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Influenza Viruses Are T Cell-Independent B Cell Mitogens

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UV-inactivated influenza virus A strains of subtypes H1, H2, H3, and H6 were shown to be mitogenic for unprimed splenic lymphocytes from BALB/c mice. Representative viruses of these four subtypes all behaved as T cell-independent B cell mitogens. The magnitude of the proliferative response was determined by the subtype of the hemagglutinin molecule: H2 and H6 viruses were the most potent mitogens, and H3 viruses were moderately mitogenic, whereas H1 viruses induced only low, but significant, levels of proliferation. Mitogenesis was inhibited by antiviral sera and by monoclonal antibodies directed against hemagglutinin.

Lymphocyte mitogens have been widely used to probe the events involved in activation, proliferation, and differentiation of T and B lymphocytes in rodents and humans. Many of the classical mitogens used are derived from plants, for example, concanavalin A (ConA), phytohemagglutinin, and pokeweed mitogen; others, like lipopolysaccharide (LPS), are of microbial origin. In recent years, a number of viruses have been shown to be mitogenic, including herpes simplex virus (9, 13), vesicular stomatitis virus (6), Sindbis virus (5), Sendai virus (10), and influenza virus (2). As well as enlarging the range of probes available for investigating lymphocyte activation, viral mitogens are of additional interest in that their study may contribute to understanding the overall response of the host to the virus in question, including the nonspecific modulation of the immune response that accompanies many virus infections (15).

Influenza virus A strains of the H2N2 subtype were reported by Butchko et al. (2) to be strong T and B lymphocyte mitogens, whereas other subtypes of influenza virus A, as well as B, strains appeared to be poorly mitogenic or nonmitogenic. We show here that in addition to the H2 viruses, influenza virus A strains of other subtypes are indeed mitogenic and, furthermore, that they are not T cell mitogens but are all T-independent B cell mitogens.

The following influenza virus A strains were used: PNG, A/Papua New Guinea/75 (H3N2); Guiyang, A/Guiyang/1/57 (H2N2); PR8, A/Puerto Rico/8/34 (H1N1); and the recombinant viruses X-31, A/Aichi/2/68 by A/PR/8/34 (H3N2); $Mem72_H$-Bel_N, A/Memphis/102/72 by A/Bel/42 (H3N1); Jap_H-Bel_N, A/Japan/305/57 by A/Bel/42 (H2N1); $Shearwater_H$-Bel_N, A/Shearwater/E. Aust/1/72 by A/Bel/42 (H6N1); and NWS_H-$Tokyo_N$, A/NWS/33 by A/Tokyo/3/67 (H1N2).

Viruses were grown for 2 days in the allantoic cavity of 10-day-embryonated hen eggs and then purified by adsorption to and elution from chick erythrocytes, followed by differential centrifugation and banding in sucrose gradients (11). Before addition to lymphocyte cultures, viruses were inactivated by exposure to UV irradiation (2 min, 14 cm from a 15 W germicidal lamp), which reduced their infectivity for eggs to below 10 50% egg infective doses per ml. Hemagglutination assays were performed by the method of Fazekas de St. Groth and Webster (4).

Spleen cells from female BALB/c mice (aged 6 to 8 weeks) were cultured in 96-well flat-bottomed microtiter trays (4×10^5 cells per well) in 0.25 ml of culture medium containing various doses of UV-inactivated influenza virus, 1 μg of ConA (Boehringer Mannheim Australia Pty. Ltd.), 10 μg of LPS (*Escherichia coli* O111:B4 LPS-W) (Difco Laboratories, Detroit, Mich.), or no additions. The culture medium was Eagle minimal essential medium plus nonessential amino acids supplemented with 2 mM glutamine, 10^{-4} M 2-mercaptoethanol, 30 μg of gentamicin per ml, and 5% heat-inactivated (56°C for 30 min) fetal calf serum (FCS) or 2% heat-inactivated goat serum. After 24, 48, or 72 h of incubation, cultures were pulsed with 0.5 μCi of [^{3}H]thymidine (5 Ci/mmol) (Amersham Australia Pty. Ltd.), and 18 h later the cells were harvested onto glass fiber filters and ^{3}H incorporation was determined by liquid scintillation counting.

