

Direct Role of Viral Hemagglutinin in B-Cell Mitogenesis by Influenza Viruses

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The mitogenic activity of influenza virus is a function of the hemagglutinin (HA) molecule. Purified HA is mitogenic for murine B lymphocytes but not T lymphocytes. Furthermore, like the intact virus, HA of the H2 (but not H3) subtype is mitogenic only for B cells expressing the class II major histocompatibility complex glycoprotein I-E. Since virus bearing uncleaved HA is as mitogenic as virus bearing cleaved HA, the membrane fusion activity of the HA molecule is not involved.

Many strains of influenza virus A are mitogenic for murine B lymphocytes in vitro (2, 4). Several observations indicate an important role for the hemagglutinin (HA) molecule in the mitogenic process: (i) mitogenesis is specifically inhibited by monoclonal anti-HA antibodies (2); (ii) mitogenic activity differs for viruses of different HA subtype (viruses of the H1 subtype are only weak mitogens, whereas viruses of the H2, H3, and H6 subtypes are moderate to strong mitogens [2]); (iii) two distinct mechanisms of mitogenesis operate, depending on the HA subtype of the virus. Viruses of the H2 and H6 subtypes are strongly mitogenic only for lymphocytes from strains of mice that express the cell surface class II major histocompatibility complex glycoprotein I-E, and competition experiments with monoclonal anti-I-E antibody suggest that these viruses interact directly with I-E molecules on the B-cell surface (15, 16). In contrast, the mitogenic response to viruses of the H3 subtype is independent of I-E expression. In no case does the particular subtype of the viral neuraminidase (NA) glycoprotein appear to affect mitogenic activity or specificity.

These observations suggest that interaction of the viral HA with the lymphocyte membrane is a critical event in the induction of mitogenesis by influenza virus. The question arises of whether this interaction alone is sufficient for transmission of a mitogenic signal. Viral infectivity is not required for the mitogenic activity of influenza virus (2, 4), but the possibility exists that some event subsequent to HA attachment, possibly fusion of viral and lymphocyte membranes, or some role for the viral NA may be essential for activation of the B lymphocyte. Armstrong et al. (3) reported that HA derived from bromelain-treated influenza virus (Brom HA) was mitogenic, even when derived from type A viruses of the H1 subtype or from influenza virus B, which themselves are poorly mitogenic or nonmitogenic. They proposed that the mitogenic interaction involved a site on HA that was inaccessible on intact virions of the H1 subtype or type B. Whether Brom HA functioned as a T-cell mitogen or as a B-cell mitogen was not examined. It is therefore not clear whether mitogenesis by Brom HA reflects the events occurring in virus-induced mitogenesis.

In this study, we examined the mitogenic activity of purified HA isolated from detergent-disrupted influenza virus. The two strains of influenza virus A used were the

recombinants Jap_H-Bel_N = A/Japan/305/57 × A/Bel/42 (H2N1), and PNG_H-Bel_N = A/Papua New Guinea/75 × A/Bel/42 (H3N1). Viruses were grown in the allantoic cavity of embryonated hen eggs and then purified by rate-zonal centrifugation. To prepare isolated HA, purified virus (1 to 3 mg) was incubated for 1 h at 37°C in phosphate-buffered saline (pH 7.3) containing 30 mM *n*-octyl-β-D-glucoside (Sigma Chemical Co.) and 0.1% sodium azide and then centrifuged in a Beckman air-driven centrifuge (100,000 × *g*, 15 min, 4°C). The supernatant fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (12) and found to contain the two viral glycoproteins HA and NA, together with a small amount of nucleoprotein (NP) (Fig. 1A, lane c). To obtain purified HA, NA was removed by passing the glycoprotein fraction over a column of Sepharose 4B to which had been coupled immunoglobulin G from a monoclonal antibody against strain Bel (N1) NA. More than 99% of the NA was removed from the HA-NA preparation by this procedure, as shown by SDS-PAGE (Fig. 1A, lane e), and by assaying for NA enzymic activity by the release of sialic acid from fetuin (14) (Fig. 1B). For some experiments, HA was also freed of contaminating NP by passage over an immunoadsorbent column containing an anti-NP monoclonal antibody that recognized the NP of both viruses (Fig. 1A, lane d). Before addition to lymphocyte cultures, the various protein fractions were dialyzed extensively against culture medium.

