeuGc) in either an SAα2,6Gal or an SAα2,3Gal linkage. All of the H3 strains were found to give strong agglutination of erythrocytes bearing the NeuAcα2,6Gal sequence but agglutinated NeuAcα2,3Gal-derivatized erythrocytes only poorly, if at all (Table 3). In keeping with previous findings (14), this pattern of binding specificity correlated with sensitivity of the viruses to HI by normal (nonimmune) horse serum. The viruses differed, however, in their ability to recognize the NeuGcα2,6Gal sequence; the early strains X-31 and Mem71$_H$-Bel$_N$ failed to agglutinate NeuGcα2,6Gal-derivatized erythrocytes, whereas all of the later H3 viruses agglutinated these cells. A distinction has been noted previously (8) between the original 1968 H3 strains and strains isolated after 1972 in their ability to bind the NeuGcα2,6Gal sequence. The present findings show that the 1971 strain resembles 1968 viruses in its receptor-binding characteristics and demonstrate a clear correlation between the ability of a virus to recognize NeuGcα2,6Gal and strong mitogenic activity.

Mitogenic activity of HS^r variants of H3 viruses. Growth of influenza virus in the presence of HS selects against virus with specificity for SAα2,6Gal, and the virus that emerges displays specificity for the SAα2,3Gal sequence (14). This change in receptor-binding specificity has been shown to result from a single amino acid substitution at residue 226 of HA$_1$ (13) which lies within the receptor-binding pocket near the tip of the HA molecule (20). To determine whether mitogenic activity is affected by this change in receptor-binding specificity, HS^r variants of a number of H3 viruses were derived as described above. The binding characteristics of the cloned HS^r variants are shown in Table 3, and as anticipated (14), all exhibited a strong preference for the SAα2,3Gal linkage; the ability to bind NeuGc was also lost. Their mitogenic activity was then compared with that of their HS^s counterparts. This change in receptor-binding specificity was accompanied by an almost complete loss of mitogenic activity of the later H3 viruses (Fig. 1).

Amino acid changes in the H3 subtype HA molecule responsible for NeuGc binding and strong mitogenic activity. The published amino acid sequences of the HA of the viruses listed in Table 3 (10, 18) were compared. Of the amino acid substitutions that distinguish the early non-NeuGc-binding strains X-31 and Mem71$_H$-Bel$_N$ from the later NeuGc-binding strains, two, at residues 155 and 188, are located adjacent to the receptor-binding pocket of the HA molecule (20) and thus might be expected to affect receptor-binding specificity. To differentiate the contribution of these two residues to NeuGc binding and to mitogenic activitiy, two additional strains of the H3 subtype isolated during the period from 1969 to 1972 were examined. The two viruses, Eng69 and Eng72, differ at residue 155 but not at 188: Eng69 resembles X-31 and Mem71$_H$-Bel$_N$ in that it has a Thr at 155 and an Asn at 188; Eng72 has a Tyr at 155 but still retains an Asn at 188, unlike the later H3 viruses (for example Texas), all of which have a Tyr at 155 and an Asp at 188 (18). Table 4 shows the 1969 strain Eng69 to be like X-31 and Mem71$_H$-Bel$_N$ in that it is only weakly mitogenic, whereas the 1972 strain Eng72 is strongly mitogenic. Furthermore, although both viruses agglutinated erythrocytes derivatized to contain the NeuAcα2,6Gal sequence, only Eng72 recognized NeuGcα2,6Gal. These findings confirm the correlation between the ability of H3 viruses to bind theNeuGcα2,6Gal sequence and strong mitogenic activity for B cells. Moreover, they rule out involvement of the amino acid substitution of Asp for Asn at residue 188 in the acquisition of NeuGc binding and suggest that the amino acid change at

