SPECIFIC MURINE B-CELL ACTIVATION BY SYNTHETIC SINGLE- AND DOUBLE-STRANDED POLYNUCLEOTIDES*

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Nonspecific mitogens have provided valuable data regarding the mechanism of lymphocyte activation and cellular cooperation. Studies of the activation of murine lymphocytes by mitogens have shown that phytohemagglutinin (PHA)[1] and concanavalin A (Con A) are able to stimulate murine thymus-derived (T) cells but not bone marrow-derived (B) cells, whereas bacterial lipopolysaccharide (LPS) activated only B cells (1–3). The selective activation of lymphocytes by mitogens is not determined by the specific adherence of these materials to only B or T cells, since their binding has been shown to be nonspecific (4–6). In addition, the usual T-cell activation seen with PHA or Con A can be altered, allowing B-cell activation, when the molecular characteristics of these mitogens are changed by complexing them to an insoluble matrix such as sepharose (5, 7). This implies that the molecular structure of a mitogen is significant in the induction of B-cell activation. In this regard, it is of note that the B-cell activation with LPS has been ascribed to its unusual molecular structure (8, 9). Certain other observations support the concept that the physical properties of the stimulant are also important for B-cell activation leading to antibody formation. Thus, B cells are stimulated to form specific antibody by thymic-independent antigens such as LPS (10, 11), polyvinylpyrrolidone (PVP) (10), pneumococcal polysaccharide (SSS III) (12, 13), polymerized flagellin (POL) (14, 15), and the synthetic double-stranded RNA, polyinosinic-polycytidylic acid (poly I·C) (16, 17). These antigens are all characterized by a polymeric structure with repeating antigenic determinants.

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; FCS, fetal calf serum; LPS, bacterial lipopolysaccharide; LT, lymphotoxin; NRS, normal rabbit sera; PHA, phytohemagglutinin; POL, polymerized flagellin; poly C, polycytidylic acid; poly I, polyinosinic acid; PVP, polyvinylpyrrolidone; SSS III, pneumococcal polysaccharide; TxBM, thymectomized, bone marrow reconstituted.

Poly I·C has been shown to share many of the biologic actions of LPS. Both induce interferon production (18, 19), are pyrogenic (20) and toxic (21, 22), and both poly I·C and LPS act as potent immunological adjuvants (8, 23, 24). In addition, poly I·C has been shown to stimulate proliferation of murine lymphoid cells in vitro and in vivo (25, 26); however, these studies did not show whether poly I·C activated T cells or B cells. The characterization of poly I·C as a mitogen is of considerable importance to the understanding of the mechanism of cell activation. Thus, if poly I·C was able to act as a B-cell mitogen, this would support the concept that it is not the nature of the determinants but the unique polymeric presentation of the determinants of thymic-independent antigens that trigger B-cell activation.

In this communication we have examined the specificity of poly I·C as a mitogen. We have shown that the double-stranded polynucleotide poly I·C and the single-stranded polynucleotides poly I and poly C are specific B-cell mitogens.

Materials and Methods

Animals.—DBA/2N mice originally derived from those of the National Institutes of Health, Bethesda, Md. were inbred in our laboratory. C57Bl/10J, C57Bl/6J, C3H/HeJ, BALB/cJ, CBA/J, and B10.D2/J mice were obtained from Jackson Laboratory, Bar Harbor, Maine. Nude mice (Nu/Nu) were obtained from the National Institutes of Health. Groups of C3H/HeJ mice were thymectomized at birth or at 6 wk of age by aspiration through a sternum-splitting incision; littermates were sham operated. Thymectomized mice were irradiated (800 R) 7 days after surgery. They were reconstituted after irradiation with 20×10^6 syngeneic bone marrow cells that had been treated with AKR anti-theta C3H (anti-θ) serum (see antisera treatment of spleen cells for further details). All mice were sacrificed at 8–12 wk of age.

Mitogens and Culture Media.—RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.) with *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid buffer (25 mM) was used throughout the experiments. Before use, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (200 mM) were added. Polyinosinic acid (poly I), polycytidylic acid (poly C), and poly I·C were obtained from P. L. Biochemicals, Inc. (Milwaukee, Wis.; lot nos. 200-15, 191-15, and 247231, respectively). The poly I·C used in these experiments was complexed by P. L. Biochemicals, Inc. from the same lots of single-stranded nucleic acids used in these studies. Using aseptic technique, they were dissolved in supplemented RPMI 1640 at a final concentration of 1 mg/ml and stored at −20°C as stock solutions. Cultures of the poly I, poly C, and poly I·C, as shipped from P. L. Biochemicals, Inc., were inoculated in thioglycolate broth and incubated for 72 h at 37°C to verify their sterility.

