THE GLYCOPROTEIN ISOLATED FROM VESICULAR STOMATITIS VIRUS IS MITOGENIC FOR MOUSE B LYMPHOCYTES*

By GAIL GOODMAN-SNITKOFF,‡ RAPHAEL J. MANNINO, and JAMES J. McSHARRY

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

For approximately the last 15 years, mitogens have been used to study lymphocyte activation. The first identified mitogens were predominantly plant lectins such as concanavalin A (Con A),[1] phytohemagglutinin, and pokeweed mitogen (1, 2). In addition, lipopolysaccharide endotoxin (LPS) extracted from the outer membrane of Gram-negative bacteria was shown to activate B lymphocytes (2–4). Subsequently, other components of the outer membranes of Gram-positive and Gram-negative bacteria were shown to be lymphocyte activators (5–10), as was purified protein derivative of tuberculin that was recovered from mycobacteria (11). In addition, membrane proteins from mycoplasma and peptidoglycans from the cell walls of yeasts were mitogenic for mouse lymphocytes (12–15). Recently, the list of mitogens has expanded to include the digitalis glycosides (16), low molecular weight chemicals such as 2-mercaptoethanol and periodate (17, 18), and the polymers of dextran sulfate and polyinosinic-polycytidylic acid (19–21).

In the late 1970s, it was reported that influenza virus (H2N2 subtype) and herpes simplex virus (HSV) types 1 and 2 stimulated mitogenesis in resting nonsensitized lymphocytes from mice (22–24). Influenza virus was stimulatory for purified B and T cells (22), whereas HSV types 1 and 2 activated B lymphocytes (23, 24). HSV was not mitogenic after ultraviolet (UV) or heat inactivation (23, 24), which suggests that infectious virus was required for activation, whereas noninfectious influenza virus was mitogenic (22), which suggests that one or more of the viral constituents was the mitogenic principle. Because the mitogenic activity of influenza viruses varied with the H_2 glycoprotein, Butchko et al. (22) suggested that this viral glycoprotein was responsible for influenza virus mitogenesis.

Recently we have demonstrated that vesicular stomatitis virus (VSV) was a B cell mitogen and that UV-inactivated VSV and defective interfering particles of VSV, which do not undergo primary transcription and translation, were mitogenic (25, 26).

* Supported by grant 1572 from the New York State Health Research Council, and grant PCM-8003126 from the National Science Foundation.

‡ Supported by Postdoctoral Fellowship SP179-14819 from the National Science Foundation.

[1] *Abbreviations used in this paper:* Con A, concanavalin A; FCS, fetal calf serum; G, glycoprotein; HSV, herpes simplex virus; L, large protein; LPS, lipopolysaccharide endotoxin; M, membrane protein; N, nucleocapsid protein; NS, nonstructural protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCA, polyclonal activation; RNP, ribonucleoprotein; SRBC, sheep erythrocytes; TNP, trinitrophenyl; TP, Triton pellet; VSV, vesicular stomatitis virus.

To determine whether the VSV surface glycoprotein (G) was responsible for the mitogenic activity of the virus particle, the G protein was extracted from the virion and tested for its ability to stimulate mitogenesis in lymphocytes. The results reported here indicate that the isolated G protein is a mitogen and a polyclonal activator for murine B cells, but neither the isolated G protein nor the virus particle is mitogenic for murine T cells. In addition, other viral proteins may also be B cell mitogens.

Materials and Methods

Mice. C3H/HeJ and CBA/J mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and maintained in our facility on Purina mouse chow and water ad libitum. Female mice between 8 and 16 wk of age were used in all experiments. BALB/c *nu/nu* female mice were obtained from GIBCO Animal Resources Laboratories (Madison, Wis.) and were maintained in a sterile laminar flow hood on mouse chow and sterile water ad libitum. These mice were used at 7–9 wk of age.

Lymphocyte Cultures and Mitogenic Assays. Lymphocytes were cultured as previously described (27). Briefly, aseptically removed spleen cells were teased into single cell suspensions in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, and washed twice in RPMI-1640. The cells were resuspended, counted for viability, and diluted to 4×10^6 cells/ml. The cell suspension (0.1 ml) was dispensed per well of microtiter plate (Microtest II with lid; Falcon Plastics, Div. of Becton, Dickinson & Co., 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 48 or 72 h. 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. (28). Briefly, 1.2 g sterile nylon wool columns were incubated at 37°C for 1 h with RPMI-1640 plus 10% fetal calf serum (FCS) in a humidified atmosphere. After this preincubation, 300×10^6 cells in 4 ml of RPMI-FCS were added to the column and allowed to flow into the nylon wool; at this time, an additional 2 ml of RPMI-FCS 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-FCS to the column and collecting 15 ml of effluent (flow rate, 1 ml/2 min). The column was rapidly washed with 100 ml RPMI-FCS and this wash was discarded. Nylon wool adherent cells (B enriched) were removed by compressing the nylon wool with the syringe plunger to express the remaining medium. The nylon wool was removed and teased with sterile forceps, resaturated with RPMI-FCS and compressed again, and a total of 50 ml was collected in 4–5 cycles of compression.

Polyclonal Activation (PCA). Mouse spleen cell suspensions were prepared at a concentration of 10×10^6 cells/ml. 1 ml of the suspensions was incubated in 35-mm plastic petri dishes at 37°C in an atmosphere of 83% N_2, 10% CO_2, and 7% O_2 with or without activators as described by Nilsson et al. (29). Antibody-forming cells were identified by a modification of the Jerne hemolytic plaque assay using trinitrophenyl (TNP)-conjugated sheep erythrocytes (SRBC; 29). TNP-SRBC were prepared by the method of Rittenberg and Pratt (30).

