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Sendai Virus Glycoproteins Are T Cell-Dependent B Cell Mitogens

SHINAE KIZAKA, GAIL GOODMAN-SNITKOFF, AND JAMES J. McSHARRY*

Department of Microbiology and Immunology, Albany Medical College of Union University, Albany, New York 12208

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UV-inactivated Sendai virus is mitogenic for murine splenocytes, whereas infectious Sendai virus kills spleen cells in vitro. The isolated hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins of Sendai virus are also mitogenic for cultured mouse spleen cells. A mixture of these glycoproteins (1 μg/well) gives maximum stimulation 96 h after culture initiation. Viral proteins remaining insoluble after Triton X-100 extraction are also mitogenic for mouse spleen cells, with maximum stimulation occurring at 72 h after culture initiation with 1 to 5 μg/well. On the basis of protein concentration, the HN and F glycoproteins are approximately three times more mitogenic than the Triton X-100-insoluble material. The mitogenic response of the HN and F glycoproteins has two components, a T cell-independent B cell proliferation, which is less than one-half of the total stimulation observed, and a T cell-dependent B cell proliferation. In contrast, the Triton X-100-insoluble material is a T cell-dependent B cell mitogen. Purified T lymphocytes do not respond to the mitogenic signal of either HN-F or Triton X-100-insoluble material.

Sendai virus, a member of the paramyxovirus family, is ideally suited for the study of host responses since it causes an influenza-like upper respiratory tract infection in mice which requires an intact cell-mediated and humoral immune system for recovery (1, 2, 23, 24). When immunosuppressive agents such as cyclophosphamide are administered to mice, the viral infection is converted from a mild, self-limiting disease to a severe and often fatal infection (24). Additionally, thymectomy and irradiation of mice, followed by challenge with Sendai virus, resulted in a disease of intermediate severity, although antibody titers to the viral hemagglutinin were intact (2). The results of these experiments indicated the need for both humoral and cell-mediated immunity in recovery from Sendai virus infection in mice.

Further study and definition of the role of cell-mediated immunity to virus infection have been pursued by a number of groups interested in cell-mediated cytotoxicity. In most cases, cytotoxic T lymphocytes (CTL) are not elicited when noninfectious virus is given to mice (4, 28). However, when inactivated virus retains the ability to fuse with host cells, CTL are elicited, suggesting that the incorporation of viral proteins into the plasma membrane is required for the development of CTL (14, 21, 27). Sendai virus mediates fusion via its two envelope glycoproteins, the hemagglutinin-neuraminidase (HN) protein and the fusion protein (F). When the F protein is active, CTL are readily induced (14), whereas when the F protein is inactive (Fo), the virus is unable to induce CTL in the host animal (7). Furthermore, HN and F can be incorporated into liposomes to stimulate both primary and secondary CTL (11, 16), whereas liposomes containing HN and Fo are not able to stimulate CTL (16). In summary, the requirements for activation of CTL have been described and include the presentation of HN and the active form of F in a phospholipid environment, either in the virus or in a liposome, to the host animal or isolated lymphocytes (11, 16).

Our interest in viral subunit vaccines has led us to study in vitro lymphocyte activation as a possible model system for in vivo immunogenicity and as a rapid screening technique for immunogenic peptides. Previous work by us and others has shown that such diverse viruses as herpes simplex virus types 1 and 2, influenza virus, vesicular stomatitis virus (VSV), and Sindbis virus act as lymphocyte mitogens (6, 9, 10, 13, 20). In addition, viral glycoproteins, including the G protein from VSV, the hemagglutinins of influenza viruses, the two envelope proteins of Sindbis virus, and gp70 from Rauscher murine leukemia virus are also mitogenic for mouse lymphocytes (3, 5, 8, 10, 18). Since it has been shown that the proper configuration of purified Sendai virus glycoproteins is

able to stimulate the production of CTL and anti-HN antibodies in vivo (16), we attempted to determine whether Sendai virus would activate lymphocytes in vitro. This paper reports that UV-inactivated Sendai virus and its isolated glycoproteins are mitogenic for mouse B lymphocytes and that this activation is modulated by T cells.

MATERIALS AND METHODS

Mice. BALB/c normal, nu/nu, and nu/+ female mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass., and were maintained in a sterile laminar flow hood on mouse chow and sterile water ad libitum. BALB/c females were used at 8 to 16 weeks of age, and the nu/nu and nu/+ mice were used at 6 to 8 weeks of age.

