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Mitogenicity of Influenza Hemagglutinin Glycoproteins and Influenza Viruses Bearing H2-Hemagglutinin

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The hemagglutinin glycoprotein is responsible for the mitogenic effect of influenza A viruses of the H2N2 subtype. This was indicated by the ability of viruses bearing the H2-hemagglutinin glycoprotein, regardless of its associated neuraminidase, to induce lymphocyte proliferation in normal spleen cell suspensions and by the ability of antisera with specificity for the H2-hemagglutinin to block this response. Moreover, purified hemagglutinin from representative viruses from the H0N1, H1N1, H2N2, H3N2, and influenza B subtypes were also shown to be mitogenic.

Since our initial report that influenza A viruses of the H2N2 subtype are potent mitogens for lymphocytes from a variety of mammals (5), we have extended the studies to determine the viral component(s) responsible for this effect. Two observations suggest that the viral surface components, either the hemagglutinin, the neuraminidase, or both, rather than internal components of the virion, mediate the response. First, all type A influenza viruses, whether mitogenic or not, possess cross-reactive matrix and nucleoprotein antigens. (7, 8, 15, 16) Second, H2N2 viruses retain mitogenicity despite ultraviolet inactivation and therefore do not induce proliferation as a result of infection, which is in contrast with the mitogenicity reported for herpes simplex virus (11).

The disparate responses of whole viruses of the H2N2 and H3N2 subtypes suggest that the hemagglutinin, rather than the common neuraminidase, is responsible for mitogenicity. Thus, the proliferative response to H2N2 was reproducible, consistent, and strong, whereas the proliferative response to H3N2 was variable and weak enough that it was appreciated only after more extensive testing. The hypothesis that the hemagglutinin of the H2N2 subtype virus is mitogenic was tested by using antisera to various influenza A subtypes. In addition, experiments with purified hemagglutinins from representative H0N1, H1N1, H2N2, H3N2, and influenza B viruses were done and indicate that these glycoproteins also were mitogenic when separated from the virion.

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MATERIALS AND METHODS

Lymphocyte cultures. Single cell suspensions were prepared from the spleen of normal BALB/c mice, using ammonium chloride buffer to lyse erythrocytes (3). The spleen cells obtained were added to microtiter plates (Microtest II; Falcon Plastics, Oxnard, Calif.), 2×10^6 viable cells per well of RPMI 1640 medium enriched with 5% heat-inactivated fetal bovine serum, as previously described (6). No further additions were made to four wells, which served as controls; similarly, 0.5 μg of concanavalin A was added to an additional four wells to evaluate the ability of these cells to undergo a proliferative response. Test preparations were added in replicates of four. After a 44-h incubation, 1 μCi of tritiated thymidine (New England Nuclear Corp., Boston, Mass.) was used for a 4-h labeling period. The microtiter plates were then harvested, and tritiated thymidine incorporation was determined by liquid scintillation counting (6). Stimulation ratios (SR) were calculated by dividing the mean counts per minute of the test groups by the mean counts per minute of the control group. The Student *t* test was used to evaluate the statistical significance of the difference between control and test groups at the 99% confidence limit.

Viruses. Virus preparations stocked by the Bureau of Biologics, Food and Drug Administration, were grown in the allantoic cavity of embryonated hen eggs by standard techniques (13). No bacterial or mycoplasma contamination could be demonstrated for the harvested pools of allantoic fluid containing virus.

Virus preparations were inactivated with ultraviolet radiation before being used to test for mitogenicity and contained less than 0.5 egg infectious dose (EID_{50}) per 0.2 ml.

Protein concentrations were measured for preparations of the following viruses: A/PR-8 (H0N1), A/USSR (H1N1), A/Japan (H2N2), A/PC (H3N2), and B/Singapore. Each virus preparation was tested for mitogenicity by adding volumes containing 0.001, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mg of

protein to replicate wells as described above. Determining the response to replicates having comparable amounts of hemagglutinating activity was facilitated by determining the hemagglutination titer of each preparation with standard techniques (13).

Antisera to viruses. Antisera were prepared to A/NWS/33 (H0N1), A/Japan/305/57 (H2N2), and recombinant strains X-7 (H0N2) and X-9L (H2N1), which are designations for recombinants of A/NWS/33 (H0N1) and A/RI/5+/57 (H2N2) (9, 10). Adult chickens were inoculated intravenously with 2.5 ml and intraperitoneally with 2.5 ml of allantoic fluid containing live virus in hemagglutination titers ranging from 1:256 to 1:2048. The injections were repeated 4 weeks later, and serum pools were obtained by bleeding the chickens after another 4 weeks.