Mitogenic activity of influenza virus and viral components for normal mouse spleen cells was determined as described previously (2). Briefly, spleen cells (4 × 10⁵/0.25 ml) were cultured in microtiter trays with virus, viral components, or the mitogens lipopolysaccharide W from *Escherichia coli* O111:B4 (Difco Laboratories) (LPS) or concanavalin A (Boehringer Mannheim Australia Pty. Ltd.) (ConA). The culture medium was RPMI 1640 supplemented with 2 mM glutamine, 2 mM sodium pyruvate, 10⁻⁴ 2-mercaptoethanol, 100 μg of streptomycin per ml, 100 IU of penicillin, and, in most experiments, 2% heat-inactivated (56°C, 30 min) normal goat serum (otherwise, 5% heat-inactivated fetal calf serum). Goat serum gives rise to lower background proliferation than does fetal calf serum in control unstimulated cultures (2). After 24 h of incubation, cultures were pulsed with 0.5 μCi of [³H] thymidine; 18 h later, cells were harvested and ³H incorporation was determined by liquid

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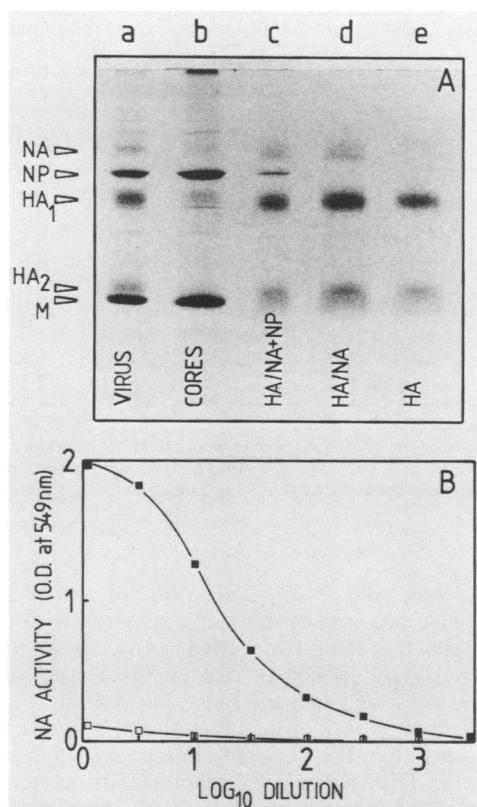


FIG. 1. (A) SDS-PAGE of Jap_H-Bel_N virus (lane a) and viral components obtained by disruption of the virion with *n*-octyl- β -D-glucoside and separation by rate-zonal centrifugation and affinity chromatography (lanes b to e). Lanes: b, insoluble viral cores released by *n*-octyl- β -D-glucoside and pelleted by centrifugation, composed largely of matrix protein (M) and NP; c, supernatant fraction containing the viral glycoproteins, HA (separated under reducing conditions into HA₁ and HA₂ chains), NA, and some NP; d, removal of NP by passage of this glycoprotein preparation over an anti-NP immunoadsorbent column; e, removal of NA by passage over an anti-NA immunoadsorbent column. (B) Removal of NA from glycoprotein fraction of Jap_H-Bel_N virus by affinity chromatography. NA enzymic activity was assayed before (■) and after (□) passage of the glycoprotein fraction over an immunoadsorbent column of monoclonal anti-Bel NA (N1) antibody coupled to Sepharose 4B.

scintillation counting. Results are expressed as counts per minute or as a stimulation index (*T/C*), where *T* is mean counts per minute incorporated in triplicate cultures containing mitogen and *C* is mean counts per minute incorporated in sextuplicate cultures containing medium alone. Standard errors of the means of replicate determinations were usually <10% and always <15% and are omitted from the tables.

Purified Jap HA and PNG HA were both found to be mitogenic for normal BALB/c spleen cells (Table 1). Virus cores containing NP and matrix (M) protein were totally inactive, and removal of contaminating NP from the glycoprotein fraction had no effect on the mitogenic activity of the latter. Removal of the NA from the glycoprotein fraction, however, slightly enhanced the mitogenicity of HA (Table 1; unpublished data). The inhibitory effect of NA may be due to enzymic destruction of sialic acid-containing receptors for HA on the B-cell membrane.