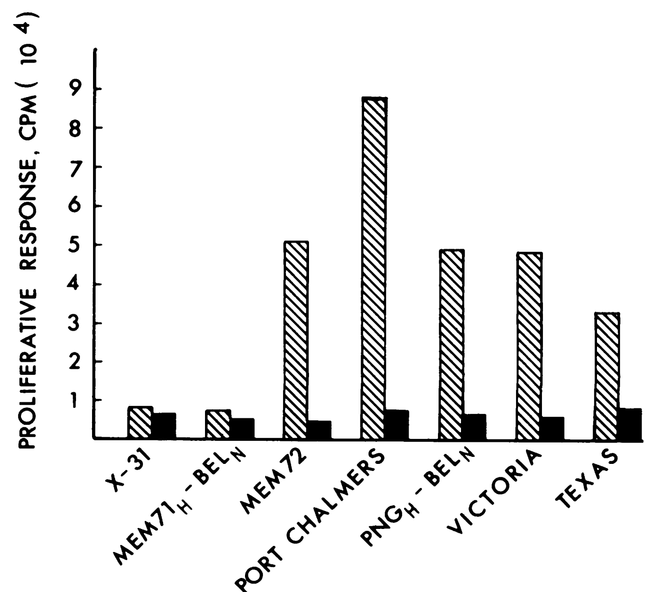

FIG. 1. Mitogenic activity of HS^s and HS^r influenza A viruses of the H3 subtype. Hatched bars, HS^s viruses; solid bars, HS^r viruses. BALB/c spleen cells (4×10^5) were cultured with 400 HAU of X-31, X-31/HS, Mem71$_H$-Bel$_N$, or Mem71$_H$-Bel$_N$/HS^r viruses or with 100 HAU of HS^s or HS^r stocks of the other virus strains shown. Culture medium contained 5% FCS; the background level of proliferation in the absence of virus was 3,315 cpm.

TABLE 3. Receptor-binding specificities of influenza A viruses of the H3 subtype determined by agglutination of derivatized erythrocytes[a]

Virus	Year of origin	Hemagglutination titer: Native	Asialo	SAα2,3Gal NeuAc	SAα2,3Gal NeuGc	SAα2,6Gal NeuAc	SAα2,6Gal NeuGc	HI titer with HS[b]
X-31	1968	512	0	0	0	512	0	111,400
Mem71$_H$-Bel$_N$	1971	512	0	0	0	512	0	90,500
Mem72 (M1/5)	1972	256	0	16	0	256	128	137,000
Port Chalmers	1973	512	0	32	0	256	1,024	90,500
Victoria	1975	512	0	16	0	512	1,024	119,400
PNG$_H$-Bel$_N$	1975	512	0	8	0	256	512	90,500
Texas	1977	512	0	8	0	256	512	128,000
Bangkok	1979	256	0	0	0	256	512	119,400
HS^r variants[c]								
X-31/HS		512	0	1,024	0	0	0	<10
Mem71$_H$-Bel$_N$/HS^r		256	0	1,024	0	2	0	<10
Mem72 (M1/HS8)		128	0	1,024	0	32	0	<10
Port Chalmers/HS^r		512	0	1,024	0	0	0	<10
Victoria/HS^r		256	0	1,024	0	2	0	<10
PNG$_H$-Bel$_N$/HS^r		256	0	1,024	0	0	0	<10
Texas/HS^r		256	0	1,024	0	64	0	<10

[a] Human erythrocytes used were either unmodified (native), sialidase treated (asialo), or asialo erythrocytes resialylated with NeuAc or NeuGc and one of two purified sialyltransferases to form the SAα2,3Gal sequence on O-linked oligosaccharides and the SAα2,6Gal sequence commonly found on asparagine-linked oligosaccharides. Hemagglutination titers were determined in the presence of DDN, as described in the text. A value of 0 indicates a titer of <2.

[b] HI by heat-inactivated (56°C, 30 min) normal (nonimmune) HS was performed as described by Fazekas de St. Groth and Webster (6) with chicken erythrocytes.

[c] Cloned HS^r variants were obtained as described in the text.

residue 155 is critical to the ability of the later H3 viruses to bind NeuGc.

Receptor-binding specificity of influenza viruses of the H2 and H6 subtypes. Influenza viruses of the H2 and H6 subtypes have been demonstrated to be strongly mitogenic for lymphocytes from strains of mice that express cell surface I-E molecules but poorly or nonmitogenic for strains of mice lacking I-E expression (16, 17). To determine whether this pattern of mitogenic activity is correlated with a particular receptor-binding specificity of the HA molecule of these viruses, they too were screened for agglutination of resialylated erythrocytes and for HI by HS (Table 5). In contrast to the strongly mitogenic H3 viruses (1972 onward), Jap$_H$-Bel$_N$ (H2N1) virus and the avian virus Shear$_H$-Bel$_N$ (H6N1) were HS^r and showed specificity for NeuAcα2,3Gal; no agglutination of derivatized cells bearing NeuGc was observed.