In initial experiments with FCS-supplemented medium, we observed low but significant ($P < 0.001$) stimulation of normal BALB/c spleen cells by the H3 viruses $Mem72_H$-Bel_N (H3N1) and X-31 (H3N2), as well as more marked stimulation by the H2 virus Jap_H-Bel_N (H2N1), as expected from the previous report of Butchko et al. (2) (Table 1). The stimulation index is (T/C), where T = mean counts per minute incorporated in triplicate cultures containing virus or mitogen and C = mean counts per minute incorporated in sextuplicate cultures containing medium alone. Standard errors of the means of replicate determinations were all <10%. T was significantly different from C in all cases ($P < 0.001$) by Student's t test (3). Since medium supplemented with FCS gave very high background levels of proliferation in control cultures lacking viruses, it was possible that the mitogenic potential of influenza viruses was being masked, and an alternative serum to FCS was sought.

Medium containing goat serum (2% [vol/vol]) gave much lower levels of background stimulation and enabled the mitogenicity of these three viruses to be seen clearly (Table 1). A number of other influenza viruses were tested, and all were found to induce proliferation; the magnitude of the responses varied widely, however, and appeared to correlate with the hemagglutinin (HA) subtype of the virus (Table 1). Thus the H2 and H6 viruses were potent mitogens, the H3 viruses were moderately mitogenic, and low but significant levels of proliferation were induced by the H1 viruses tested. In later experiments, the absolute magnitude of the proliferative response to the different viruses varied, depending on the batch of goat serum used, but the general hierarchy in mitogenic activity of the viruses remained the same. This hierarchy could also be seen in a comparison of the minimum dose of virus needed to elicit a proliferative response (Table 2). Differences in mitogenic activity did not reflect differences in the kinetics of the response to the different viruses;

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TABLE 1. Mitogenicity of influenza viruses for BALB/c spleen cells

Virus (subtype)[a] or mitogen	Stimulation index in medium supplemented with:		
	5% FCS	2% FCS	2% Goat serum
$Mem72_H$-Bel_N (H3N1)	3.1	1.8	9.3
X-31 (H3N2)	2.3	1.8	10.0
PNG (H3N2)	—[b]	—	6.7
Jap_H-Bel_N (H2N1)	4.9	3.7	53.2
Guiyang (H2N2)	—	—	67.7
NWS_H-$Tokyo_N$ (H1N2)	—	—	3.6
PR8 (H1N1)	—	—	2.4
$Shearwater_H$-Bel_N (H6N1)	—	—	27.8
LPS	—	—	23.0
ConA	5.7	3.7	54.8

[a] Viruses were tested at concentrations from 1,000 to 1 HAU per culture. Data given are for 1,000 HAU per culture and represent maximum responses. Cultures were harvested after 42 h (goat serum) or 66 h (FCS). The counts per minute with each serum supplement were as follows for the control culture: 5% FCS, 9,488; 2% FCS, 10,938; 2% goat serum, 650.

[b] —, Not tested.

in each case maximum proliferation was observed in cultures pulsed from 24 to 42 h (not shown).

The nature of the cells proliferating in response to the viruses Jap_H-Bel_N, X-31, $Mem72_H$-Bel_N, $Shearwater_H$-Bel_N, and PR8 was investigated. The viruses were equally stimulatory for spleen cells from specific pathogen-free mice as for spleen cells from conventionally raised mice (Table 3), indicating that proliferation represents a true mitogenic response of unprimed lymphocytes and not a specific immune response. The viruses were mitogenic for spleen cells from BALB/c nu/nu mice and for normal spleen cells that had been depleted of T cells by treatment with antithymocyte serum in vivo, followed by anti-T cell serum and complement in vitro (1, 8) (Table 3). The lack of functional T cells in these cell preparations was indicated by their complete failure to respond to ConA. These five influenza virus A strains thus all behave as T cell-independent B cell mitogens.

None of the viruses was mitogenic for BALB/c thymocytes. Background proliferation in medium containing goat serum was extremely low (42 cpm), and stimulation indices for the viruses ranged from 0.6 to 1.4 over the entire dose range (1,000 to 1 units of hemagglutination [HAU] per culture). ConA (1 μg per culture), on the other hand, gave a stimulation index of 140.

To test the ability of the viruses to act as mitogens for peripheral T cells, normal spleen cells were depleted of B cells by passage over nylon wool columns (7). This procedure reduced the proportion of surface immunoglobulin-positive cells from 40% in the untreated spleen cell population to 5% after one passage and 1.4% after two passages over nylon wool, as monitored by immunofluorescence with fluoresceinated rabbit anti-mouse immunoglobulin (DAKOPATTS a/s, Denmark).

