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Mitogenic Activity of Sindbis Virus and Its Isolated Glycoproteins

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Purified preparations of Sindbis virus, a member of the togavirus family, are mitogenic for lymphocytes from a number of different mouse strains. Cell separation techniques, as well as studies using lymphocytes from the congenitally athymic BALB/c nu/nu mouse, showed that Sindbis virus is a T-cell-independent B-cell mitogen. Additionally, the envelope glycoproteins of Sindbis virus, isolated by Triton X-100 extraction and butanol precipitation, stimulated lymphocytes to incorporate five times as much [^{3}H]thymidine into their DNA as did the Sindbis virion. These results are similar to those previously reported for vesicular stomatitis virus and herpes simplex virus types I and II and for the purified glycoproteins of vesicular stomatitis virus and influenza virus.

Although viral infection has been recognized to be a nonspecific modulator of the host's immune response (27), until recently the response of lymphocytes to viruses has been studied as a specific interaction involving the production of host protective antibodies, cytolytic T cells, or both. However, in the last 5 years, a number of groups have reported that viruses as diverse as influenza virus, herpes simplex virus (HSV) types I and II, and vesicular stomatitis virus (VSV) are able to induce nonspecific lymphocyte activation, as measured by mitogenesis (5, 9, 14, 20). Both types of HSV and VSV are exclusively B-lymphocyte mitogens, while influenza virus serotype H2N2 stimulates both B- and T-cell activation. Additionally, viral infectivity is not required for stimulation of lymphocytes by either influenza virus or VSV (5, 9), a result which suggested that the mitogenic potential of the intact virion resides in a viral component. Mitogenic activity was demonstrated with the isolated glycoprotein of VSV (8, 19) and with the hemagglutinin of influenza viruses (1). It was also observed that the isolated envelope glycoproteins from these viruses are much more stimulatory for lymphocytes than is the intact virion (1, 8, 19).

Mitogens are not only important tools for the study of lymphocyte activation, but also, because of their intimate relationship with the cell surface, they may be important as modulators of the immune response. We have, therefore, continued our study of viral mitogens for lymphocytes by extending this study to Sindbis virus, a member of the togavirus family. Sindbis virus is an enveloped RNA virus consisting of two surface glycoproteins, a lipid bilayer, an internal non-glycosylated core protein, and a single segment of positive-stranded RNA (24). Large quantities of purified Sindbis virus can be obtained readily, and purified viral proteins and peptides can be isolated from the virus for studying their interactions with lymphocytes. This paper reports that Sindbis virus is mitogenic for lymphocytes from several mouse strains and is a T-cell-independent B-cell mitogen as determined in BALB/c nu/nu mice. Furthermore, data are presented which demonstrate that the glycoproteins of Sindbis virus are more stimulatory for mouse splenocytes than is the intact virion.

MATERIALS AND METHODS

Mice. C3H/HeJ and CBA/J mice were purchased from Jackson Laboratories (Bar Harbor, Maine), and Swiss Webster mice were purchased from Taconic Farms (Germantown, N.Y.). These animals were maintained in our facility on Purina mouse chow and water ad lib. Female mice between 8 and 16 weeks of age were used in all experiments. BALB/c 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 lib. These mice were used at 7 to 9 weeks of age.

Lymphocyte cultures and mitogenic assays. Lymphocytes were cultured as previously described (7). Briefly, aseptically removed spleen cells were teased into single-cell suspensions in RPMI 1640 medium supplemented with 100 U of penicillin 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. A 0.1-ml amount of the cell suspension was dispensed into each well of

the microtiter plate (Falcon Microtest II with lid), 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 48 or 72 h. At 24 h before harvesting, 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 using 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. (11). Briefly, 1.2-g sterile nylon wool columns were incubated at 37°C for 1 h with RPMI 1640 medium–10% fetal bovine serum (RPMI-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 T cells 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. Nylon wool-adherent (B-enriched) cells 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, and a total of 50 ml was collected in four to five cycles of compression.