Lymphocyte cultures and mitogenic assays. Lymphocytes were cultured as described previously (8, 9). Briefly, aseptically removed spleen cells were teased into single cell suspensions in RPMI 1640 medium supplemented with 100 U of penicillin per ml and 100 μg of streptomycin per ml and washed twice in RPMI 1640 medium. The cells were resuspended, tested for viability, and diluted to 4×10^6 viable cells per ml. The cell suspension (0.1 ml/well) was dispensed in microtiter plates (Microtest II with lid; Falcon Plastics, Oxnard, Calif.), and an equal volume of mitogen or medium was added to each well. The cultures were incubated in an atmosphere of 10% CO_2 in humidified air at 37°C for various amounts of time. Twenty-four hours before harvest, 1 μCi of [^{3}H]thymidine (42 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added to each culture. At the end of the culture period, cells were harvested onto glass fiber filters with a multiple automated sample harvester (Brandel, Gaithersburg, Md.), and [^{3}H]thymidine incorporation was determined by liquid scintillation counting.

Nylon wool separation of spleen cells. Purified T lymphocytes were recovered from nylon wool by the method of Hodes et al. (12). Briefly, 1.2-g sterile nylon wool columns were incubated at 37°C for 1 h with RPMI 1640 medium plus 10% fetal bovine serum (FBS) in a humidified atmosphere. After this preincubation, 300×10^6 cells in 4 ml of RPMI-FBS were added to the column and allowed to flow into the nylon wool; at this time, an additional 2 ml of RPMI-FBS was added to the column. The column and cells were incubated for 45 min at 37°C in humidified air and 10% CO_2. After incubation, nonadherent cells (T enriched) were removed by adding RPMI-FBS to the column and collecting 15 ml of effluent (flow rate, 1 ml/2 min). The column was rapidly washed with 100 ml of RPMI-FBS, and this wash was discarded. Adherent cells (B enriched) were removed by compressing the nylon wool with the syringe plunger to express remaining medium. The nylon wool was removed and teased with sterile forceps, resaturated with RPMI-FBS, and compressed again; a total of 50 ml was collected in four to five cycles of compression.

Mitomycin C treatment of lymphocytes. Lymphocytes to be treated with mitomycin C were suspended at a concentration of 1×10^7 to 2×10^7 cells per ml in balanced salt solution (BSS). Mitomycin C (Sigma Chemical Co., St. Louis, Mo.) was dissolved at 1 mg/ml in BSS and filter sterilized; 25 μl of mitomycin C solution was added per ml of cells. The mitomycin C-treated cells or cells with medium were then incubated at 37°C for 30 min, followed by three BSS washes. The cells were counted and resuspended to a final concentration of 4×10^6 cells per ml; after treatment, about 50% recovery was obtained, and 99% of recovered cells were viable.

Immunofluorescence. Mouse splenocytes were cultured in plastic tissue culture tubes (16 by 75 mm; Falcon) at a concentration of 10^6 cells per ml. At culture initiation, 0.1 ml of mitogen or medium was added to each tube, and the cells were cultured at 37°C in a humidified atmosphere of 10% CO_2 for 72 h. At this time, the cells were labeled with fluoresceinated goat anti-mouse immunoglobulin G F(ab')2 fragments (Cappel Laboratories, Cochranville, Pa.) as follows. Five tubes of cells were pooled, centrifuged, washed in phosphate-buffered saline (PBS) with 10 mM sodium azide in PBS (PBSA), and resuspended in 0.1 ml of PBSA. Fluoresceinated antibody (10 μg of antibody protein in 0.1 ml) was added to 5×10^6 cells, and the mixture was incubated on ice for 30 min. The cells were diluted in 5 ml of 0.5% bovine serum albumin (BSA) in PBSA and centrifuged through 0.5 ml of 5% BSA; the cells were washed an additional three times in this manner. The cell pellet was resuspended in 0.1 ml of FBS, streaked onto glass slides, stained with Wright-Giemsa stain, counted on an Olympus Vanox fluorescent microscope, and scored for blastogenesis and fluorescence.

Mitogens. *Salmonella typhosa* lipopolysaccharide endotoxin (LPS) was prepared by the phenol-water method of Westphal et al. (26) and contained less than 1% protein. Concanavalin A (ConA) was purchased from Sigma Chemical Co.

Virus propagation. Sendai virus was grown from stock virus kindly donated by R. Mannino, Albany Medical College, Albany, N.Y. Briefly, 0.1 ml of Sendai virus (10^5 hemagglutinating units per ml) was inoculated onto the allantoic membrane of 11-day-old embryonated eggs. The infected eggs were incubated at 37°C for 48 to 72 h and then transferred to 4°C for 24 h. The allantoic fluid was removed by pipette and clarified by centrifugation at 2,000 rpm for 20 min at 4°C. Virus was recovered from the supernatant by centrifugation at 23,000 rpm in an SW27 rotor (Beckman Instruments, Palo Alto, Calif.) for 60 min at 4°C. The virus pellet was resuspended in PBS, layered onto 15 to 35% potassium tartrate gradients, and spun at 23,000 rpm in an SW27 rotor for 16 h at 4°C. The virus band was collected from the gradients and diluted, and the virus was recovered by pelleting at 30,000 rpm in an SW40 rotor (Beckman) for 60 min at 4°C. The virus preparations were resuspended in PBS and assayed for protein, hemagglutination of chicken erythrocytes, and purity by Coomassie blue-stained polyacrylamide gel electrophoresis.