Mitogens. Salmonella typhosa LPS was prepared by the phenol-water method of Westphal et al. (31) and contained <1% protein. Con A was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Cells. Monolayers of the BHK-21F line of Syrian hamster kidney cells and the MDBK line of bovine kidney cells were grown in Falcon tissue culture flasks (75 mm^2) or plastic tissue cultures dishes (100 × 20 mm) in Dulbecco's medium supplemented with 10% fetal bovine serum (32).

Virus. Stocks of the Indiana serotype of VSV were initiated from single plaques in BHK-

21F cells, grown in stationary cultures of MDBK cells and assayed on BHK-21F cell monolayers as previously described (33).

Virus Propagation. VSV was grown in confluent monolayers of BHK-21F cells in tissue culture plates at a multiplicity of infection of 0.05–0.1 plaque-forming U/cell and purified as previously described (25). The purity of the viral preparations was determined by polyacrylamide gel electrophoresis (PAGE) followed by Coomassie Blue staining. Only preparations that showed no contaminating proteins were used for mitogen assays.

Preparation of Purified G and Triton-insoluble Pellet (TP). The procedure for isolating the G protein from VSV has been previously described (34). Briefly, purified VSV (~1 mg/ml) was diluted 1:5 in 2% (vol/vol) Triton X-100 (Sigma Chemical Co.) in 0.01 M Tris, 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 Instruments, Inc., Palo Alto, Calif.). The supernate was decanted into 10 vol of ice-cold butanol, and G protein was precipitated in the cold for 0.5 h and recovered by centrifugation at 1,000 rpm for 15 min at 4°C. The pelleted G was washed with ice-cold acetone, recovered by low speed centrifugation, dried under nitrogen, and resuspended in phosphate-buffered saline (PBS) by sonification for two 15-s intervals at 30 W on a Bronson sonifier (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). Purity of preparations was determined by Coomassie Blue-stained PAGE. Only preparations that had no other proteins present were used for these assays. The Triton-insoluble material was washed repeatedly in 5–10 ml PBS 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.

Protease Digestion of VSV. The glycoprotein of VSV was enzymatically removed as previously described (35). In brief, purified virus was resuspended in 0.1 M Tris (pH 7.2) to a concentration of between 1 and 2 mg/ml. One part of *Streptomyces griseus* protease type VI (Sigma Chemical Co.) at a concentration of 1 mg/ml in Tris buffer was added to nine parts virus. The virus-enzyme mixture was incubated at 37°C for 1.5 h and then repurified on potassium tartrate gradients. Control preparations were treated as described, but no enzyme was added.

Preparation of Reconstituted G Protein-Lipid Vesicles. VSV glycolipids, phospholipids, and G protein were extracted with the nonionic detergent octyl-β-D-glucoside (Sigma Chemical Co.) as previously described (36, 37). Briefly, a pellet of VSV was suspended in 10 mM phosphate buffer, pH 8.0, containing 50 mM octyl-β-D-glucoside to a final concentration of 2.5 mg viral protein/ml. The suspension was vortexed for 30 min at room temperature to disperse clumps, followed by an additional incubation for 30 min at room temperature. Insoluble material was removed by centrifugation at 40,000 rpm for 40 min at 15°C, and the supernate was used for the preparation of reconstituted vesicles. Vesicles with a high protein:lipid ratio were prepared by dialysis of the octyl-β-D-glucoside supernate against 2 × 100 vol of Dulbecco's PBS. Low-protein lipid vesicles were prepared by adding an additional 0.4 mg lipid consisting of cholesterol, phosphatidylethanolamine, and phosphatidylcholine (1.6:1.2:1; Avanti Biochemicals, Inc., Birmingham, Ala.) per mg viral protein to the octyl-β-D-glucoside supernate of the virus. The solution was vortexed to dissolve the lipid and then dialyzed as described above. Reconstituted vesicles were removed from the dialysis tubing and tested for mitogenicity.

Protein Determination. Protein was determined by the method of Lowry et al. (38).

Results

Mitogenic Activity of the G Protein. The ability of the isolated G protein to stimulate mitogenesis was tested in CBA/J mouse splenocytes. The results presented in Fig. 1 show that the VSV glycoprotein is mitogenic. After 48 h incubation of the G protein with spleen cells, the optimal stimulatory concentration of the G protein is between 5 and 10 μg/well; higher concentrations of G protein are inhibitory. Both VSV and PBS-washed TP were also able to induce mitogenesis in these cells. However, the mitogenic activity of the isolated G protein is at least two times greater than that of either VSV or TP. Because the G protein represents between 20 and 30% of the total protein present in VSV (39), 25–50 μg of virus contains between 5 and 15 μg of G protein. Although there was as much or more G present in VSV as in the purified G