To determine whether the I-E-dependent mitogenic activity of H2 viruses was a property only of HS^r, SAα2,3Gal-binding variants, the mitogenic activity of RI/5$^+$ (HS^s) and RI/5$^-$ (HS^r) variants of RI/5 (H2N2) virus was compared. Carroll et al. (3) have shown these viruses to have specificity for SAα2,6Gal and SAα2,3Gal sequences, respectively; and this was confirmed for the virus stocks used in this study (Table 5). The mitogenic activity of these viruses was examined with lymphocytes from B10.D2 (I-E$^+$) and B10 (I-E$^-$) mice. RI/5$^+$ (HS^s) displayed the same mitogenic activity and specificity as the HS^r variant RI/5$^-$ (Table 6), both being mitogenic only for lymphocytes of I-E$^+$ origin. Furthermore, as found previously for other H2 viruses (17), the mitogenic response of I-E$^+$ lymphocytes to both RI/5$^+$ (HS^s) and RI/5$^-$ (HS^r) variants was inhibited by anti-I-E MAb 14-4-4 at concentrations that had no effect on the response to the H3 virus PNG$_H$-Bel$_N$, which is not I-E restricted in its mitogenicity (Table 6).

To verify that the mitogenic activity of the RI/5$^+$ virus stock was not due to a low level of contamination with SAα2,3Gal-binding variants, the mitogenic activity of RI/5$^+$ (HS^s), RI/5$^-$ (HS^r), and PNG$_H$-Bel$_N$ (HS^s) virus stocks was tested for sensitivity to inhibition by HS. Mitogenesis in-

TABLE 4. Effect of amino acid substitutions at residues 155 and 188 of H3 subtype HA on NeuGc binding and mitogenesis

Virus	Amino acid at residue[a]: 155	188	Hemagglutination titer[b]: Native	Asialo	SAα2, 6Gal NeuAc	SAα2, 6Gal NeuGc	Mitogenicity (stimulation index)[c]
Eng69 (H3N2)	Thr	Asn	128	0	128	0	3.9
Eng72 (H3N2)	Tyr	Asn	16	0	32	64	38.0
Texas (H3N2)	Tyr	Asp	512	0	256	512	36.5

[a] See reference 18.

[b] Receptor-binding specificity was determined by agglutination of derivatized erythrocytes as described in footnote a to Table 3.

[c] BALB/c spleen cells were cultured with 50 HAU of UV-inactivated influenza virus or medium alone. Culture medium contained 2% normal sheep serum. The background level of proliferation in the absence of virus was 808 cpm.

TABLE 5. Receptor-binding specificities of influenza viruses of the H2 and H6 subtypes

Virus	Hemagglutination titer[a]						HI titer with HS[b]
	Native	Asialo	SAα2,3Gal		SAα2,6Gal		
			NeuAc	NeuGc	NeuAc	NeuGc	
Jap_H-Bel_N (H2N1)	>256	0	512	0	0	0	<10
$Shear_H$-Bel_N (H6N1)	>256	0	256	0	0	0	<10
Receptor variants							
RI/5$^+$ (H2N2)	16	0	0	0	32	0	78,000
RI/5$^-$ (H2N2)	32	0	128	0	0	0	<10

[a] Receptor-binding specificity was determined by agglutination of derivatized erythrocytes as described in footnote a to Table 3.
[b] HI titers were determined as described in footnote b to Table 3.

duced by the HS^s viruses RI/5$^+$ and PNG_H-Bel_N was sensitive to inhibition by HS, whereas RI/5$^-$ (HS^r) virus-induced mitogenesis was not affected (Table 7).

DISCUSSION

The results of this study show that the receptor-binding specificity of HA plays a critical role in determining the mitogenic activity and specificity of influenza A viruses.

For viruses of the H3 subtype, strong mitogenic activity was shown to correlate with the ability of the virus to recognize the sialyloligosaccharide sequence NeuGcα2,6Gal. Viruses that arose early in the H3 subtype (from 1968 to 1971) bind NeuAcα2,6Gal but fail to bind NeuGcα2,6Gal and are weakly mitogenic. Viruses isolated from 1972 onward bind NeuGcα2,6Gal as well as NeuAcα2,6Gal and are strongly mitogenic.