Preparation of glycoproteins and Triton pellet (TP). The procedure for isolating the glycoproteins from Sendai virus was essentially that which has been previously described for VSV (17). Briefly, purified Sendai virus (approximately 1 mg/ml) was diluted 1:5 in 2% (vol/vol) Triton X-100 (Sigma) in 0.01 M Tris buffer (pH 8.0). The solution was mixed gently at room temperature for 45 min. Insoluble material was removed by centrifugation at 30,000 rpm for 1 h at 4°C in

an SW50.1 rotor (Beckman). The supernatant was decanted into 10 volumes of ice-cold butanol, and glycoproteins were precipitated on ice for 0.5 h and recovered by centrifugation at 1,000 rpm for 15 min at 4°C. The pelleted glycoproteins (HN and F) were resuspended in ice-cold acetone, repelleted, dried under nitrogen, and resuspended in PBS by sonification twice for 15 s each time at 30 W on a Heat Systems sonifier. The Triton-insoluble material was washed repeatedly in 5 to 10 ml of PBS and pelleted by centrifugation at 30,000 rpm for 1 h at 4°C in an SW50.1 rotor. Purity of preparations was determined by Coomassie blue-stained polyacrylamide gel electrophoresis. Preparations containing only Sendai virus proteins were used for these assays.

Protein determination. Protein was determined by the method of Lowry et al. (15).

RESULTS

Mitogenic activity of Sendai virus. Previous studies have shown that a number of infectious viruses are mitogenic for murine spleen cells (6, 9, 10, 13, 20). To study the mitogenic potential of Sendai virus, various concentrations of virus ranging from 0.001 to 2.5 μg/well were incubated with mouse splenocytes for 48, 72, 96, and 120 h and then assayed for mitogenesis. Concentrations of virus greater than 0.1 μg/well resulted in [^{3}H]thymidine incorporation at levels below background, and lower concentrations of virus did not result in significant stimulation (data not shown). The results of this experiment suggest that Sendai virus is inhibiting the transport of [^{3}H]thymidine into lymphocytes, inhibiting DNA synthesis, or destroying the cells. To determine the effect of Sendai virus on the viability of lymphocytes, infectious virus (0.5 μg/ml) was added to splenocytes, and the number of viable cells, as well as the total number of cells, was measured after incubation for 24, 48, 72, or 96 h. The data in Table 1 show that, in the presence of 0.5 μg of Sendai virus per ml, the number of viable cells as compared to control cultures was decreased by 30% at 24 h and by 70% at 48, 72, or 96 h. The total number of cells was also significantly decreased as compared to the uninfected cultures. The results of this experiment show that infectious Sendai virus kills lymphocytes and provide a reasonable explanation for the failure of infectious Sendai virus to induce mitogenesis in murine splenocytes. In contrast, treatment of murine lymphocytes with UV-inactivated Sendai virus (0.5 μg/ml) did not lead to a decrease in the number of viable cells or in the number of total cells as compared to controls (Table 1). At all time points, there was a slight increase in the number of viable and total cells, suggesting that UV-inactivated Sendai virus may stimulate cell division and may be a more useful mitogen than infectious virus.

TABLE 1. Viability of lymphocytes cultured in the presence of Sendai virus

Sendai virus added	No. of viable cells × 10^5 (total no. of cells recovered × 10^5)[a]			
	24 h	48 h	72 h	96 h
None	6.0 (10.2)	3.6 (5.2)	3.2 (4.8)	1.3 (4.0)
0.5 μg/ml	4.0 (6.6)	1.2 (1.8)	1.6 (2.6)	0.6 (1.8)
0.5 μg of UV-irradiated virus per ml[b]	7.6 (11.6)	4.4 (5.2)	3.4 (5.6)	3.1 (4.6)

[a] BALB/c spleen cells (2 × 10^6) were added to each tube. Results are the arithmetic means of four tubes at each time point.

[b] Sendai virus was UV inactivated under a General Electric germicidal lamp at 23 cm for 1.5 h. The energy emitted at this distance was 120 μW/cm^2.

Mitogenic activity of UV-inactivated Sendai virus. The ability of UV-inactivated Sendai virus to stimulate mitogenesis was tested in mouse splenocytes. The data presented in Table 2 are representative of at least three experiments and show that UV-inactivated Sendai virus stimulated mitogenesis in murine lymphocytes. Maximum stimulation occurred with 10 μg of UV-inactivated Sendai virus per well at all times tested and decreased with higher concentrations of virus. The optimum time for mitogenesis was 72 h after culture initiation. At all time points examined, lymphocyte activation by UV-inactivated Sendai virus was less than that observed with LPS or ConA, a result consistent with those in other viral mitogen systems (6, 9, 10, 13, 20). These results show that, although infectious Sendai virus is not mitogenic for mouse spleen cells, noninfectious, UV-inactivated Sendai virus can act as a mitogen for splenic lymphocytes, with maximal stimulation occurring at 72 h after culture initiation.