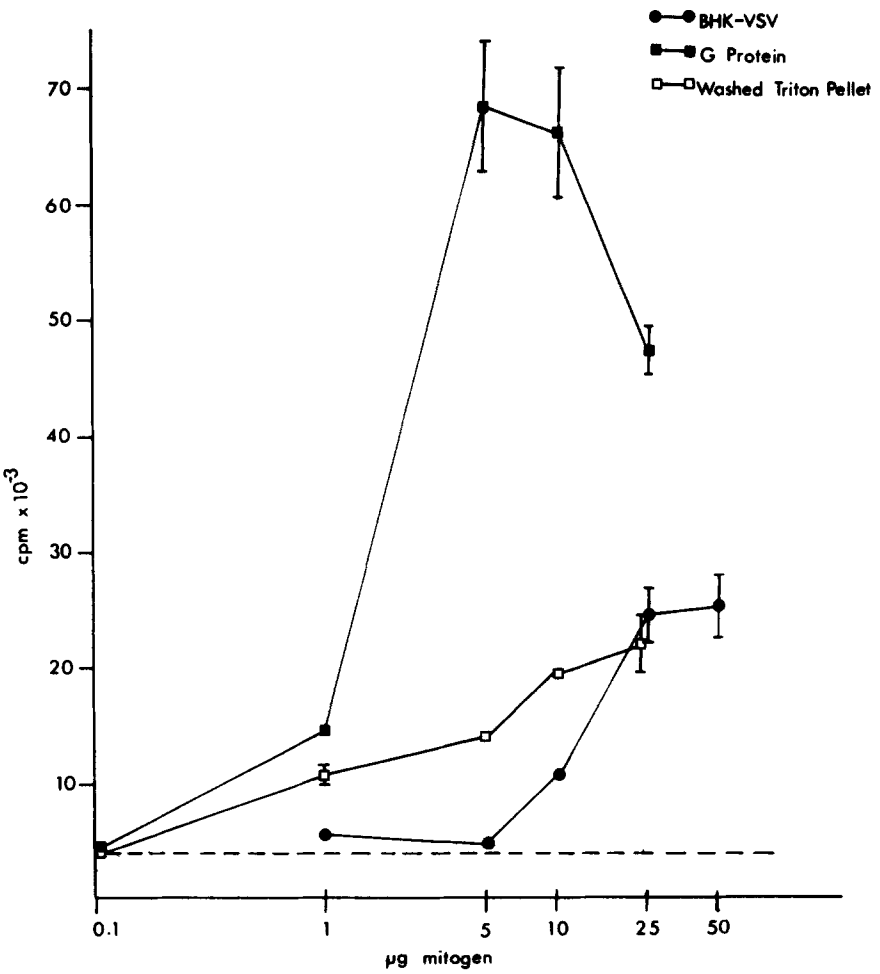

FIG. 1. The response of CBA/J spleen cells to BHK-VSV, G protein, or the TP after 48 h of culture in microtiter plates (37°C, 100% humidity, 7% CO_2 in air).

preparation, maximal stimulation by VSV is <50% that of isolated G protein, and increasing the concentration of VSV did not enhance stimulation. This result shows that the isolated G protein is a more efficient mitogen than the virus particles. Furthermore, the amount of [^{3}H]thymidine incorporated in the presence of an optimal concentration of G protein (5–10 μg) was very similar to that incorporated by stimulation with 50 μg LPS, a known B cell mitogen (see Fig. 3).

Mitogenic Activity of the G Protein in C3H/HeJ Mouse Spleen Cells. We have reported previously that VSV mitogenesis is not due to contamination with LPS by demonstrating that VSV is mitogenic in spleen cells isolated from C3H/HeJ mice, which are low responders to mitogenesis by LPS (25, 26). To demonstrate that the mitogenic activity illustrated in Fig. 1 is due to G protein and not LPS, we compared the ability of the G protein, TP, and VSV to stimulate incorporation of [^{3}H]thymidine into spleen cells isolated from C3H/HeJ and CBA/J mice. The data presented in Fig. 2 demonstrate that the G protein, VSV, and the TP stimulate mitogenesis in spleen

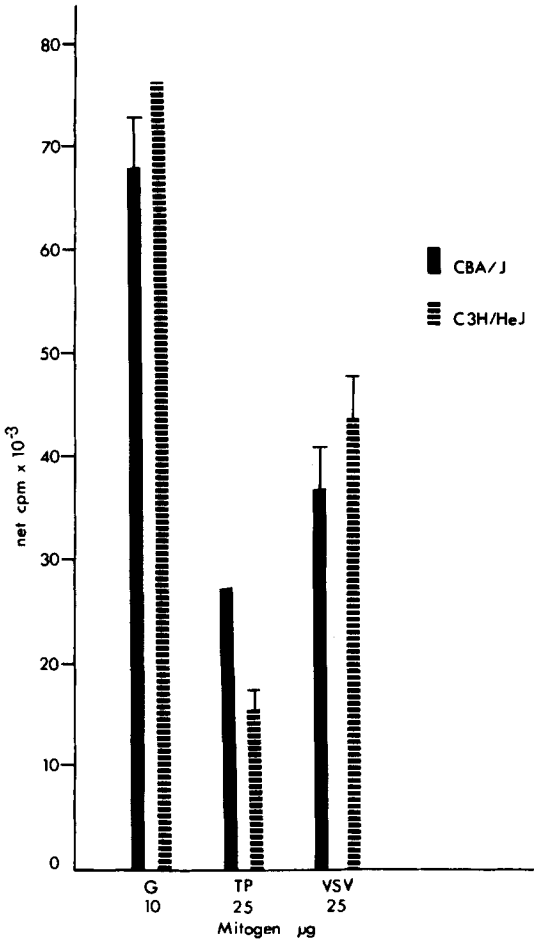

FIG. 2. Response of CBA/J and C3H/HeJ to G protein, TP, and BHK-VSV 48 h after culture initiation.

cells isolated from both strains of mice. PAGE of these preparations showed only viral proteins, indicating the absence of any detectable contaminating protein. On the basis of these experiments, we conclude that the G protein is a mitogen for murine spleen cells.