Compared with untreated spleen cells, nylon wool-passed T cells showed an enhanced responsiveness to ConA (Fig. 1). In contrast, cells obtained after a single passage over nylon wool showed a markedly diminished response to the influenza viruses, and this was reduced still further in cells which had been passed twice over nylon wool. Failure of nylon wool-passed T cells to respond to influenza viruses was not due to depletion of accessory cells, since a similar lack of response was seen whether or not an equal number of irradiated (2,200 R) normal syngeneic spleen cells was included in the cultures. The loss in responsiveness to influenza virus as B cells were depleted closely paralleled the loss in responsiveness to the known B cell mitogen LPS. Thus we obtained no evidence that these influenza viruses are T cell mitogens, although the possibility that a nylon wool-adherent subpopulation of T cells may proliferate has not been excluded.

These findings contrast with the conclusion of Butchko et al. (2) that influenza virus A/Singapore/1/57 (H2N2) is mitogenic for nylon wool-passed T cells as well as for B cells. Their conclusion regarding T cell mitogenicity was based on the observation that spleen cells enriched for T cells by passage over nylon wool remained responsive to influenza virus A/Singapore, giving a stimulation index of 4.4, compared with 6.2 for unseparated spleen cells. The extent of B cell depletion achieved by nylon wool-passage was not monitored by the authors, but we have found that after a single passage of spleen cells over nylon wool, sufficient B cells remained to give a significant response to LPS. Furthermore, comparison of stimulation indices in this situation may be misleading if the background proliferation of nylon-passed cells is markedly lower than that of unseparated spleen cells, as we find to be so, particularly when FCS-containing medium is used. The two cell populations can then give rather similar stimulation indices, when in reality nylon wool passage has led to a marked reduction in overall response to a mitogen.

Mitogenesis induced by Jap_H-Bel_N and X-31 viruses was inhibited in the presence of mouse antiserum to the homologous virus (Table 4). The blocking was specific in that the same concentration (1%) of normal mouse serum or of the heterologous antiserum failed to inhibit (indeed often slightly enhanced) the response to each virus.

The ability of monoclonal antibodies against the HA (H3) glycoprotein to inhibit mitogenesis by X-31 virus was also examined. Ascitic fluids containing monoclonal antibodies H14/A2, H14/A20, and H14/A21 against the HA of X-31 (H3N2) virus were kindly provided by W. Gerhard, The Wistar Institute, Philadelphia, Pa. These antibodies bind to different regions of the HA molecule, selecting variants with substitutions in the hinge (site C), loop (site A), and tip-interface (sites B and D) regions, respectively (12, 17). Additional monoclonal antibodies, 138/1 against the loop and 12/1 against the tip-interface of X-31 HA (16), were the gift of R. G. Webster, St. Jude Children's Research Hospital, Memphis, Tenn. These antibodies all blocked mitogenesis induced by X-31 virus, whereas they had no effect on, or

TABLE 2. Relationship between virus dose and mitogenic response[a]

Virus dose (HAU/culture)	Stimulation index[b]				
	Jap_H-Bel_N	$Shearwater_H$-Bel_N	$Mem72_H$-Bel_N	X-31	PR8
1,000	97.2	82.3	52.4	16.4	3.1
100	94.5	79.6	24.7	8.2	2.2
10	89.5	47.1	4.3	3.3	1.7
1	37.7	5.0	1.8	1.5	1.0
0.1	2.0	1.3	1.0	1.1	1.1

[a] Cultures were harvested after 42 h.

[b] [^{3}H]thymidine incorporation in control cultures was 560 cpm.

TABLE 3. Influenza viruses are B cell mitogens

Mouse spleen cells	Stimulation index							Control values (cpm)[b]
	Virus[a]					Mitogen[a]		
	Jap_H-Bel_N	X-31	$Mem72_H$-Bel_N	$Shearwater_H$-Bel_N	PR8	ConA	LPS	
Conventional	49.1	14.0	21.6	24.4	3.5	39.1	—[c]	503
SPF[d]	48.4	24.9	29.4	—	3.2	42.0	—	400
nu/nu	53.5	7.9	13.5	21.0	4.5	1.2	—	504
Untreated	42.1	8.2	7.9	25.1	—	45.4	16.5	837
T depleted	61.2	8.5	9.4	39.8	—	0.8	18.4	602

[a] Data represent stimulation index in response to 1,000 HAU virus or 1 μg of ConA or 10 μg of LPS per culture.
[b] cpm incorporated by control cultures lacking mitogen.
[c] —, Not tested.
[d] SPF, Specific pathogen-free conditions.

somewhat enhanced, mitogenesis induced by Jap_H-Bel_N (H2N1) virus (Table 4). The inhibitory effect of anti-HA monoclonal antibody on X-31-induced mitogenesis did not extend to a concurrent LPS response occurring in the same wells (data not shown). The mechanism of inhibition thus does not appear to be one involving a general, nonspecific effect of antigen-antibody complexes on B cell proliferation.