Growth of influenza viruses in the presence of HS selects for receptor variants which show preferential binding to SAα2,3Gal rather than SAα2,6Gal sequences (14; Table 3); the ability to bind NeuGc is also lost (Table 3). Sequence analysis of the HA molecule of such variants has shown this change in receptor-binding specificity to result from a single amino acid substitution at residue 226 of HA_1, which lies within the receptor-binding pocket of HA (13, 20). The finding that this change in receptor-binding specificity is accompanied by the loss of mitogenic activity of the later H3 virus strains further illustrates the important role of receptor-binding specificity of HA in determining mitogenic activity and supports the correlation between strong mitogenic activity of H3 viruses and the ability to bind NeuGcα2,6Gal.

The pattern of binding of NeuGcα2,6Gal-derivatized erythrocytes by the various strains of H3 viruses (Table 3) confirms and extends the findings of Higa et al. (8), who showed that although all strains tested could agglutinate erythrocytes derivatized with the NeuAcα2,6Gal sequence, only strains isolated from 1972 onward, and not the original 1968 strains, could agglutinate NeuGcα2,6Gal-derivatized erythrocytes. It was proposed (8) that the ability of the later viruses to bind NeuGc could be due to an amino acid change at residue 155 or 188 of HA_1, both of which are located adjacent to the receptor-binding pocket in the three-dimensional structure of the HA molecule (20). The inclusion of Eng72 in this study allowed the contribution of these two residues to be assessed. Eng72 resembles the later H3 viruses in that it has a Tyr at 155 (the early [from 1968 to 1971] strains have a Thr at 155) but resembles the early strains in that it has an Asn at 188 (the later viruses have an Asp at 188) (18). The ability of Eng72 to agglutinate NeuGcα2,6Gal-derivatized erythrocytes and its strong mitogenic activity indicate that the change at residue 188 (Asn → Asp) in subsequent strains is not responsible for their change in receptor-binding specificity and mitogenic activity. Rather, the findings suggest a critical role for the change at residue 155 from Thr to Tyr in conferring the ability to bind the NeuGcα2,6Gal sequence and the consequent strong mitogenic activity. A further four amino acid substitutions, at residues 122 (Thr → Asn), 207 (Arg → Lys) 242 (Val → Ile), and 275 (Asp → Gly) in the globular head region of the HA molecule, also distinguish the early non-NeuGc-binding strains (X-31, $Mem71_H$-Bel_N, and Eng69) from the later NeuGc-binding strains (Eng72 onward) (10, 18); but the distance of these residues from the receptor-binding pocket (20) makes it less likely that they are involved.

TABLE 6. Requirement for I-E expression for B-cell mitogenesis by receptor variants of the H2 subtype

Virus[a]	Stimulation index of[b]:		
	B10.D2 (I-E$^+$)		B10 (I-E$^-$)
	Without antibody	With anti-I-E MAb[c]	
RI/5$^+$ (HS^s) (H2N2)	43.4	7.2	4.7
RI/5$^-$ (HS^r) (H2N2)	43.4	3.4	3.9
PNG_H-Bel_N (H3N1)	16.0	14.2	18.5

[a] All virus preparations used were UV-inactivated allantoic fluid stocks used at 10 HAU per culture.
[b] Culture medium contained 2% normal sheep serum. Background responses in the absence of virus were as follows: B10.D2 spleen cells without antibody, 298 cpm, and with antibody, 296 cpm; B10 spleen cells, 288 cpm.
[c] Anti-I-E MAb 14-4-4 IgG (1 μg/ml) was added to B10.D2 spleen cells 1 h before the addition of virus.

TABLE 7. Sensitivity of mitogenic activity to inhibition by HS[a]

Virus	Dose (HAU/culture)	Stimulation index[b]	
		Without HS	With HS
RI/5$^+$ (HS^s)	1	42.9	5.4
RI/5$^-$ (HS^r)	1	38.0	37.0
PNG_H-Bel_N (HS^s)	10	34.2	4.9

[a] UV-inactivated viruses (50 μl) were incubated in microtiter wells in the presence of 100 μl of 2.5% heat-inactivated (56°C, 30 min) normal HS or medium alone for 30 min at 37°C, before the addition of 4 × 10^5 BALB/c spleen cells in 100 μl. Cultures contained 2% normal sheep serum, with or without HS.
[b] Background responses in the absence of virus were as follows: without HS, 808 cpm; with 1% HS, 610 cpm.