Mitogenic Activity of the G Protein in Lipid Vesicles. To determine whether the isolated G protein could be mitogenic when it was incorporated into a membrane, the G protein and viral lipids were extracted from virus with octyl-β-D-glucoside, the insoluble material was removed by centrifugation, and vesicles containing the G protein and viral lipids were formed while removing the octyl-β-D-glucoside by dialysis. Various concentrations of vesicles were incubated with spleen cells isolated from CBA/J mice and mitogenesis was determined at 48 h. The data in Table I show that the G protein in lipid vesicles reconstituted with viral lipids was as mitogenic as VSV, whereas the G protein in lipid vesicles containing twice as much lipid as those consisting of viral lipids alone, is considerably less mitogenic. Lipid vesicles containing only cholesterol, phosphatidylethanolamine, and phosphatidylcholine are not mito-

TABLE I

Mitogenic Activity of the G Protein in Lipid Vesicles

Mitogen	μg/ml	[^{3}H]Thymidine incorporation ± SE*
None	—	2,683 ± 181
VSV	1	4,028 ± 384
	5	12,133 ± 372
	10	16,792 ± 338
	25	13,719 ± 1,262
Reconstituted VSV	0.8	5,783 ± 178
	4	11,863 ± 1,223
	8	22,610 ± 1,015
	21	19,204 ± 1,637
Reconstituted VSV + lipid	0.8	4,807 ± 359
	4	4,742 ± 278
	8	5,274 ± 549
	21	1,107 ± 309
Lipid‡	1.25	2,407 ± 284
	1.5	2,658 ± 44
	2.5	1,667 ± 143

* SE, standard error of the mean of triplicate samples.
‡ Dilutions of lipid vesicle without protein comparable to the dilution of vesicles containing G protein.

TABLE II

Activation of B Lymphocytes by G Protein

Mitogen	μg/well	[^{3}H]Thymidine incorporation (cpm) ± SE*	
		CBA/J spleen cells	BALB/c *nu/nu* spleen cells
None		2,597 ± 231	3,194 ± 301
G	5	87,947 ± 1,770	61,885 ± 5,889
	10	120,286 ± 9,845	116,153 ± 8,889
VSV	25	31,425 ± 2,132	26,190 ± 3,663
TP	25	30,196 ± 2,416	27,169 ± 2,169
LPS	50	95,183 ± 5,900	89,574 ± 6,588
Con A	0.125	133,835 ± 6,642	1,450 ± 239

* See legend to Table I.

genic, and at higher concentrations may inhibit mitogenesis because the amount of [^{3}H]thymidine incorporated in the presence of these vesicles is less than in the absence of mitogen. These results show that the G protein is mitogenic when it is found in a proper configuration in a lipid bilayer and that the amount of stimulation is dependent on the spacing of the G protein on the surface of the lipid vesicles. Thus, both the G protein isolated by Triton extraction and used in an aggregated form, and the G protein isolated by octyl-β-D-glucoside extraction and reconstituted into the bilayer of lipid vesicles, are mitogenic for murine spleen cells.

G Protein Is a B Cell Mitogen. We have previously demonstrated that VSV is a T cell-independent, B cell mitogen (25). To determine whether the G protein is also a B cell mitogen, Triton-extracted G protein was incubated with splenocytes isolated from CBA/J or congenitally athymic BALB/c *nu/nu* mice. The data presented in Table II show that the G protein is equally stimulatory to spleen cells isolated from both strains of mice. In addition, the data show that VSV and TP are also T cell-

independent, B cell mitogens because they were mitogenic in both spleen cell preparations. LPS, a recognized T cell-independent, B cell mitogen (2, 3), stimulates spleen cells from both strains of mice. However, Con A, a T cell mitogen (1, 2), was mitogenic in the CBA/J mouse spleen cells but not active in the BALB/c *nu/nu* cells, indicating that there are no functional T cells in the nude spleen cell preparation. The results of these experiments demonstrate that the G protein is a T cell-independent, B cell mitogen.

G Protein and VSV Are Polyclonal Activators. Because most nonspecific B cell mitogens are also polyclonal activators (20), the ability of G protein, VSV, and TP to stimulate nonspecific antibody production was examined. As shown in Table III, VSV, G protein, and TP all activated lymphocytes to make antibody against TNP-SRBC, although not as efficiently as LPS. In contrast to the greatly enhanced proliferation induced by isolated G protein as compared with the intact virion, both G protein and VSV stimulated similar numbers of plaque-forming cells. The ability of TP to induce PCA is considerably decreased compared with either G protein or VSV, suggesting that G protein is required for PCA.

G Protein and VSV Do Not Activate T Cells. Preliminary results with VSV had indicated that it did not stimulate mitogenesis in T lymphocytes (G. Goodman-Snitkoff, unpublished observations). Because others have demonstrated the ability of VSV to replicate in a small percentage of the T cell population after being activated by Con A (40–42), the possibility existed that VSV was activating these cells and then replicating in them, resulting in cell death before the time of our mitogenesis assay. To test the possibility that VSV or an active moiety of the virion was able to stimulate T lymphocytes, normal spleen cells were separated on nylon wool columns into nonadherent (T enriched) and adherent (B enriched) cell populations and assayed for their ability to be stimulated by TP, VSV, or G protein. As shown in Fig. 3, the untreated spleen cells responded to all the mitogens assayed. The response of unseparated mouse spleen cells to G protein, TP, and VSV peaked at 48 h and was lower at 72 h. The response to LPS was the same at 48 and 72 h; and the response to Con A was evident at 48 h and increased at 72 h. At 48 h, the unseparated cells responded to G protein and to LPS essentially equally; however, the mitogenic activity of G protein was ~4 times greater than that of VSV or TP. Nonadherent T-enriched cells

TABLE III

Stimulation of Plaque-forming Cells by G Protein

Mitogen	μg/ml	Anti-TNP-SRBC/10^6 cells*
G	10	82
	25	77
	50	70
VSV	25	70
	50	84
	100	101
TP	25	7
	50	41
	100	48
LPS	100	186

* This is a representative experiment of four; background levels were <5 PFC.