Mitogens. *Salmonella typhosa* lipopolysaccharide endotoxin (LPS) was prepared by the phenol-water method of Westphal et al. (32) and contained less than 1% protein. Concanavalin A (ConA) was purchased from Sigma Chemical Co., St. Louis, Mo.

Cells. Monolayers of the BHK-21F line of Syrian hamster kidney cells were grown in Falcon plastic tissue culture dishes (100 by 20 mm) in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum.

Virus. Stocks of Sindbis virus were initiated from single plaques in BHK-21F cells, grown in stationary cultures of BHK-21F cells, and assayed on Vero cell monolayers as previously described for VSV (9).

Virus propagation. Sindbis virus was grown in confluent monolayers of BHK-21F or Vero cells in tissue culture plates at a multiplicity of infection of 0.05 to 0.1 PFU per cell. The virus was purified by clarifying the medium at 5,000 rpm for 15 min and then pelleting the virus at 23,000 rpm for 1 h in an SW27 rotor. The virus was resuspended and layered onto 15 to 35% potassium tartrate gradients and spun in an SW40 rotor at 30,000 rpm for 90 min. The virus band was collected, dialyzed overnight against phosphate-buffered saline, and pelleted at 35,000 rpm for 45 min. The purity of the viral preparations was determined by polyacrylamide gel electrophoresis (PAGE) followed by Coomassie blue staining. Preparations which contained only Sindbis virus proteins were used for mitogen assays.

Preparation of glycoproteins and Triton pellet. The procedure for isolating the glycoproteins from Sindbis virus was essentially that which has been previously described for VSV (18). Briefly, purified Sindbis virus (approximately 1 mg/ml) was diluted 1:5 in 2% (vol/vol) Triton X-100 (Sigma Chemical Co.) 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. The supernatant was decanted into 10 volumes of ice-cold butanol, and glycoproteins were precipitated in the cold for 0.5 h and recovered by centrifugation at 1,000 rpm for 15 min at 4°C. The pelleted glycoproteins were washed with ice-cold acetone, recovered by low-speed centrifugation, dried under nitrogen, and resuspended in phosphate-buffered saline by sonification for 2 × 15 s at 30 W on a Bronson Sonifier. Purity of preparations was determined by Coomassie blue-stained PAGE. Only preparations which had no other proteins present were used for these assays. The Triton-insoluble material was washed repeatedly in 5 to 10 ml of phosphate-buffered saline and pelleted by centrifugation at 30,000 rpm for 1 h at 4°C in an SW50.1 rotor. Its polypeptide composition was determined by PAGE.

Protein determination. Protein was determined by the method of Lowry et al. (16).

RESULTS

Mitogenicity of Sindbis virus. The mitogenicity of Sindbis virus was determined in splenocytes isolated from Swiss Webster, BALB/c, and CBA/J mice. Optimal stimulation occurred at 48 h after initiation of culture. The data in Table 1 show that Sindbis virus is mitogenic for splenic lymphocytes from all three mouse strains. At 48 h after initiation of culture, the optimal stimulation of lymphocytes from BALB/c and CBA/J mice is achieved at concentrations of 1 to 10 μg of virus per well, and stimulation drops off at higher concentrations. In contrast, with lymphocytes from the Swiss Webster mice, significant activation is seen with as little as 0.01 μg of virus per well, and the optimal concentration is 1 μg per well. Furthermore, with Swiss Webster spleen cells the dose-response curve is not as sharp as that seen with lymphocytes from the other mouse strains. For cells from all of the mouse strains tested, optimal stimulation by Sindbis virus is less than that observed with LPS or ConA.