The dose of HA required to elicit a mitogenic response was substantially lower when HA was presented in the intact virion than when it was presented as isolated HA. Since 1 mg

of virus is equivalent to ca. 3×10^5 HA units (HAU) and HA makes up 30% (by mass) of the virion, it can be calculated that 100 HAU of virus contains 0.1 μ g of HA, a dose which, in the form of isolated HA, was only barely mitogenic (data not shown). This difference in dose response may reflect a higher avidity of interaction of HA with cell surface receptors when HA is presented as closely packed spikes in the virion envelope rather than as isolated spikes or rosettes.

To identify the class of lymphocyte that proliferates in response to the mitogenic action of influenza viral HA, BALB/c spleen cells were depleted of T or B lymphocytes. T-cell depletion (B-cell enrichment) was achieved by first treating mice in vivo with anti-thymocyte serum 2 days before sacrifice and then treating the spleen cells in vitro with anti-T-cell serum plus complement (1). B-cell depletion (T-cell enrichment) was achieved by passing spleen cells twice through nylon wool columns under conditions that remove most of the B cells and adherent accessory cells (7); accessory cells were then replaced in the form of γ -irradiated (2,200 R) syngeneic spleen cells (1, 2). The effectiveness of each procedure is shown in Fig. 2. The T-cell-depleted (B-cell-enriched) population proliferated normally in response to the B-cell mitogen LPS but showed no response to the T-cell mitogen ConA, while the B-cell-depleted (T-cell-enriched) population proliferated normally in response to ConA but almost not at all in response to LPS. HA purified from both Jap_H-Bel_N (H2) and PNG_H-Bel_N (H3) viruses, like the parent viruses themselves, were mitogenic for B cells but not at all for T cells. There is some indication that the mitogenic response of B cells to isolated HA may not be as absolutely T-cell independent as is their response to intact virions, since the response of B cells to HA was somewhat lower than the response of unseparated spleen cells.

We showed previously that influenza viruses of the H2 subtype are strongly mitogenic only for strains of mice that express cell surface I-E molecules, namely, strains of mice bearing the Ia.7 marker. In contrast, the mitogenic response to viruses of the H3 subtype is independent of I-E expression (15, 16). To investigate whether the same holds for purified HA as mitogen, Jap HA (H2) and PNG HA (H3) were tested

TABLE 1. Mitogenicity of purified influenza viral HA

Expt no.	Mitogen	Dose per culture	Proliferative response:	
			cpm	Stimulation index
1	None		250	1.0
	Jap _H -Bel _N virus	100 HAU	8,753	35.0
	Jap HA	3 μ g	2,798	11.1
		1 μ g	1,237	4.9
	PNG _H -Bel _N virus	100 HAU	3,943	15.8
	PNG HA	3 μ g	1,460	5.8
		1 μ g	1,524	6.1
2	LPS	20 μ g	9,116	36.5
	None		1,088	1.0
	Jap _H -Bel _N virus	100 HAU	28,228	25.9
	cores	— ^a	852	0.8
	HA-NA and NP	—	6,047	5.6
	HA-NA	—	6,226	5.8
	HA	—	8,838	8.1

^a —, The various protein fractions were brought to the same volume, and the dose used per culture corresponded to the material recovered from 10 μ g (3,000 HAU) of virus; 3,000 HAU of Jap_H-Bel_N virus stimulates a similar level of proliferation as 100 HAU (data not shown).

for mitogenicity for spleen cells from a number of different strains of mice, some expressing I-E and others not. The data in Table 2 show that each of the HA preparations displayed the same pattern of activity as the intact virus, PNG HA being mitogenic for all the mouse strains tested but Jap HA being mitogenic only for Ia.7⁺ strains of mice.