Mitogenic activity of the isolated HN and F glycoproteins and TP of Sendai virus. Since it has been shown that isolated glycoproteins from VSV, Sindbis virus, Rauscher murine leukemia virus, and influenza viruses are mitogenic for mouse lymphocytes (3, 5, 8, 10, 18), we examined the ability of the isolated Sendai virus glycoproteins (HN and F) and the proteins associated with the TP to stimulate mitogenesis in lymphocytes. The results in Table 3 are representative of data from at least three experiments and show that both the isolated HN-F and the proteins remaining in the TP are mitogenic for mouse spleen cells. At 96 h after culture initiation, 1 μg of the HN-F complex per well stimulated maximum incorporation of [^{3}H]thymidine into mouse lymphocytes. At this time, both 1.0 and 5.0 μg of the TP per well was significantly stimulatory for murine lymphocytes. At a concentration of 1.0 μg/well, the glycoproteins are approximately three times more stimulatory than the TP, a finding consistent with those of

TABLE 2. Stimulation of BALB/c lymphocytes by UV-inactivated Sendai virus

Mitogen	Amt of mitogen (μg/well)	cpm [^{3}H]thymidine incorporated ± SE[a]			
		48 h	72 h	96 h	120 h
None		2,044 ± 161	1,228 ± 126	551 ± 20	504 ± 70
LPS	25	40,926 ± 3,469	43,709 ± 2,465	27,266 ± 1,022	12,646 ± 134
ConA	0.125	96,601 ± 2,605	176,208 ± 6,645	127,562 ± 13,929	61,991 ± 4,519
UV-inactivated	1	2,685 ± 215	1,346 ± 232	287 ± 50	242 ± 94
Sendai virus[b]	5	3,085 ± 46	2,530 ± 750	1,502 ± 21	931 ± 29
	10	3,817 ± 244	8,751 ± 815	4,539 ± 383	1,440 ± 130
	50	ND[c]	5,469 ± 120	1,937 ± 152	904 ± 58
	100	ND	2,570 ± 185	1,138 ± 33	364 ± 93

[a] SE, Standard error of the mean of counts derived from three replicate wells.
[b] UV-inactivated Sendai virus was prepared as described in footnote *b* of Table 1.
[c] ND, Not done.

other isolated viral glycoproteins and TPs (3, 5, 8, 10, 18).

To determine the kinetics of the response to the mitogenic signal for these isolated proteins, optimal concentrations of HN-F (1 μg/well) and TP (5 μg/well) were incubated with mouse splenocytes for 48, 72, 96, and 120 h. The data presented in Fig. 1, which are representative of more than one experiment, show that the maximal mitogenic response of the glycoprotein preparation (HN-F) occurs at 96 h and declines to background at 120 h after culture initiation. The maximal mitogenic response of the TP occurs at 72 h and declines at 96 and 120 h after initiation of culture (Fig. 1). These results suggest slightly different responses of lymphocytes to these mitogens and may suggest that the HN-F and TP are interacting with different cell populations.

TABLE 3. Dose response of lymphocyte activation by isolated viral proteins

Mitogen	Amt of mitogen (μg/well)	[^{3}H]thymidine incorporated ± SE[a] at 96h	SI[b]
None		1,918 ± 12	–
LPS	25	38,015 ± 3,230	19.8
ConA	0.125	197,455 ± 11,073	102.9
HN-F	0.1	13,512 ± 2,081	7.0
	0.5	18,714 ± 870	9.8
	1.0	27,527 ± 1,310	14.4
	5.0	13,773 ± 2,777	7.2
TP	0.1	6,866 ± 478	3.6
	0.5	5,846 ± 837	3.0
	1.0	10,749 ± 1,026	5.6
	5.0	11,608 ± 1,997	6.0

[a] SE, Standard error of the mean of counts derived from three replicate wells.
[b] SI, Stimulation index = cpm experimental/cpm control.

Mitogenic activity of the HN-F glycoproteins and TP in separated B and T cell populations. Previous work with viral mitogens has demonstrated that, whereas most are B cell activators (9, 10, 13, 20), influenza virus and Rauscher murine leukemia virus can stimulate both B and T cells (5, 6). To determine which cell populations responded to Sendai virus proteins, mouse splenocytes were separated into B cell- and T cell-enriched populations, incubated with optimal concentrations of mitogens, and then assayed for incorporation of [^{3}H]thymidine into lymphocytes at 72 and 96 h. As shown in Fig. 2,