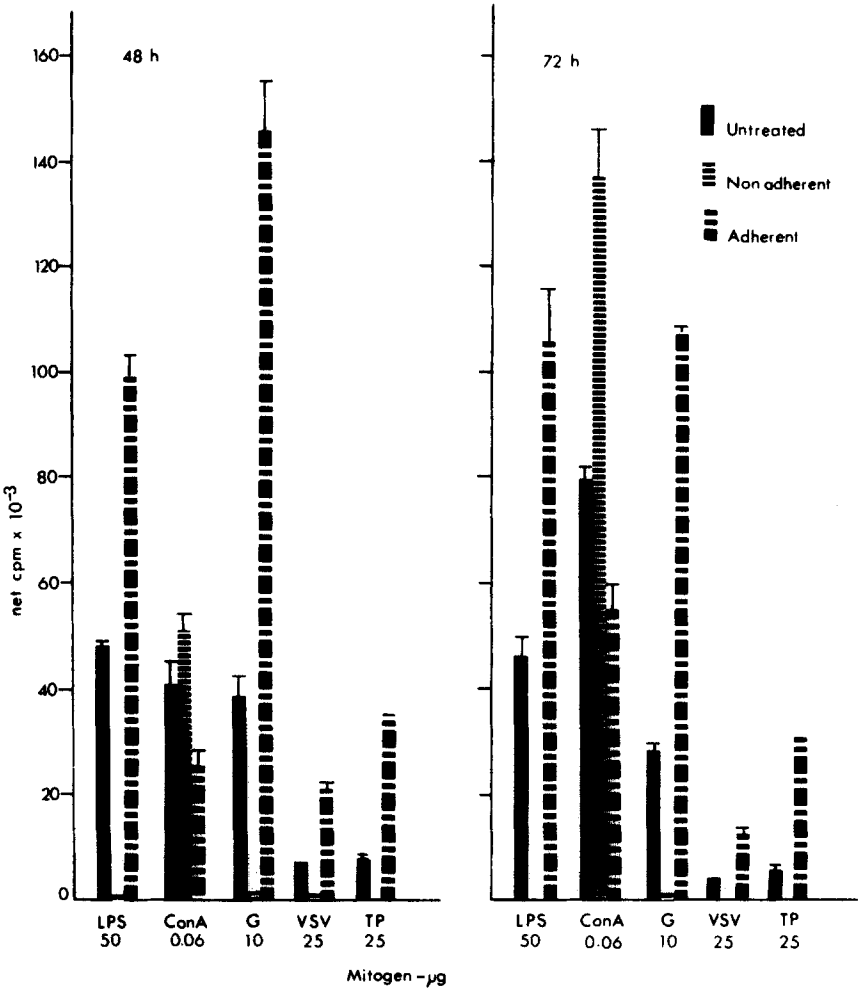

FIG. 3. The response of nylon-wool separated spleen cells to different mitogens at 48 and 72 h. Abbreviations as in Fig. 2.

were unresponsive to LPS, the G protein, VSV, or TP at either time, suggesting that these are not T cell mitogens, T-enriched cells were stimulated by Con A at both 48 and 72 h, and their response to this mitogen was greater than that of unseparated cells. The response of adherent, B-enriched lymphocytes to VSV, isolated G protein, TP, and LPS is enhanced compared with that of untreated cells, which suggests that G protein, TP, and VSV are B lymphocyte activators. The results of these experiments show that the G protein, TP, and VSV are not T cell mitogens, but are B cell mitogens as shown before in Table II.

TP Has Mitogenic Activity. The data presented in this paper have demonstrated that the VSV G protein is a mitogen for murine spleen cells. In all the data we presented, TP, which contains the viral membrane (M) protein, the ribonucleoprotein (RNP) complex containing large protein (L), nucleocapsid protein (N), nonstructural protein (NS), and RNA, very small amounts of G protein (<5%), and no lipid, is also

mitogenic. These results suggest that there is more than one mitogen in VSV. Possibly, the membrane or nucleocapsid proteins or both are mitogens. Further evidence that there are mitogens in VSV other than G protein comes from mitogenesis experiments involving protease-treated VSV. On the basis of PAGE, these particles contain no detectable G protein but have the hydrophobic tail of the G protein and all other viral proteins (M, L, N, NS) and lipid. The data in Table IV show that protease-treated VSV is mitogenic in CBA/J spleen cells; however, it is about one-half as mitogenic as virions, which suggests that half of the mitogenic activity has been removed. The protease itself is not mitogenic. Taken together, these results suggest that some component(s) of the virion, in addition to the G protein, is mitogenic for murine spleen cells.

Discussion

The data presented here demonstrate that VSV, TP, and purified G protein are T cell-independent, B cell mitogens, but do not activate nylon wool nonadherent T cells. In addition, VSV and the isolated G protein are polyclonal activators for murine spleen cells.