The function of HA in the infection of a host cell by influenza virus is not only to mediate virus adsorption but also to mediate fusion of the viral envelope with the membrane of a phagolysosome, bringing about uncoating of the virion. Fusion activity is associated with the N terminus of the HA₂ chain of proteolytically cleaved HA, which is the form of HA existing in egg-grown virus (11). Virus bearing uncleaved HA lacks fusion activity (6, 18). To determine whether the mitogenic activity of influenza virus depends upon the fusion function of the HA molecule, stocks of Jap_H-Bel_N and PNG_H-Bel_N viruses bearing uncleaved HA (HA₀) were prepared by growth in Madin-Darby canine kidney (MDCK) cells in the absence of trypsin (11) followed by differential centrifugation of the culture supernatants collected after 24 h. To verify that the viral HA produced was in fact uncleaved, some of the cultures received [³⁵S]methionine (30 μCi/ml). A portion of the ³⁵S-labeled and unlabeled virus stocks was subsequently treated in vitro with 50 μg of trypsin (Difco) per ml for 1 h at 37°C. The unlabeled virus preparations were used in a proliferation assay, described below. The ³⁵S-labeled virus preparations were disrupted with lysis buffer (0.05 M Tris [pH 7.5], 0.6 M KCl, 0.5% Triton X-100), immunoprecipitated with monoclonal antibodies directed against the HA of each virus, and subjected to SDS-PAGE under reducing conditions, as described by Kida et al. (8). The HA in the MDCK-grown Jap_H-Bel_N and PNG_H-Bel_N virus stocks was present in the form of the uncleaved precursor HA₀, whereas HA from trypsin-treated virus was entirely in the cleaved form, migrating on a reducing gel as the two constituent polypeptide chains HA₁ and HA₂ (Fig. 3).

The mitogenic activities of virus preparations bearing cleaved and uncleaved HA were then compared (Fig. 4). For

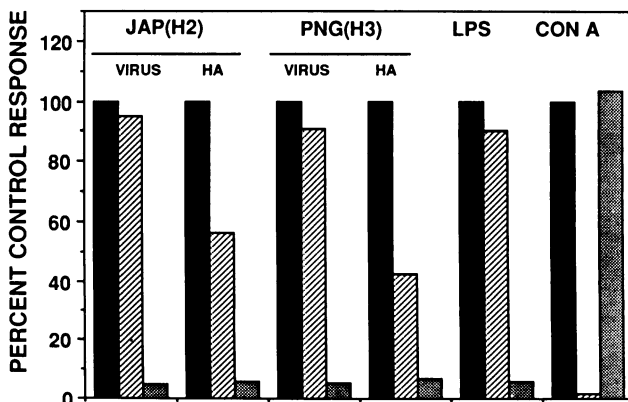


FIG. 2. Proliferative response of T and B cells to influenza virus and purified HA. Symbols: ■, response of 4×10^5 unseparated spleen cells; ▨, response of 4×10^5 T-depleted spleen cells (B cells plus accessory cells); ▩, response of 3×10^5 B-depleted spleen cells (T cells) cultured in the presence of 3×10^5 irradiated (2,200 R) normal spleen cells as accessory cells. Dose of mitogen per well: virus, 100 HAU; HA, 3 μg; LPS, 20 μg; ConA, 1 μg. Results are expressed as a percentage of the response of unseparated spleen cells, which was as follows: Jap_H-Bel_N virus, 17,876 cpm; PNG_H-Bel_N virus, 12,978 cpm; Jap HA, 4,370 cpm; PNG HA, 3,324 cpm; LPS, 15,129 cpm; ConA, 66,121 cpm.

TABLE 2. Mitogenic activity of Jap HA is restricted to I-E-expressing (Ia.7⁺) spleen cells

Mouse strain	Ia.7 ^a	Stimulation index ^b				
		Jap _H -Bel _N virus	Jap HA	PNG _H -Bel _N virus	PNG HA	LPS
BALB/c	+	15.6	3.7	9.9	2.7	14.7
B10.D2	+	7.4	3.8	5.5	4.2	11.8
B10.BR	+	7.3	6.4	5.1	4.8	17.0
B10.A(2R)	+	11.8	4.8	7.3	4.9	32.6
B10.A(5R)	+	8.0	4.0	11.6	5.8	41.0
B10	-	1.4	1.1	8.3	5.9	26.1
B10.A(4R)	-	1.1	0.9	8.6	5.7	17.8
B10.Q	-	1.4	1.2	7.8	6.8	16.7
B10.S	-	1.4	1.3	12.3	7.5	26.5

^a See reference 10.