The mitogens employed in these experiments were used at levels determined by preliminary experiments that gave optimum stimulation with mouse spleen cells. All the mitogens were diluted in RPMI 1640 stock solution and stored at −20°C until used. Phytohemagglutinin-P (PHA-P) (Difco Laboratories, Inc., Detroit, Mich.) was used at 0.05%; bacterial lipopolysaccharide *Escherichia coli* 0111:B4 (LPS) (Difco) was used at a concentration of 50 μg/ml; and concanavalin A (Con A) (Calbiochem, San Diego, Calif.) was used at a concentration of 1.25 μg/ml.

Cell Suspensions.—Mice were killed by cervical dislocation and spleens removed using aseptic technique. Spleen cells were obtained by gentle teasing with a rubber policeman. Cell aggregates were then dispersed by passing them through a 26 gauge needle, and single-cell suspensions were washed twice with media. Histological study of blastogenic transformation

induced by poly I, poly C, and poly I·C was studied by staining smears of cell suspensions (25×10^6 cells/ml) with Wright's Giemsa stain. Primitive cells were identified by their loose chromatin staining, large nucleolus, and size greater than 15 μm. Cell suspensions were incubated with 250 μg/ml of the polynucleotides for 48 h before staining and 500 mononuclear cells were counted to express an index of blastogenesis.

Spleen Cell Cultures.—Cultures were set up in sterile tissue culture microtiter plates (no. 3040; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) (27). Briefly, 100 μl of 8×10^6 spleen cells/ml in media was added to each microculture well. In addition, 50 μl of 20% heat-inactivated (56°C, 30 min) fetal calf serum (FCS) (Grand Island Biological Co.) in media, and 50 μl of media alone or media containing the mitogen being studied were added. Each well contained a total of 800,000 spleen cells in a total volume of 200 μl with 5% FCS final concentration. The microtiter plates, covered with sterile raised lids (Linbro no. 55; Bellco Glass, Inc., Vineland, N.J.), were cultured for the appropriate lengths of time in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. 18 h before harvesting, 20 μl of media containing 1 μCi of [*methyl*-^{3}H]thymidine ([^{3}H]TdR) 1.9 Ci/mM (Schwarz/Mann, Orangeburg, N.Y.) was added to each well. Unless otherwise noted, the cultures were harvested at 48 h using the multiple automated sample harvester (MASH), described in previous reports (27). Samples were then transferred to 1-dram vials and 3 ml of scintillation fluid, toluene containing Liquifluor (New England Nuclear, Boston, Mass.), was added. The samples were counted in a Beckman Model L-S 250 liquid scintillation counter. Data were analyzed using a Wang 700C program calculator. The final data, counts per minute (cpm), are reported as the mean of triplicate samples, plus or minus the standard error (±SE).

Antisera.—Rabbit anti-poly I·C was a gift of Dr. L. Parker of the National Institutes of Health. It was prepared by immunization of New Zealand White rabbits with a complex of 100 μg of poly I·C and 100 μg of methylated bovine gamma globulin, emulsified with an equal volume of complete Freund's adjuvant (28). Rabbit anti-lipid A was a gift of Dr. W. Grace of the National Naval Medical Center and was prepared by immunization of New Zealand White rabbits with a pure preparation of lipid A complexed to bovine serum albumin and emulsified with an equal volume of complete Freund's adjuvant. Rabbit anti-MBLA sera (anti-MBLA) was prepared according to the method of Raff (29), and AKR anti-theta C3H sera (anti-θ) was prepared according to the method of Reif and Allen (30). All antisera were inactivated at 56°C for 30 min. The anti-lipid A and anti-poly I·C were absorbed by three incubations at 37°C for 30 min with an equal volume of mouse liver powder.

Antisera Treatment of Cell Populations.—Spleen cells (25×10^6) were incubated with 0.1 ml of anti-θ sera or 0.2 ml of anti-MBLA at 37°C for 30 min to a final volume of 1 ml in media. Controls consisted of normal AKR mouse sera or normal rabbit sera (NRS) that had been similarly heat inactivated and absorbed with mouse liver powder. After the initial incubation, 0.2 ml of NRS, which had been shown to be not cytotoxic to mouse spleen cells, was added as a source of complement, and the cells were incubated at 37°C for an additional 30 min. Viable cells were recovered from cellular debris by layering the treated cells, suspended in 2 ml of media, over a 7 ml Ficoll-Hypaque gradient (sp gr 1.099). The layered cells were centrifuged at 450 *g* for 40 min. The cells at the interface were recovered, washed twice, and resuspended at 8×10^6 cells/ml.