The isolated G protein is highly stimulatory for mouse spleen cells. At the optimal concentration (5–10 μg/well), the G protein stimulates as much [^{3}H]thymidine incorporation as does an optimal concentration of LPS (25–50 μg/well) and is 2–3 times as active as either VSV or TP. Lipid vesicles consisting of the G protein and viral lipids isolated using octyl-β-D-glucoside were also stimulatory. The purification technique used for the isolation of the G protein yields a highly aggregated preparation of G protein, which is the most stimulatory preparation of G protein. Virions and lipid vesicles composed of viral lipids and G protein are less mitogenic than aggregated G protein. Lipid vesicles with a low protein/lipid ratio are less mitogenic than viruses and lipid vesicles with a high protein/lipid ratio. This proportionality of activity is also seen when haptenated liposomal membranes are used to study in vivo or in vitro immunogenicity (43, 44). It has been found that liposomes that contain <2% hapten are not immunogenic and that a threshold level of 2.5% hapten was required for any significant response and an optimal response required a minimum of 5–10% hapten

TABLE IV

Mitogenic Activity of Protease-treated VSV

Mitogen	μg/well	[^{3}H]Thymidine incorporation ± SE*
None	—	2,683 ± 181
VSV	1	2,727 ± 234
	5	8,711 ± 1,093
	10	14,957 ± 299
	25	21,212 ± 2,215
Protease-treated VSV	1	3,994 ± 91
	5	6,881 ± 181
	10	7,601 ± 245
	20	11,427 ± 1,028
Protease	0.01	1,528 ± 186
	0.05	3,336 ± 242
	0.1	1,628 ± 15

* See legend to Table I.

liposome. These data correlate well with what we have shown regarding the mitogenicity of G protein, the aggregated material being the most stimulatory, and the activity decreasing as the G protein:lipid ratio decreases. These results suggest that the purified G protein is more mitogenic than G protein in association with membranes, possibly because it is aggregated. It is possible that aggregated G protein presents more mitogen to the cell surface than the G protein in virus or lipid vesicles. Alternatively, different portions of the G protein are presented to the cell surface with aggregated G protein than with G protein in its native configuration. Although these possibilities are unresolved, it is clear that the G protein, either as an aggregation of purified glycoproteins or in lipid vesicles consisting of viral lipids and G protein, is mitogenic for murine spleen cells.

On the basis of the data presented here, it is unlikely that the observed mitogenesis is due to a contaminant in the preparation. The data supporting this conclusion are as follows. Coomassie Blue-stained gels of G protein contain no resolvable proteins other than G, whereas those of the intact virion or TP contain only the appropriate viral proteins. Activation of C3H/HeJ lymphocytes by G protein or VSV is equal to that seen in CBA/J splenocytes; stimulation of C3H/HeJ spleen cells by naturally occurring endotoxin (LPS) is generally lower than the activation seen in CBA/J lymphocytes (6). Because endotoxin is a complex of 8–10% protein and ~90% LPS (6), one would expect that any endotoxin protein present in these preparations would be resolved on the stained gels. Additionally, G protein stimulates as much mitogenesis as does LPS, but at an optimal concentration 1/5 of that required for maximal activation by LPS. The other probable contaminant of these preparations is mycoplasma. Mycoplasma proteins have been found to be mitogenic for lymphocytes at concentrations equal to or greater than those required for activation by G protein (12, 13). Again, as with endotoxin, if the observed stimulation was due to mycoplasma mitogens, proteins other than those of VSV or the purified G would be resolved by PAGE. Furthermore, the response of lymphocytes to mycoplasma mitogens is optimal at 72 h (12, 13), whereas the response to G protein or VSV peaks at 48 h. It is our conclusion, therefore, that G protein, VSV, and TP are responsible for the stimulation described here.

It is interesting that G, which is significantly more mitogenic than VSV, activates essentially the same number of plaque-forming cells. Also, TP is much less stimulatory in this system than in the induction of mitogenesis when compared with VSV. This lack of correlation between mitogenesis and PCA has been demonstrated with other B cell mitogens, such as dextran sulfate (19, 20) and lantoside C (16), which stimulate good mitogenesis but poor PCA when compared with LPS. VSV contains these different activities in the intact virion, but the disruption of the virus results in the separation of the activities into a strong mitogen and good PCA (G), and a good mitogen and weak PCA (TP). The reason for the lack of correlation between mitogenesis and PCA by G protein requires additional investigation. Whether this is due to disruption of the lipid membrane, resulting in loss of ability to stimulate plaque-forming cells, or simply to the subset of B lymphocytes being stimulated, remains to be studied. The questions of what the active portion of TP is, and whether or not the mitogenic and PCA signals are on the same or different components, needs to be addressed.

It has been demonstrated that VSV is unable to stimulate mitogenesis in T

lymphocytes. One possible explanation was that VSV was activating these cells and then multiplying in them, because Bloom et al. (40, 41) had shown that VSV was able to replicate in mitogenized T lymphocytes, and more recently, data from Minato and Katsura (42) has demonstrated that it is the nylon wool-adherent T cell that supports the replication of VSV and not the nonadherent T cell. Because nonadherent T lymphocytes do not incorporate [^{3}H]thymidine in the presence of VSV, the G protein, or TP, we conclude that this virus is not mitogenic for T cells. Whether purified virion or viral components can stimulate any activity in the cellular arm of the immune response, such as interferon production, requires further investigation.