To show that the observed mitogenesis was not due to contamination by LPS, we tested the ability of Sindbis virus to activate lymphocytes from C3H/HeJ mice. The data in Table 2 show that Sindbis virus was equally mitogenic in splenocytes from C3H/HeJ and CBA/J mice, whereas LPS was only significantly mitogenic for splenocytes from CBA/J mice. Lymphocytes from C3H/HeJ mice are essentially unresponsive to purified LPS and are only about one-half as responsive to whole endotoxin as are lymphocytes from CBA/J mice (29, 30). Furthermore, virus preparations were analyzed for LPS by the *Limulus* amoebocyte lysate assay (Microbiologi-

TABLE 1. Lymphocyte activation by Sindbis virus

Mitogen	Amt (μg per culture)	[^{3}H]thymidine incorporation (cpm ± SEM) into the DNA of mouse cells		
		CBA/J	Swiss Webster	BALB/c
None		4,359 ± 254	5,838 ± 882	3,726 ± 40
LPS	10	83,265 ± 5,879	47,241 ± 3,761	84,067 ± 8,924
ConA	0.125	86,416 ± 395	124,415 ± 8,571	128,457 ± 2,107
Sindbis virus	0.01	ND[a]	19,521 ± 455	3,167 ± 207
	0.1	5,818 ± 518	19,100 ± 1,786	10,496 ± 925
	1.0	11,936 ± 964	31,881 ± 629	22,101 ± 68
	5.0	18,783 ± 1,281	29,798 ± 230	31,602 ± 4,132
	10.0	26,881 ± 2,234	14,661 ± 1,552	23,510 ± 1,684
	25.0	8,270 ± 543	ND	ND

[a] ND, Not done.

cal Associates, Walkersville, Md.), and the results indicated that there was less than 5 ng of LPS per ml of virus. This is an insufficient amount of LPS to cause the mitogenic stimulation seen with Sindbis virus. These two experiments show that the observed activity of Sindbis virus is not due to LPS contamination. Another possible contaminant, mycoplasma, can be ruled out for the following reasons. First, our viral preparations are optimally active at mitogen concentrations which are suboptimal for mycoplasma, which is optimal at 100 μg/ml (2, 6, 22), and second, there is no evidence of proteins other than those of Sindbis virus on our PAGE. We are confident in concluding, therefore, that Sindbis virus itself is mitogenic for mouse spleen cells.

Identification of the responding cell. The viral mitogens so far described have been either B-cell or both B- and T-cell activators (5, 8, 14, 20). The data in Table 1 show that for Sindbis virus the greatest stimulation occurs 48 h after the addition of mitogen to cells and that stimulation is significant over a 10-fold range of mitogen concentration. These results are similar to those obtained with B-cell mitogens such as LPS and purified protein derivative, but not with T-cell mitogens such as ConA. To identify which cell population was responding to the Sindbis virus mitogenic stimulation, CBA/J spleen cells were first separated into nonadherent- and adherent-cell populations by passage over nylon wool and were then exposed to various mitogens. The data in Fig. 1 show that the nylon wool-nonadherent cells contained predominantly T cells, as determined by the response to ConA, and very few, if any, B lymphocytes, as assayed by their lack of response to LPS stimulation. In contrast, the adherent-cell (B-cell-enriched) population contains predominantly B cells, as determined by the LPS response, as well as residual T lymphocytes which responded significantly to stimulation by ConA. When we examine the response of the B- or T-enriched cells to Sindbis virus, we find that the nonadherent cells are unable to respond significantly to Sindbis virus, but the adherent cells respond as well as, or better than, the unseparated cells (Fig. 1). This suggests that Sindbis virus is a B-cell mitogen, but not a T-cell mitogen.

To establish whether Sindbis virus is a T-cell-independent B-cell mitogen and to confirm our data suggesting that it is a B-cell mitogen, we examined the ability of spleen cells from congenitally athymic BALB/c nu/nu mice to respond to the virus. As these cells lack functional T lymphocytes, any observed mitogenic response can be attributed to B cells. The data in Table 3 show that when cultured with Sindbis virus, lymphocytes from the BALB/c nu/nu mice are stimulated to incorporate [^{3}H]thymidine in a manner identical to that of their normal littermates. Cells from both types of mice are optimally stimulated at 5 μg of virus per well. In addition, cells from the nude mouse are fully responsive to LPS, but are unresponsive to ConA, indicating that there are no functional T cells. This ability of lymphocytes from the nu/nu mouse to be activated by Sindbis virus to the same degree as are normal splenocytes indicates that Sindbis virus acts as a T-cell-independent B-cell mitogen.