Treatment of Mitogens with Antisera.—In order to determine if the mitogenicity observed with poly I·C was due to LPS contamination, LPS and poly I·C at different concentrations were incubated with either anti-lipid A antibody or anti-poly I·C antibody in media in a total volume of 0.6 ml for 1 h at 37°C followed by 4°C for 18 h. The tubes were then spun in an ultracentrifuge (Damon/IEC, motor head A-321) at 150,000 *g* for 1 h, and the supernatant fluid was passed through a 0.22 μm Swinnex filter and assayed for mitogen activity. Control tubes containing NRS were run in parallel.

Lymphotoxin Assay.—10 million DBA/2N mouse spleen cells/ml were suspended in media

containing 10% FCS. To this was added an equal volume of media containing 0.1% PHA-P, 25 μg/ml Con A, 100 μg/ml LPS, and 500 μg/ml poly I·C, or media containing no mitogen to a total final volume of 5 ml. The cells were incubated for 4 days at 37°C in a 5% CO^2 humidified atmosphere. Supernatant fluids were obtained by centrifugation of the cultures at 800 *g* for 15 min. The supernatant fluids were dialyzed in media using a minicon-B (B-10) clinical sample concentrator (Amicon Corp., Lexington, Mass.). Samples were stored at −20°C until used.

Lymphotoxin (LT) was assayed by a recently developed microassay procedure.[2] Mouse L-929 cells were maintained and used in media containing 10% FCS and 2.5 μg per ml Amphotericin B (Grand Island Biological Co.). Monolayers of L cells were detached by trypsinization, suspended in media, counted, and diluted to 20,000 L cells/ml. 50 μl of L cells (1,000 cells) was added to each well of a microtiter plate using a Hamilton microsyringe fitted with a repeating dispenser. After allowing the L cells to attach for 3–4 h, 50 μl of twofold serial dilutions of the spleen cell supernatant fluids was added to triplicate cultures. The plates were incubated for a total of 96 h and 24 h before harvesting 1 μCi of [^{3}H]thymidine was added to each well as noted before. The harvesting procedure consisted of removing the media from each well by aspiration using a 20 ml syringe fitted with a 26 gauge needle. To each well was added 50 μl of a 0.5% solution of trypsin 1:200 (Difco Laboratories) and plates were incubated at 37°C for 15 min. Cells in each well were harvested and counted for [^{3}H]thymidine as previously described. Data are presented as the dilution that shows 50% inhibition of growth of L cells (ID_{50}) when mitogen-stimulated or unstimulated lymphocyte supernatant fluids were compared with control wells containing only media.

RESULTS

Thymidine Incorporation Induced by Poly I, Poly C, and Poly I·C.—In a previous study using murine spleen cells, poly I·C was shown to be a mitogen at 10, 25, and 50 μg/ml (26). In order to study the response to poly I·C in the microculture system, female DBA/2N and C3H/HeJ mouse spleen cells were incubated with varying concentrations of poly I·C for 48 h as shown in Fig. 1. In the spleen cells from the C3H/HeJ strain, there were only minor increases in the thymidine incorporation with concentrations of poly I·C greater than 5 μg/ml. The spleen cells from the DBA/2N strain showed a gradual increase in incorporation that reached a plateau at 250 μg/ml of poly I·C. Increasing the number of DBA/2N cells from 200,000 to 800,000 per culture caused a linear increase in responsiveness to poly I·C (Fig. 2). All further studies were carried out with 800,000 cells per culture unless indicated.

It has been noted (25) that the single-stranded polynucleotides, poly I and poly C, when given in vivo at 200 μg daily for 3 days, did not cause an increase in the percentage of primitive lymphoid cells in blood films. Under similar conditions, poly I·C caused 30% of the lymphoid cells to appear as primitive cells. These findings suggested that poly I and poly C might not stimulate thymidine incorporation in vitro. For this reason and to help define the possibility of LPS contamination of poly I·C (which was prepared from the single-stranded poly I and poly C), cultures were set up using poly I, poly C, and poly I·C at 5, 50,

[2] Knudsen, R. C., A. Ahmed, and K. W. Sell. 1973. A microassay for lymphotoxin using microtiter plates and a multiple automated sample harvester. Manuscript in preparation.