TP, which contains the viral M protein, the RNP consisting of proteins L, N, NS, and RNA, and a small amount of residual G protein (<5%), but no lipid, is mitogenic for murine spleen cells. Its mitogenic activity is considerably less than that of aggregated G protein, but is similar to that of virions and lipid vesicles containing G protein. Because TP contains only traces of G protein, the mitogenic activity must be attributed to some other component of the virion. Additional evidence to suggest that subvirus particles lacking the complete G protein are mitogenic is derived from the studies that demonstrated the mitogenic activity of protease-treated VSV. These particles lack detectable G protein, but retain the hydrophobic tail of the G protein and contain the M protein and the RNP complex. These particles are approximately half as active as whole virions in mitogenic assays. In this case, the mitogenic activity could be due to the hydrophobic tail of G protein or some lipid or protein of the subvirus particle. These results suggest that there are additional mitogens in VSV. Because TP does not contain lipid and retains mitogenic activity, lipids are probably not the mitogens. The M and N proteins are the major proteins that remain in these subparticles. These will be isolated and tested for mitogenic activity in murine splenocytes.

Although the role of the glycoprotein in stimulating nonspecific mitogenesis was suggested by the work of Butchko et al. (22) with influenza virus, the work reported here is the first time, to our knowledge, that a purified viral protein has been shown to activate murine lymphocytes.

Summary

The glycoprotein (G protein) of VSV was purified from the intact virion by Triton X-100 extraction. The isolated G protein has been shown to be a T cell-independent, B lymphocyte mitogen and polyclonal activator. Neither G protein nor the intact virion are stimulatory for murine T lymphocytes. The greater the density of G protein in lipid vesicles or the degree of aggregation of isolated G protein, the more highly stimulatory it is for murine splenocytes. As G protein is spread out in artificial vesicles, it becomes less mitogenic. It is probable that other viral components are also stimulatory since the Triton-insoluble pellet and VSV from which the G protein has been enzymatically removed retain mitogenic activity. To our knowledge, this is the first time a purified viral component has been demonstrated to be a lymphocyte mitogen.

We wish to thank Ms. Lise Winters for technical assistance and Kathleen Cavanagh for manuscript preparation.

Received for publication 11 February 1981.

References

1. Stobo, J. D. 1972. Phytohemagglutinin and concanavalin A probes for murine "T"-cell activation and differentiation. *Transplant. Rev.* **11**:60.
2. Greaves, M., and G. Janossy. 1972. Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. *Transplant. Rev.* **11**:87.
3. Anderson, J., O. Sjoberg, and G. Moller. 1972. Mitogens as probes for immunocyte activation and cellular cooperation. *Transplant. Rev.* **11**:131.
4. Peavy, D. L., J. W. Shands, Jr., W. H. Adler, and R. T. Smith. 1973. Mitogenicity of bacterial endotoxins: characterization of the mitogenic principle. *J. Immunol.* **111**:352.
5. Melchers, F., V. Brown, and C. Galanos. 1975. The lipoprotein of the outer membrane of *Escherichia coli*: a B-lymphocyte mitogen. *J. Exp. Med.* **142**:473.
6. Sultzer, B. M., and G. W. Goodman. 1976. Endotoxin protein: a B-cell mitogen and polyclonal activator of C3H/HeJ lymphocytes. *J. Exp. Med.* **144**:821.
7. Moller, G., and P. Landwall. 1977. The polyclonal B-cell-activating property of protein A is not due to its interaction with the Fc part of immunoglobulin receptors. *Scand. J. Immunol.* **6**:357.
8. Sakane, T., and I. Green. 1978. Protein A from *Staphylococcus aureus*—a mitogen for human T lymphocytes and B lymphocytes but not L lymphocytes. *J. Immunol.* **120**:302.
9. Goodman, G. W., and B. M. Sultzer. 1979. Endotoxin protein is a mitogen and polyclonal activator of human B lymphocytes. *J. Exp. Med.* **149**:713.
10. Damais, C., C. Bona, L. Chedid, J. Fleck, C. Nauciel, and J. P. Martin. 1975. Mitogenic effect of bacterial peptidoglycans possessing adjuvant activity. *J. Immunol.* **115**:268.
11. Sultzer, B. M., and B. S. Nilsson. 1972. PPD-tuberculin—a B-cell mitogen. *Nature (Lond.).* **24**:198.
12. Biberfeld, G., and E. Gronowicz. 1976. *Mycoplasma pneumoniae* is a polyclonal B-cell activator. *Nature (Lond.).* **261**:238.
13. Naot, Y., and H. Ginsberg. 1978. Activation of B lymphocytes by mycoplasma mitogen(s). *Immunology.* **34**:715.
14. Ciorbaru, R., A. Adam, J. F. Petit, E. Lederer, C. Bona, and L. Chedid. 1975. Isolation of mitogenic and adjuvant active fractions from various species of *Nocardiae. Infect. Immun.* **11**: 257.
15. Brochier, J., C. Bona, R. Ciorbaru, J. P. Revillard, and L. Chedid. 1976. A human T-independent B lymphocyte mitogen extracted from *Nocardia opaca. J. Immunol.* **117**:1434.
16. Hammarstrom, L., and C. I. E. Smith. 1978. Lantoside C, a new polyclonal B-cell activator. *Cell. Immunol.* **36**:377.
17. Goodman, M. G., and W. O. Weigle. 1977. Nonspecific activation of murine lymphocytes. I. Proliferation and polyclonal activation induced by 2-mercaptoethanol and α-thioglycerol. *J. Exp. Med.* **145**:473.
18. Kendall, P. A., and D. Hutchins. 1978. Effect of thiol compounds on lymphocytes stimulated in culture. *Immunology.* **35**:189.
19. Gronowicz, E., and A. Coutinho. 1974. Selective triggering of B-cell subpopulations by mitogens. *Eur. J. Immunol.* **4**:771.
20. Gronowicz, E., and A. Coutinho. 1976. Heterogeneity of B-cells. Direct evidence of selective triggering of distinct subpopulations by polyclonal activators. *Scand. J. Immunol.* **5**:55.
21. Sher, I., D. M. Strong, A. Ahmed, R. C. Knudsen, and K. W. Sell. 1973. Specific murine B-cell activation by synthetic single and double-stranded polynucleotides. *J. Exp. Med.* **138**: 1545.
22. Butchko, G. M., R. B. Armstrong, W. J. Martin, and F. A. Ennis. 1978. Influenza A viruses of the H2N2 subtype are lymphocyte mitogens. *Nature (Lond.).* **271**:66.
23. Mochizuki, D., S. Hendrick, J. Watson, and D. T. Kingsbury. 1977. The interaction of