Lymphocyte activation by Sindbis virus glycoproteins. To determine if the Sindbis virus glycoproteins are mitogenic, we studied the ability of

TABLE 2. Activation of C3H/HeJ and CBA/J lymphocytes by Sindbis virus

Mitogen	Amt (μg per culture)	[^{3}H]thymidine incorporation (cpm ± SEM) into the DNA of mouse cells	
		C3H/HeJ	CBA/J
None		3,458 ± 165	3,169 ± 202
LPS	10	7,077 ± 110	94,248 ± 3,949
ConA	0.125	104,355 ± 578	100,947 ± 4,354
Sindbis virus	10	41,165 ± 1,223	44,951 ± 4,847

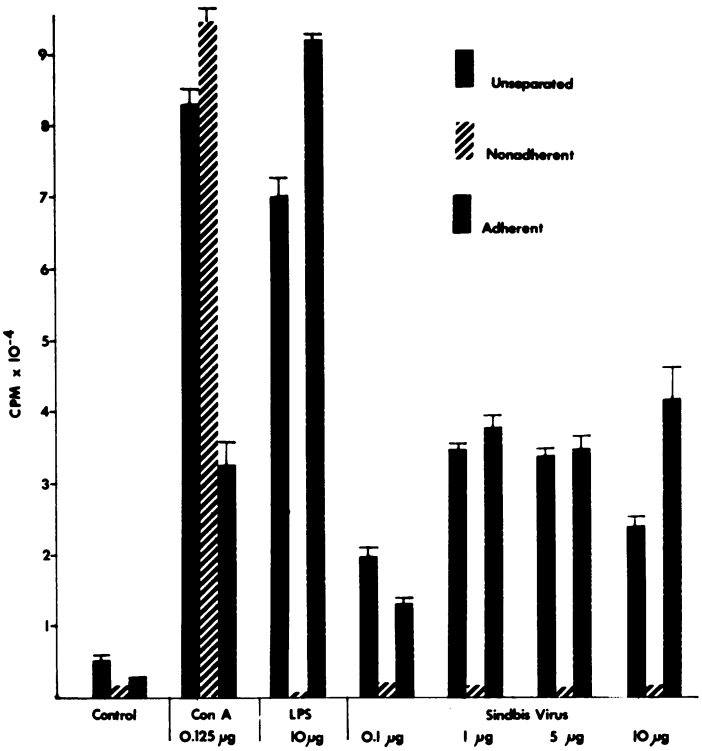

FIG. 1. Response of unseparated, nylon wool-adherent and -nonadherent spleen cells from CBA/J mice to LPS, ConA, and Sindbis virus. Lymphocytes (4×10^5) were cultured with mitogen at the concentrations indicated in a total volume of 0.2 ml. The cultures were incubated at 37°C and 100% humidity for 48 h. [^{3}H]thymidine (1 μCi) was added to each culture 24 h before the cells were harvested for scintillation counting.

these isolated glycoproteins to induce lymphocyte activation. As can be seen in Fig. 2, the isolated glycoproteins are indeed highly stimulatory for these cells, and 1 μg induces [^{3}H]thymidine incorporation into lymphocytes at a level five times greater than that stimulated by 1 μg of the intact virus. On the basis of Coomassie blue-stained gels, the Sindbis virus preparations used for these experiments contained about 40% glycoproteins and 60% core protein. Therefore, for every 1 μg of Sindbis virus, there was approximately 0.4 μg of glycoprotein. The data in Fig. 2 show that 0.5 μg of isolated glycoproteins gives essentially the same amount of incorporation of [^{3}H]thymidine as does 1 μg of glycoproteins. Both concentrations of glycoproteins give approximately five times more stimulation than 0.5 and 1 μg of virus. It appears, therefore, that the glycoproteins of the virus make up the major mitogenic moieties of the virion. The greater mitogenic activity of the isolated glycoproteins, as compared with that of virions, could be due to the high degree of aggregation associated with the Triton X-100-extracted and butanol-precipitated glycoproteins. Similar results have been reported for the isolated glycoproteins of influenza viruses (1) and VSV (19). The Triton pellet, which contains predominantly core protein and essentially no glycoproteins, is as mitogenic as the virion (Fig. 2), suggesting that other components of the virus core are also mitogens.