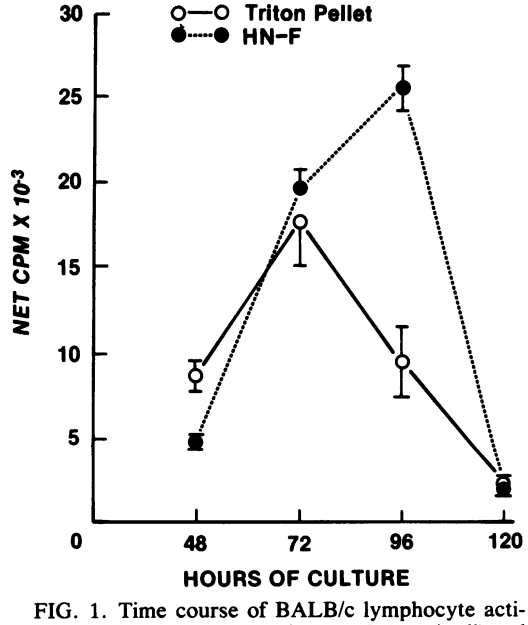

FIG. 1. Time course of BALB/c lymphocyte activation by isolated glycoproteins HN-F (1 μg/well) and the TP (5 μg/well) of Sendai virus. Bars represent standard deviation from mean of counts from three replicate wells.

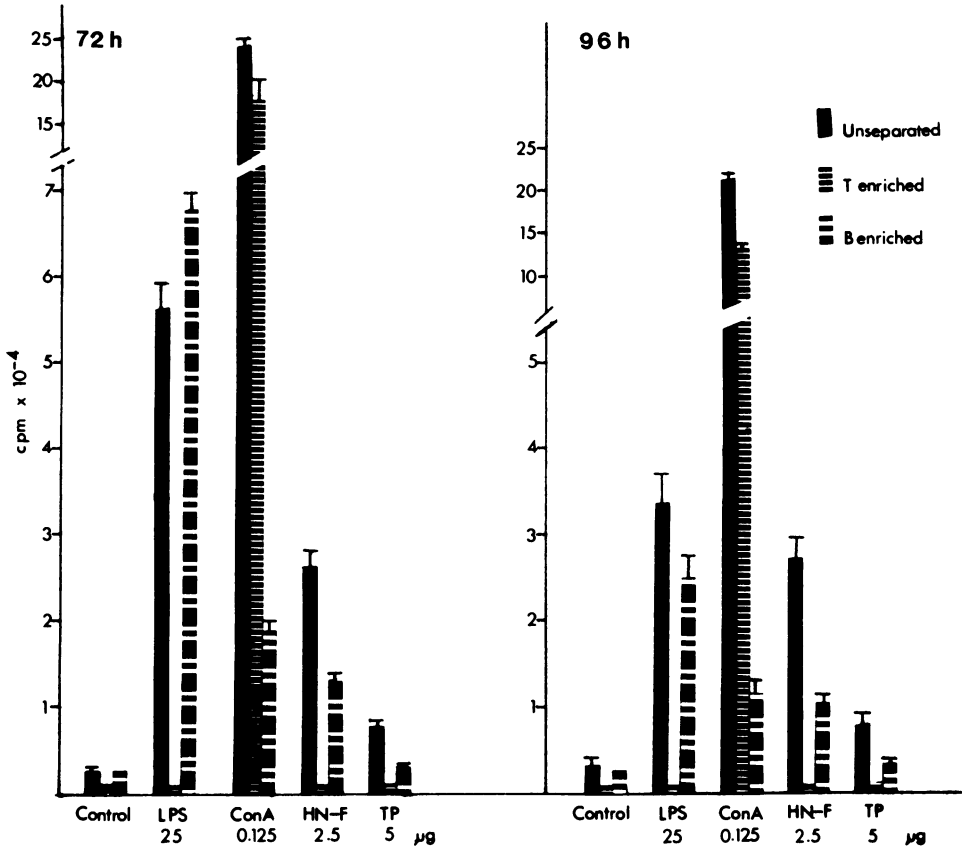

FIG. 2. Activation of separated cell populations at 72 and 96 h by LPS, ConA, isolated glycoproteins (HN-F), and TP. Bars represent standard deviation from mean of counts from three replicate wells.

which is representative of a number of experiments, when the abilities of adherent and nonadherent cells to respond to HN-F or TP were assayed and compared with those of unseparated cells, it was observed that T-enriched cells were not activated by either HN-F or TP, suggesting that they are not T cell mitogens. The B cell-enriched population responded to both the HN-F and TP preparations, but the response of the B-enriched cells to these mitogens was only about one-half of that observed with unseparated cells. Since in this system LPS, a T cell-independent B cell mitogen, stimulates B cells as well as unseparated cells, and ConA, a T cell mitogen, stimulates T cells as well as unseparated cells, the data showing that the response of B cells to the Sendai virus mitogens, HN-F and TP, was only about one-half that of unseparated cells suggest that T cell-B cell cooperation is required for good stimulation by these mitogens. Thus, Sendai virus proteins are B cell mitogens which seem to require some T cell help. The data in Fig. 2 also show that the nonadherent (T cell-enriched) population responded well to ConA at 72 and 96 h after culture initiation, but did not respond to LPS, indicating that the T cell-enriched population lacked B cells. The adherent (B cell-enriched) population was highly responsive to LPS at both 72 and 96 h after culture initiation, with the 72-h response approximately three times greater than the 96-h response, which is typical of B cells. The responsiveness of the adherent (B cell-enriched) population to ConA indicates the presence of residual T cells. These results show that the separation technique used yielded an essentially pure T cell population and an enriched B cell population.