The observation that mitogenic potency of influenza virus A strains appears to depend on the viral HA subtype strongly implies that mitogenesis is induced by the virus itself and not by a contaminating B cell mitogen such as LPS or some unidentified component of allantoic fluid. This conclusion is reinforced by the specificity of the blocking of mitogenesis by antibody just described. The basis for the different mitogenic potencies of the different virus subtypes is not known, but it may reflect differences in binding of the viruses to mouse B cells, conceivably to different B cell subsets. Woodruff and Woodruff (18, 19) have shown that rat lymphocytes show considerable heterogeneity in their ability to bind different influenza virus A strains. We are currently investigating whether viruses of the various subtypes show quantitative differences in binding to mouse B lymphocytes which correlate with differences in mitogenicity. Alternatively, a binding difference could be more subtle; viruses of high and low mitogenic potency may show similar levels of binding to B lymphocytes overall, but differ in their ability to bind to a critical membrane component.

The biological relevance of B cell mitogenicity for the immune response to influenza virus in vivo is at present unknown. It will be of particular interest to compare the magnitude of the primary antibody response of mice to viruses of differing mitogenic capacity and the abilities of these viruses to cause polyclonal activation of B cells to immunoglobulin secretion and to act as adjuvants.

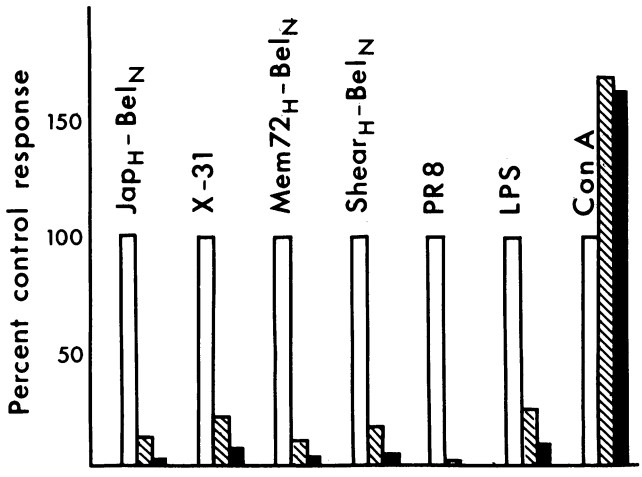

FIG. 1. Proliferative response of nylon wool-passed spleen cells to influenza viruses. Open bars, response of 2×10^5 unseparated spleen cells. Hatched and solid bars, responses of 2×10^5 spleen cells obtained after one or two passages, respectively, through nylon wool columns and cultured in the presence of 2×10^5 irradiated (2,200 R) normal spleen cells. Virus dose, 1,000 HAU per culture. Results are expressed as a percentage of the responses of unseparated spleen cells, which were as follows (net counts per minute): Jap_H-Bel_N, 10,020; X-31, 1,035; $Mem72_H$-Bel_N, 2,518; $Shearwater_H$-Bel_N, 7,700; PR8, 1,025; LPS, 9,156; and ConA, 22,877.

TABLE 4. Inhibition of influenza virus-induced mitogenesis by antiviral sera and by monoclonal antibodies against HA

Antibody[a]	Stimulation index[b]	
	X-31 (H3N2)	Jap_H-Bel_H (H2N1)
None	9.6	82.7
Anti-X-31 serum	2.0	98.9
Anti-Jap_H-Bel_N serum	12.1	0.9
Normal mouse serum	11.7	117.0
None	9.1	83.6
Monoclonal anti-HA (H3)		
H14/A2	2.3	92.0
H14/A20	2.8	169.2
138/1	1.6	101.9
H14/A21	1.4	87.3
12/1	1.6	97.7
Monoclonal anti-rotavirus A3/J7	10.5	59.4

[a] Antibody was added to virus or medium in the wells of a microtiter tray before the addition of 4×10^5 spleen cells. Mouse antisera had HA inhibition titers of 800 by microassay (14). These antisera and normal mouse serum were used at 1/100 final dilution; monoclonal antibodies (ascitic fluids) were used at 1/300 final dilution.
[b] In each experiment, [^{3}H]thymidine incorporation in cultures lacking virus was ca. 200 cpm, both in the presence and absence of added antibody.

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