Antisera were adjusted to have an hemagglutination inhibition titer of 1:128 against 4 units of homologous virus. The adjusted antiserum (10 μl) was added to either concanavalin A or A/Japan/305/57 virus and incubated for 1 h. Aliquots (10 μl) of the respective mixtures were added to microtiter wells containing spleen cells from normal animals to test for mitogenicity.

Hemagglutinin purification. Allantoic fluid stocks containing virus were clarified at 1000 × *g* for 15 min. The supernatant was centrifuged at 54,000 × *g* for 3 h, and the virus-enriched pellet was suspended in phosphate-buffered saline at pH 7.2. This suspension was twice centrifuged through a continuous 20 to 60% (wt/vol) gradient of sucrose in phosphate-buffered saline for 16 h at 130,000 × *g*. The virus found in the 38 to 54% sucrose band was collected, and the purified virus was stored in this liquid at −80°C.

Previously described methods for isolating hemagglutinin were employed (14). Purified virus was treated with bromelain (Sigma Chemical Co., St, Louis, Mo.) and filtered through a 0.45-μ millipore filter (Millipore Corp., Bedford, Mass.). Bromelain-solubilized hemagglutinin was isolated by centrifugation through a continuous gradient of 5 to 30% sucrose. The gradient was fractionated with a displacement fractionator (Isco Model 640; Lincoln, Nebr.) equipped with 254-nm monitor and was examined for purity by slab polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. Qualitative protein examinations were done in 10% acrylamide slab gels under reducing conditions with 2-mercaptoethanol (12). Identification of virus polypeptides was facilitated by coelectrophoresis of markers of known molecular weight (lysozyme, 14,000; soy bean trypsin inhibitor, 21,000; carbonic anhydrase, 30,000; ovalbumin, 43,000; bovine serum albumin, 68,000; and phosphorylase B, 94,000).

Neuraminidase activity. Hemagglutinin preparations were screened for neuraminidase activity in the presence of Fetuin. (1, 13) A 1-h incubation period at 37°C in the presence of Fetuin was used for screening purposes.

Protein concentration determinations. The method for determining protein concentration by using a fluorescent *o*-phthalaldehyde assay (Aldrich Chemical Co., Milwaukee, Wis.), has been previously described (14). The fluorescence of each mixture was measured at 455 nm after excitation at 340 nm. Serial dilutions of bovine serum albumin were treated in a similar manner to permit calculation of protein concentration by comparison.

RESULTS

Direct testing of viruses demonstrated statistically significant differences in thymidine incorporation between control spleen cells and spleen cells exposed to 1 μg of H2N2 virus (SR, 5.8) but not to H0N1 (SR, 1.9), H1N1 (SR, 1.8), H3N2 (SR, 0.4), or B/Singapore (SR, 3.8) (Table 1). When the different virus preparations were contrasted in terms of comparable amounts of hemagglutinin activity, H2N2 virus (SR, 5.8) was again the only one for which the difference between test and control was statistically significant (SR for other viruses, <1.7). Moreover, testing with a range of virus concentrations assured that these differences were not dose related (data not shown).

The effect of pretreating A/Japan/305/57 with various antisera correlated with the specificity of the antisera for the hemagglutinin glycoprotein. Data from representative experiments are presented in Table 2. Thus, virus pretreated with antisera to either H2N2 or H2N1 virus did not induce a proliferature response (SR, 0.7), whereas the response was not inhibited by virus pretreated with antisera to H0N1 or H0N2 virus (SR, 15.5 and 13.2 respectively). The antisera alone neither induced proliferation (data not shown) nor interfered with proliferation induced by concanavalin A (SR, >20.5).

The purified preparations of hemagglutinins from the various viruses were evaluated for polypeptide content by polyacrylamide electropho-

TABLE 1. *Response of normal spleen cells to incubation with whole influenza virus of different subtypes*

Virus (subtype)	SR at amount of virus preparation	
	1 μg of protein[a]	HA adjusted[b]
A/PR-8/8/34 (H0N1)	1.87	0.73
A/USSR (H1N1)	1.83	1.12
A/Japan/305/57 (H2N2)	5.80[c]	5.80[c]
A/Port Chalmers/1/73 (H3N2)	0.42	1.56
B/Singapore	3.77	1.56

[a] SR produced by adding 1 μg of protein of virus preparation added to each replicate well.

[b] SR produced by adding an amount of virus preparation adjusted to have a hemagglutination titer comparable to 1 μg of A/Japan/305/57.

[c] Difference from control statistically significant at 99% confidence limit.

resis. No contamination of hemagglutinin fractions by other molecular species was found within the limits of sensitivity of this technique. No neuraminidase activity was detected in these preparations.

The results of testing with purified hemagglutinins from various influenza viruses (Table 3) revealed the interesting and unanticipated result that all of the strains tested yielded a glycoprotein capable of inducing a proliferative response (SR, >14) when they were not an integral part of the virion, even if the intact virus did not have a similar effect.