In addition to the cell separation experiment, the ability of cells from the congenitally athymic BALB/c nu/nu mouse to respond to isolated viral proteins (HN-F and TP) was also examined. Figure 3, which is representative of three experiments, shows that when nude mice were compared to their nu/+ littermates, the response of the T cell-deficient mice to HN-F was less

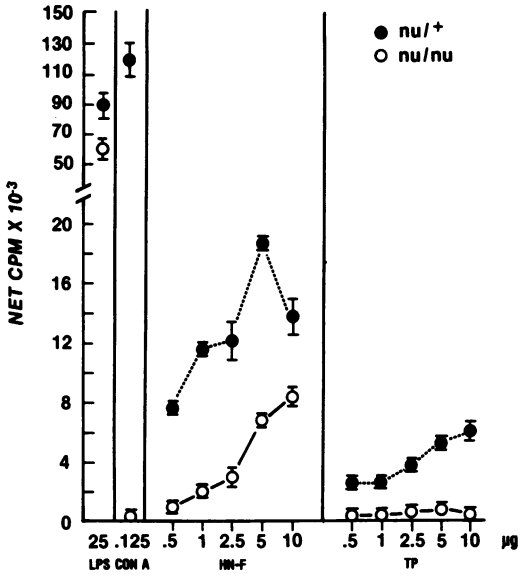

FIG. 3. Activation of BALB/c nu/+ and nu/nu spleen cells by LPS, ConA, isolated glycoproteins (HN-F), and TP at 72 h. Bars represent standard deviation from mean of counts from three replicate wells.

than one-half of that observed with normal mouse spleen cells. At 72 h and 5 μg/well, the net counts per minute of [^{3}H]thymidine incorporated into nu/nu spleen cells in response to HN-F was 38% of that incorporated into nu/+ spleen cells, suggesting that this portion of the HN-F glycoproteins is a T cell-independent B cell mitogen. The TP does not stimulate lymphocytes from BALB/c nu/nu mice, suggesting that the components of TP are not T cell-independent B cell mitogens. Similar results were obtained at 96 h (results not shown). As expected, lymphocytes from nude mice were unresponsive to ConA, showing a functional lack of T cells, but were fully responsive to LPS (Fig. 3).

Requirement of helper cells for Sendai virus protein mitogenesis. The inability of B lymphocytes either enriched over nylon wool or from a T cell-deficient mouse to respond to HN-F or TP with values equal to those of unseparated cells, coupled with the total lack of response from enriched T cells to the virus proteins, suggested that optimal stimulation of mouse splenocytes might require cooperation between B and T cells. This hypothesis was first tested by mixing separated, enriched B and T lymphocyte populations and determining whether they would respond to stimulation by HN-F or TP. Optimal stimulation was obtained with 5 μg of the preparation of HN-F per well; thus, this concentration was used for this experiment. As shown in Table 4, which is representative of more than one experiment, at 72 h the response of unseparated cells to HN-F and TP was 40,000 to 50,000 cpm. Similar to the results shown in Fig. 2 and 3, the response of enriched B cells to HN-F and TP was less than one-half that of control cells, and enriched T cells were unresponsive to the viral proteins. However, when the cells were mixed together and cultured with HN-F or TP, the measured stimulation was equal to that seen in unseparated cells. Since optimal mitogenicity is seen only when B and T cells are cultured together and the total counts per minute of [^{3}H]thymidine incorporated is greater than that which would be observed by adding the counts per minute of [^{3}H]thymidine incorporated by separated B cells and T cells, it is clear that a helper cell was involved in the observed proliferative response.

Sendai virus proteins require T cell help for B cell mitogenesis. The need for helper cell activity

TABLE 4. Activation of mixed T and B cell populations by isolated virus proteins

Cell population[a]	cpm [^{3}H]thymidine incorporated ± SE[b]				
	None	LPS (25)	ConA (0.125)	HN-F (5)	TP (5)
Unseparated	1,599 ±166	37,263 ±2,122	125,358 ±2,059	49,126 ±1,101	41,082 ±2,201
B enriched	1,503 ±505	30,042 ±1,941	6,642 ±296	20,497 ±1,019	14,109 ±1,388
T enriched	705 ±58	1,853 ±539	111,971 ±1,469	1,604 ±164	1,213 ±120
B + T	2,971 ±264	36,201 ±1,372	132,965 ±4,510	48,914 ±2,603	49,455 ±3,648

[a] Each cell population had a total of 4.0×10^5 cells per well. B + T cell mixtures consisted of 2×10^5 B cells and 2×10^5 T cells.