The nature of the receptor molecule on B cells, with which H3 viruses interact to trigger B-cell proliferation, is not known. The correlation of mitogenic activity with recognition of the sequence NeuGcα2,6Gal suggests, however, that this sequence may be present on the critical receptor molecule. That murine lymphoid cells do bear high levels of NeuGc has been demonstrated by Kaufmann et al. (9) for T lymphocytes, but they did not examine B cells in that study.

In contrast to the H3 viruses, which are mitogenic for all inbred strains of mice tested, influenza viruses of the H2 and H6 subtypes are mitogenic only for strains of mice that express cell surface I-E molecules (16). The ability of anti-I-E MAb to block H2 influenza virus-induced mitogenesis and the ability of H2 virus to inhibit the binding of anti-I-E MAb to spleen cells indicate that the B-cell receptor for mitogenic activity of these viruses may be the I-E molecule itself (17).

Two features of the receptor-binding specificity of H2 and H6 viruses distinguish them from the strongly mitogenic H3 viruses. First, the H2 and H6 viruses included in this study failed to bind NeuGcα2,6Gal and, therefore, presumably do not bind to the particular receptor molecule with which the mitogenic H3 viruses interact. This would account for the failure of H2 and H6 viruses to be mitogenic for strains of mice not expressing I-E. Second, there is no difference in mitogenic activity or specificity (I-E restriction) between the HS^s and HS^r receptor variants of the H2 virus RI/5, which bind preferentially to NeuAcα2,6Gal and NeuAcα2,3Gal, respectively. This led us to consider whether for H2 (and H6) viruses the mitogenic interaction of viral HA with B lymphocytes may occur not via the receptor-binding site of HA at all but, perhaps, by direct protein-protein interaction of the receptor molecule (I-E?) with viral HA. However, evidence for the involvement of SA as an essential component of the receptor was provided by two experimental approaches (unpublished data). (i) Pretreatment of spleen cells with bacterial sialidase was shown to reduce their proliferative response to H2 viruses (and also to H3 viruses) but not to LPS. (ii) The mitogenic response to H2 viruses (but not to LPS) was enhanced threefold in the presence of the neuraminidase inhibitor DDN (1 mM). This suggests that the viral neuraminidase activity normally limits the degree of mitogenic stimulation by cleaving SA from the receptor molecule on the B cell.

It is possible that the receptor molecule (I-E?) on B lymphocytes that is specifically triggered by the binding of H2 (or H6) viruses bears both NeuAcα2,6Gal and NeuAcα2,3Gal sequences, and so can interact with viruses of both receptor specificities. This does not explain, however, why the early H3 viruses (NeuAcα2,6Gal-binding) or HS^r variants of H3 viruses (NeuAcα2,3Gal-binding) are not similarly mitogenic. An alternative possibility is that the specific sialyloligosaccharide determinant recognized by H2 and H6 viruses on the critical B-cell receptor molecule is neither NeuAcα2,6Gal nor NeuAcα2,3Gal but is a third sequence, as yet unidentified, which is not recognized by viruses of the H3 subtype. The fact that this structure is recognized equally well by H2 viruses of either receptor-binding specificity could be explained if the interaction of this ligand with the receptor-binding site were not affected by the particular amino acid change in HA that affects binding to NeuAcα2,6Gal and NeuAcα2,3Gal sequences (14).

Viruses of the H1 subtype were not included in this study as they are only very weakly mitogenic (2). Results of previous studies on the receptor-binding specificity of H1 viruses, however, suggest further differences in specificity compared with H2 and H3 viruses because plaque-purified A/PR/8/34 (H1N1) virus was able to utilize either NeuAcα2,3Gal or NeuAcα2,6Gal sequences as receptor-binding determinants (12).

While many different sialylated membrane glycoproteins and glycolipids may function as cell surface receptors for influenza viruses, presumably only few such receptors are capable of transducing an activation signal to the B cell. The results of this study suggest that differences in mitogenic activity between influenza viruses relate to differences in their ability to recognize the particular sialyloligosaccharide sequence(s) present on these critical triggering receptor molecules.

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