^b Data represent stimulation index in response to 100 HAU of virus, 3 μg of HA, or 20 μg of LPS per culture. [³H]thymidine incorporation in control cultures lacking mitogen ranged from 670 to 2,080 cpm for the different mouse strains.

both Jap_H-Bel_N and PNG_H-Bel_N viruses, virus bearing uncleaved HA was just as mitogenic as virus with cleaved HA, indicating that the fusion activity of HA is not involved in mitogenesis by influenza viruses. McSharry and co-workers (9, 13) found that the mitogenic activity of Sendai virus glycoproteins resides mainly with the fusion (F) glycoprotein, rather than the hemagglutinin-neuraminidase (HN) glycoprotein. The question of whether fusion activity is actually required for mitogenesis by F glycoprotein was not addressed in those reports, however, since the glycoproteins were derived only from egg-grown Sendai virus which bears the cleaved, fusion-active form of F (5, 17).

In summary, the mitogenic activity of influenza virus is mediated by the HA molecule, does not require the fusion activity of HA, and most probably results from a lectinlike interaction of the viral HA with particular sialylated B-cell surface receptor molecules, which, on cross-linking, are capable of transducing an activation signal into the B cell; for HA of the H2 subtype, the receptor may be the I-E molecule (15, 16). In support of this concept, we have recently shown

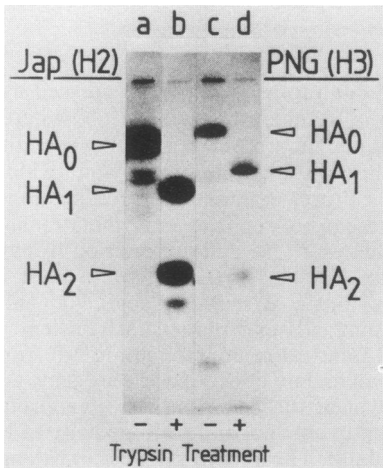


FIG. 3. SDS-PAGE of immunoprecipitated HA molecules which had been extracted from [³⁵S]methionine-labeled Jap_H-Bel_N and PNG_H-Bel_N viruses that had been grown in MDCK cells in the absence of trypsin and subsequently either treated with trypsin in vitro (lanes b and d) or untreated (lanes a and c).

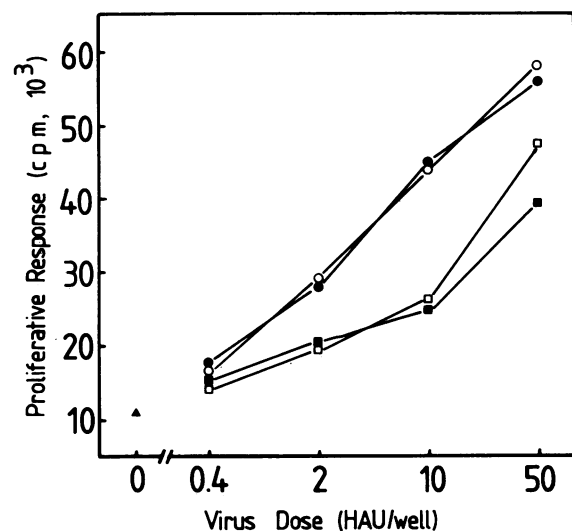


FIG. 4. Mitogenic response of BALB/c spleen cells to MDCK-grown JapH-BelN (●, ○) and PNGH-BelN (■, □) viruses bearing cleaved HA (solid symbols) or uncleaved HA (open symbols). Culture supernatant from uninfected MDCK cells had no mitogenic activity (data not shown). The high background stimulation in control culture lacking virus (▲, 11,440 cpm) was due to the use of fetal calf serum rather than goat serum in the culture medium in this experiment.

that the differing mitogenic activities of different strains of influenza virus are directly related to the receptor-binding specificity of their HA molecules (1a).

We thank Mary Jones and Del Dickson for expert technical assistance and Lorena Brown and Robert Webster for supplying the monoclonal antibodies against HA, NA, and NP. Some of the recombinant mouse strains used in this study were kindly provided by I. F. C. McKenzie, Department of Pathology, University of Melbourne.

This work was supported by Project Grant No. 840626 from the National Health and Medical Research Council of Australia.

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