[b] SE, Standard error of the mean of counts derived from three replicate wells. Numbers in parentheses after various mitogens are micrograms of mitogen per well.

TABLE 5. Lymphocyte activation in mitomycin C-treated cell populations

Cells[a]	cpm [^{3}H]thymidine incorporated ± SE[b]				
	None	HN-F (10 μg)	TP (25 μg)	ConA (0.125 μg)	LPS (10 μg)
Unseparated	1,320 ± 123	8,870 ± 51	7,614 ± 369	236,068 ± 6,719	56,265 ± 2,108
B	1,413 ± 138	4,051 ± 252	2,905 ± 163	1,930 ± 60	61,377 ± 2,966
T	152 ± 18	137 ± 13	13 ± 2	288,475 ± 17,795	181 ± 13
B + T	677 ± 67	9,433 ± 567	4,136 ± 327	257,534 ± 28,880	72,558 ± 4,289
B_{mc}	377 ± 118	455 ± 192	232 ± 65	713 ± 71	2,234 ± 81
T_{mc}	187 ± 39	223 ± 3	182 ± 30	1,002 ± 8	134 ± 38
B_{mc} + T	388 ± 72	1,549 ± 264	945 ± 114	206,259 ± 5,419	2,373 ± 185
B + T_{mc}	1,448 ± 15	16,844 ± 449	5,870 ± 689	3,846 ± 715	89,955 ± 94

[a] Total number of cells was 4.0×10^5 cells per well. When cells were mixed, equal numbers of B and T cells were added to give a total of 4.0×10^5 cells per well. MC, Mitomycin treated.

[b] SE, Standard error of the mean of counts derived from three replicate wells.

in response to stimulation by proteins left unresolved the question of whether it was a helper B cell or T cell. To answer this question, lymphocytes were separated, mock treated or treated with mitomycin C to inhibit proliferation, mixed, cultured, and assayed for responsiveness to HN-F and TP. The results at 72 h after culture initiation are reported in Table 5 and are representative of three experiments. The HN-F and TP mitogen preparations used in this experiment and that shown in Table 6 gave optimal stimulation at 10 and 25 μg/well, respectively. B lymphocytes responded to HN-F and TP only one-half as well as unseparated cells, and T cells were unresponsive. Mixtures of B and T cells gave a response to HN-F equal to that of unseparated control cells, whereas the response of B and T cell mixtures to TP was about half that of unseparated cells. As expected, the B cell population was activated by LPS and the T cell population by ConA. After mitomycin C treatment, B cells and T cells were unresponsive to mitogenic stimulation by LPS, ConA, HN-F, or TP. The cell mixture consisting of mitomycin-treated B + T was activated by ConA, but not LPS, HN-F, or TP. In contrast, that consisting of B + mitomycin-treated T cells was highly responsive to LPS and, in culture with HN-F or TP, incorporated as much as or more [^{3}H]thymidine than did unseparated or B + T reconstituted cells. These results were strongly suggestive of a T helper cell.

TABLE 6. Blastogenesis by Sendai virus proteins

Mitogen	Amt of mitogen (μg/well)	% of cells			
		Small lymphocytes		Blast cells	
		sIg−	sIg+	sIg−	sIg+
None		34	59	2.5	4.5
LPS	10	26	42	8	24
ConA	0.125	18	45	31	6
HN-F	10	29	42	6	22
TP	25	33	46	4	17

To confirm this finding of T cell help, 5×10^6 cells at 1×10^6 cells per ml were cultured in tubes and stained, first with fluoresceinated goat anti-mouse immunoglobulin G (F(ab')2 fraction for surface antibodies and then with Wright-Giemsa stain. These cells were counted and scored for both blastogenesis and immunofluorescence. A total of 400 cells per group was counted (Table 6). After 72 h of culture, the number of surface immunoglobulin-positive (sIg+) blasts was increased more than five times in the presence of LPS, whereas ConA stimulated sIg− blast cells more than 10-fold. Culture with HN-F resulted in a fivefold increase in sIg+ blast cells, whereas culture with TP caused a fourfold increase in sIg+ blast cells. Both HN-F and TP caused a small increase in the number of sIg− lymphoblasts, indicating that the cells which proliferated in response to the viral proteins were B cells, but this stimulation was enhanced by the presence of T lymphocytes. Thus, optimal mitogenesis of B lymphocytes by HN-F or TP required a helper T cell.

DISCUSSION

The results presented here demonstrate that, although infectious Sendai virus kills mouse lymphocytes, UV-inactivated virus is mitogenic for these cells. Furthermore, the Triton X-100-extracted and butanol-precipitated HN-F proteins are stimulatory for these cells, as is the TP of Sendai virus. The mitogenicity of HN-F appears to have two components, a T cell-independent B cell mitogen function and a T cell-dependent B cell mitogen function. The TP appears to be a T cell-dependent B cell mitogen. Separation of the HN and F proteins will be necessary to determine whether the two mitogenic activities reside in different proteins.