DISCUSSION

Direct testing of intact viruses confirmed that naturally occurring H2N2 influenza virus regularly exerted a strong mitogenic effect. This effect has also been observed, albeit sporadically and at lower levels, with intact H3N2 virus (data not shown), but not with H0N1, H1N1, or the B strains tested.

TABLE 2. *Response of normal spleen cells to incubation with A/Japan/305/57 or concanavalin A pretreated with antisera of selected specificities*

Specificity of antiserum used for pretreatment (viral subtype used to immunize)	SR from incubation with pretreated:	
	A/Japan/305/57	Concanavalin A
No antiserum used	11.5[a]	17.8[a]
A/NWS/33 (H0N1)	15.5[a]	20.5[a]
X-7 (H0N2)	13.2[a]	20.5[a]
X-9L (H2N1)	0.7	22.7[a]
A/Japan/305/57 (H2N2)	0.7	22.7[a]

[a] Difference from control statistically significant at 99% confidence limit.

The importance of the hemagglutinin in the lymphocyte proliferation produced by intact H2N2 viruses was also indicated by the effect of antisera of varying specificity. Antisera to H2N1 or H2N2 viruses blocked the mitogenic effect of A/Japan/305/57 on normal spleen cells, whereas antisera to H0N1 or H0N2 viruses did not.

The most direct means of assessing the effect of influenza hemagglutinin on normal spleen cells is to obtain purified preparations of the glycoprotein for testing independent of other constituents of the virus. When this was done for H0N1, H1N1, H2N2, H3N2, and B/Singapore viruses, all of the hemagglutinins obtained were found to be capable of inducing proliferation of normal spleen cells. The reason for the difference in effects produced by isolated hemagglutinin compared with the hemagglutinin attached to the intact virus is not clear, although the simplest explanation would be that the effect site(s) is masked on A/PR-8, A/USSR, and B/Singapore, partially masked on A/PC, but not masked on A/Japan/305/57 and other H2N2 viruses.

In vitro interaction between A/Japan/305 and lymphocytes from rodents has been reported to alter the circulatory patterns of reinjected lymphocytes (18). In this instance, heat treatment sufficient to inactivate neuraminidase activity could be shown to interfere with the homing pattern of lymphocytes but not with binding between virus and lymphocyte. These results were interpreted to indicate the presence of receptors capable of binding A/Japan/305 on the surface of rat lymphocytes.

The mechanism of the mitogenic effect of influenza hemagglutinins is not known. How-

TABLE 3. *Response of normal spleen cells to incubation with purified hemagglutinin from influenza viruses or concanavalin A*

Mitogen added (μg of hemagglutination preparation)	SR after incubation of cells with hemagglutinin obtained from influenza virus (subtype):				
	A/PR-8 (H0N1)	A/USSR (H1N1)	A/Japan (H2N2)	A/PC (H3N2)	B/Singapore
None	1.0	1.0	1.0	1.0	1.0
Concanavalin A	16.3[a]	26.6[a]	17.9[a]	23.4[a]	14.7[a]
2.000	5.7[a]	16.1[a]	0.6	7.9[a]	19.4[a]
1.000	29.3[a]	31.7[a b]	25.1[a]	8.4[a]	33.7[a b]
0.500	42.6[a b]	26.2[a]	50.7[a]	9.1[a]	29.8[a]
0.200	20.3[a]	14.7[a]	51.9[a]	32.2[a b]	11.8[a]
0.100	13.2[a]	5.8[a]	69.2[a b]	16.6[a]	6.7[a]
0.050	5.8[a]	2.7	46.5[a]	10.2[a]	3.6[a]
0.020	2.2	1.7	20.5[a]	5.4	1.3
0.010	1.4	1.1	10.9[a]	1.4	1.1
0.005	1.1	1.0	3.1	2.0	1.0
0.001	1.0	0.8	1.9	1.4	0.8

[a] Difference from control statistically significant at 99% confidence limit.
[b] Maximum stimulation observed for this hemagglutinin.

ever, the interaction between virus hemagglutinin and cell surface glycoprotein is believed to be responsible for the attachment of influenza viruses to susceptible cells (9). If this is correct, the mitogenicity of A/Japan/305/57 may be analogous to the effect of lectins, which bind to lymphocyte cell surface glycoproteins to induce a proliferative response (17). The ability of monosaccharides to block the mitogenic effect of lectins is established (2), and we have observed that *N*-acetyl-D-glactosamine, but not D-galactose, blocks the proliferative response to H2N2 virus (data not shown).

The biological significance, of this interaction, if any, remains to be demonstrated. It is possible that the action of H2N2 viruses may be similar to that exhibited by lectins, which bind to lymphocyte cell surface glycoproteins.

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