DISCUSSION

The data presented in this paper demonstrate that both Sindbis virus and the isolated viral

TABLE 3. Activation of lymphocytes from BALB/c nu/+ and nu/nu mice by Sindbis virus

Mitogen	Amt (μg per culture)	[^{3}H]thymidine incorporation (cpm ± SEM) into the DNA of mouse cells	
		BALB/c nu/+	BALB/c nu/nu
None		4,291 ± 268	4,234 ± 190
LPS	10	113,055 ± 10,554	133,219 ± 3,628
ConA	0.125	123,589 ± 5,427	4,164 ± 289
Sindbis virus	0.01	8,647 ± 1,166	9,966 ± 512
	0.1	17,788 ± 994	26,148 ± 2,201
	1.0	33,441 ± 1,183	32,170 ± 3,448
	5.0	58,952 ± 1,801	53,509 ± 989
	10.0	36,508 ± 3,755	39,496 ± 1,019

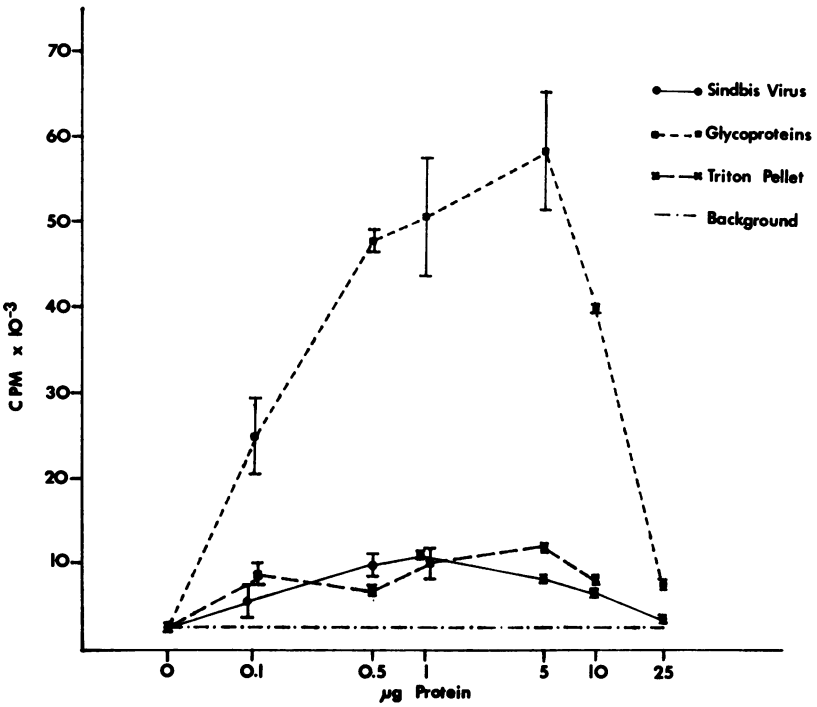

FIG. 2. Response of CBA/J spleen cells to Sindbis virus, the isolated glycoproteins, and the Triton pellet. Cells were cultured as described in the legend to Fig. 1.