Our report that Sendai virus was not mitogenic for mouse lymphocytes contrasts greatly to

results from virus systems, including herpes simplex virus types 1 and 2 (13, 20), influenza virus (6), VSV (9), and Sindbis virus (10), which do not kill lymphocytes when incubated in a mitogenesis assay. To understand this difference, we examined the viability of lymphocytes cultured in the presence of Sendai virus and found that it was greatly decreased at all time points tested. At the same time, we found that not only was UV-inactivated Sendai virus not toxic for lymphocytes, but it actually increased the number of viable cells when compared to medium controls and stimulated the mitogenicity as measured by [^{3}H]thymidine incorporation. Previously, two laboratories have investigated viral inhibition of mitogenesis by using both mouse and human lymphocytes (22, 25). It was demonstrated that Sendai virus could inhibit a C3H and BALB/c mixed lymphocyte reaction by almost 80% (25). In addition, pretreatment of either lymphocytes or lymphocytes and macrophages with Sendai virus could reduce the response of human peripheral blood lymphocytes to phytohemagglutinin by more than 60% (22). In neither case did the authors test the viability of the lymphocytes cultured in the presence of Sendai virus. We believe that previous reports of Sendai virus inhibition of lymphocyte activation and its lack of mitogenicity were due to cell death in the presence of Sendai virus.

Since inactivated Sendai virus was mitogenic for murine cells, we isolated the glycoproteins (HN and F) by Triton X-100 extraction and tested the ability of HN-F and TP to act as lymphocyte mitogens. The data presented in this paper show that both HN-F and TP are mitogenic for mouse lymphocytes. The response of whole spleen cells to HN-F was similar to that observed by us with VSV G protein (8, 18) and Sindbis virus envelope glycoproteins (10) and by others with the hemagglutinin of influenza viruses (3) and gp70 of Rauscher murine leukemia virus (5). Additionally, the TP was as stimulatory for lymphocytes as the HN-F glycoproteins. This contrasts with previous observations with Sindbis virus and VSV, in which the TP was only about one-fifth as active as the envelope glycoproteins (8, 10). The reason for these differences is not understood at this time.

It is of considerable interest that HN-F are mitogenic for murine splenocytes, since it has been shown that intraperitoneal immunization of mice with Sendai virus results in the ability of spleen cells from immunized mice to respond to both UV-inactivated Sendai virus and cyanogen bromide peptide fragments with proliferation (19). In contrast to the results given here, in which the B cell proliferates with T cell help, in the immunized spleen the proliferation was due exclusively to T cells (19). Thus, the response of nonimmunized cells is different from that of lymphocytes previously immunized to Sendai virus.

The VSV G protein and Sindbis virus glycoproteins are T cell-independent B cell mitogens, whereas the Rauscher murine leukemia virus gp70 stimulated both B and T lymphocytes (5, 8, 10, 18). It is of interest, therefore, that HN-F and TP require a helper cell for expression of optimal mitogenicity. The isolated viral proteins stimulate B lymphocytes to incorporate DNA synthesis at levels less than one-half that of control cells, and the Sendai virus proteins do not activate T cells at all. However, in mixing experiments with B and T cells, activation equal to that of control cell populations was observed. When mitomycin C-treated cell populations were used in mixing experiments, if untreated B cells were mixed with treated T cells, HN-F and TP stimulated levels of [^{3}H]thymidine incorporation equal to that of unseparated cells, but if treated B cells were mixed with untreated T cells, no lymphocyte stimulation was observed by culture with HN-F and TP. Furthermore, most of the blast cells stimulated by HN-F and TP were sIg+ cells or B cells. We conclude that, although a portion of the HN-F stimulation is T cell-independent B cell activation, a larger portion is T cell-dependent B cell activation. Although this type of activation is common for specific immune responses and some mitogens, such as pokeweed mitogen, to our knowledge it is the first time it has been observed with a viral mitogen.

Our long-term goal has been to find an in vitro assay which would correlate with in vivo immunogenicity. Sendai virus has been well studied with respect to its in vivo immunogenicity. It has been shown that antibodies can be elicited by purified Sendai virus HN and F proteins, but the production of CTL requires that the HN and F proteins be present together in a membrane (16). The presence of HN and F in the liposome can stimulate primary and secondary CTL, as well as antibodies which inhibit hemagglutination (11, 16). In vitro, a Triton X-100 isolate of HN-F can stimulate both B and T cells, B cells to proliferation and T cells as helper cells. Since the elucidation of the immune response to Sendai virus is under way, combined with our in vitro assay, it is possible that Sendai virus might be the prototype for studies designed to correlate our in vitro assay with in vivo immunogenicity.

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