herpes simplex virus with murine lymphocytes. I. Mitogenic properties of herpes simplex virus. *J. Exp. Med.* **146:**1500.

24. Kirchner, H., G. Darai, H. M. Hirt, K. Keyssner, and K. Munk. 1978. *In vitro* mitogenic stimulation of murine spleen cells by herpes simplex virus. *J. Immunol.* **120:**641.
25. Goodman-Snitkoff, G. W., and J. J. McSharry. 1980. Activation of mouse lymphocytes by vesicular stomatitis virus. *J. Virol.* **35:**757.
26. McSharry, J. J., and G. W. Goodman-Snitkoff. 1981. *In vitro* activation of murine lymphocytes by the isolated glycoprotein of vesicular stomatitis virus. *In* 4th International Symposium of Negative Strand Viruses. R. W. Compans and D. H. L. Bishop, editors. Elsevier North-Holland Press, New York. In press.
27. Goodman, G. W., and B. M. Sultzer. 1977. Mild alkaline hydrolysis of lipopolysaccharide endotoxin enhances its mitogenicity for murine B cells. *Infect. Immun.* **17:**205.
28. Hodes, R. J., B. S. Handwerger, and W. D. Terry. 1974. Synergy between subpopulations of mouse spleen cells in the *in vitro* generation of cell mediated cytotoxicity: evidence for the involvement of a non-T cell. *J. Exp. Med.* **140:**1646.
29. Nilsson, B. J., B. M. Sultzer, and W. W. Bullock. 1973. Purified protein derivative of tuberculin induces immunoglobulin production in normal mouse spleen cells. *J. Exp. Med.* **137:**127.
30. Rittenberg, N. B., and K. L. Pratt. 1969. Antitrinitrophenyl(TNP) plaque assay primary response of Balb/c mice to soluble and particulate immunogen. *Proc. Soc. Exp. Biol. Med.* **132:**575.
31. Westphal, O., O. Luderitz, and F. Bister. 1952. Uber die Extraktion von Bacterien mit Phenol-Wasser. *Z. Naturforsch. Teil B. Anorg. Chem. Org. Chem. Biochem. Biophys. Biol.* **7:**148.
32. Choppin, P. W. 1969. Replication of influenza virus in a continuous cell line: high yield of infective virus from cells inoculated at a high multiplicity. *Virology.* **39:**130.
33. Choppin, P. W., and R. W. Compans. 1970. Phenotypic mixing of envelope proteins of the parainfluenza virus SV5 and vesicular stomatitis virus. *J. Virol.* **5:**609.
34. McSharry, J. J., and P. W. Choppin. 1978. Biological properties of the VSV glycoprotein. I. Effects of the isolated glycoprotein on host macromolecular synthesis. *Virology.* **84:**172.
35. McSharry, J. J., R. W. Compans, and P. W. Choppin. 1971. Proteins of vesicular stomatitis virus and of phenotypically mixed vesicular stomatitis virus-simian virus 5 virions. *J. Virol.* **8:**722.
36. Petri, W. A., and R. R. Wagner. 1979. Reconstitution into liposomes of the glycoprotein of vesicular stomatitis virus by detergent dialysis. *J. Biol. Chem.* **254:**4313.
37. Miller, D. K., B. I. Fever, R. Vanderoef, and J. Lenard. 1980. Reconstituted G protein-lipid vesicles from vesicular stomatitis virus and their inhibition of VSV infection. *J. Cell Biol.* **84:**421.
38. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193:**265.
39. Wagner, R. R. 1975. Reproduction of rhabdoviruses. *In* Comprehensive Virology. H. Fraenkel-Conrat and R. R. Wagner, editors. Plenum Press, New York. **4:**1.
40. Bloom, B. R., L. Jimenez, and P. Marcus. 1970. A plaque assay for enumerating antigen-sensitive cells in delayed type hypersensitivity. *J. Exp. Med.* **132:**16.
41. Bloom, B. R., A. Senik, G. Stoner, M. Ju, K. Nowakowski, S. Kano, and L. Jimenez. 1976. Studies on the interactions between viruses and lymphocytes. *Cold Spring Harbor Symp. Quant. Biol.* **41:**73.
42. Minato, N., and Y. Katsura. 1978. Virus-replicating T cells in the immune response of mice. II. Characterization of T-cells capable of replicating vesicular stomatitis virus. *J. Exp. Med.* **148:**837.
43. Dancey, G. F., P. C. Isakson, and S. C. Kinsky. 1979. Immunogenicity of liposomal model

membranes sensitized with dinitrophenylated derivatives containing different length spacers. *J. Immunol.* **122:**638.

44. Tadakuma, T., T. Yasuda, S. C. Kinsky, and C. W. Pierce. 1980. The effect of epitope density on the *in vitro* immunogenicity of hapten-sensitized liposomal model membranes. *J. Immunol.* **124:**2175.