envelope glycoproteins are mitogenic for mouse splenic lymphocytes. These results are not due to contamination with LPS or mycoplasma. Sindbis virus is specifically mitogenic for murine B cells, but not T lymphocytes, and furthermore, activation was independent of T-cell help. This pattern of stimulation was also observed for VSV (9) and HSV types I and II (14, 20). In contrast, influenza virus serotype H2N2 has been shown to stimulate both B and T cells (5). Therefore, all viruses previously reported to be mitogenic for lymphocytes stimulate B cells. As with HSV types I and II and VSV, Sindbis virus is a T-cell-independent B-cell mitogen. [^{3}H]thymidine incorporation by Sindbis virus-activated lymphocytes is significantly less than that observed with LPS, an observation similar to that found with other viruses (5, 9, 14, 20). Since the Triton pellet of Sindbis virus, which contains essentially no glycoproteins, is as mitogenic as the untreated virus, one or more of the constituents of the core may also be mitogenic. Characterization of the core constituents for mitogenic activity is underway.

Butchko et al. (5) suggested that the mitogenicity observed with influenza virus serotype H2N2 was due to the hemagglutinin glycoprotein of the virion because infectivity was not required for influenza virus mitogenicity, and the major difference between mitogenic and nonmitogenic subtypes of influenza viruses was the glycoproteins (5). We have previously reported that the G protein of VSV is mitogenic for murine spleen cells and, on a microgram-of-protein basis, is a much more potent mitogen than the intact virion (8, 19). Subsequently, the hemagglutinins from a number of different types of influenza viruses were found to be mitogens, and these too were much more potent mitogens than the intact virus (1). In this study we report that the envelope glycoproteins of Sindbis virus are mitogenic for mouse lymphocytes and are much more stimulatory than the intact virion. This greater activity may be due to the high degree of aggregation characteristic of viral glycoproteins isolated by Triton X-100 extraction followed by butanol precipitation. Further study is necessary to determine whether only one or both of the Sindbis virus glycoproteins are mitogens.

It is important to know whether this in vitro stimulation of the immune response can be correlated with or related to the in vivo immunomodulation observed with so many viral infections. Viruses have been recognized modulators of immune responsiveness since early in this

century, when it was observed by Pirkquet that measles patients transiently lost their ability to respond to tuberculin (26). Since that time over 40 viruses have been shown to alter the host response to heterologous antigens (27). Although in most cases virus infection depresses the immune response (27), there have been studies demonstrating enhanced responsiveness to heterologous antigens (12, 23, 27, 31). Enhancement can be found for both humoral and cellular responses. It appears that the type of immunomodulation observed varies not only with the virus, but also with the time interval and administration sequence of virus and antigen (27, 31). It is also true that suppression of some types of stimulation is accompanied by an enhancement of other types (13). Although in most cases of immunomodulation by viruses an active infection is required, there are reports of changes in the immune response with either noninfectious virus or isolated viral proteins (17, 28). Since both humoral and cell-mediated immunity are affected (27), perhaps by altered immunoregulation, and since it has been shown that viral infection can act as an adjuvant for other antigens (27, 28, 31), it is possible that the virally induced nonspecific mitogenicity which we observed in vitro is related to this postulated in vivo regulation.

It had been previously suggested that viral mitogenicity was related to the infectious process and pathogenicity of the virus (5, 9) since a number of viruses can grow in stimulated, but not resting, lymphocytes (3, 4, 15, 33, 34). Therefore, stimulation of the cell by the virus would increase the number of cells which can be productively infected. In addition, activated lymphocytes can express endogenous type C RNA viruses (10, 21, 25). It is also possible that the ability of the virus to act as a lymphocyte mitogen affects the regulation of the immune system. Nonspecific lymphocyte activation and clonal expansion might aid in maturation of the immune responsiveness of the individual or it may produce modulation of the immune response to a heterologous antigen. Modulation of the immune response to heterologous antigen may be direct, through the interaction of virus and cell, or indirect, through the production of lymphokines by virus-stimulated lymphocytes. We believe that nonspecific viral mitogenicity will be found for many viruses and that this in vitro observation may provide a model system for the study of immunomodulation by viruses.

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