250, and 500 μg/ml for 48 h (Fig. 3). Poly I and poly C both were mitogenic at all concentrations from, 5 to 500 μg/ml, and poly I was more stimulatory than poly I·C at all concentrations studied with a maximum at 250 μg/ml. Poly C was less stimulatory than poly I·C at the concentrations studied. In efforts to determine the optimum incubation time, 250 μg/ml of poly I·C was incubated with DBA/2N spleen cells for 24, 48, 72, and 96 h. As shown in Fig. 4, mitogenic

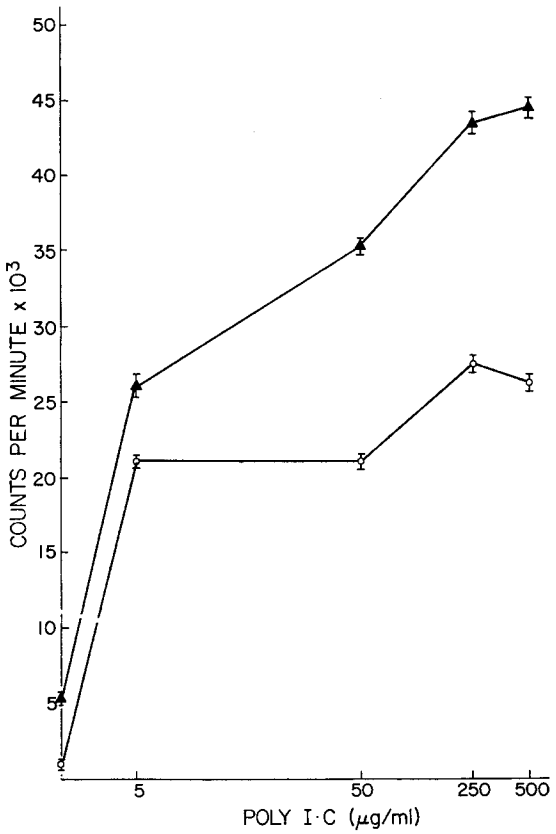

FIG. 1. The uptake of [³H]TdR by C3H/HeJ (○—○) and DBA 2N (▲—▲) spleen cells (800,000 cells/culture) in response to varying concentrations of poly I·C at 48 h.

activity was seen as early as 24 h and continued up to 96 h with the maximum activity at 48 and 72 h.

The ability of these polynucleotides to stimulate thymidine incorporation paralleled their ability to transform murine spleen cells as determined by histologic examination. Thus, poly I transformed 33.6% of the mononuclear cells, whereas poly I·C and poly C transformed 20.4 and 18.6%, respectively. The response to LPS was in the same range at 24.4%, and control cultures had 6% primitive cells.

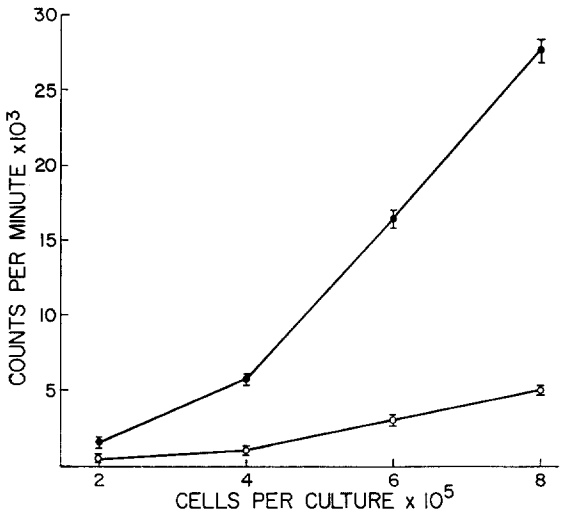

FIG. 2. The uptake of [^{3}H]TdR by varying numbers of DBA/2N spleen cells in response to 250 μg/ml of poly I·C cultured for 48 h. (Control culture, ○—○; poly I·C culture, ●—●.)

Demonstration of the Lack of Contamination of Poly I·C with LPS.—The synthetic polyribonucleic acids are prepared with a *Micrococcus lysodeikticus* polynucleotide phosphorylase. In view of the enzyme source and the ability of these polynucleotides to stimulate mouse cells, we were concerned that they were contaminated with LPS. In order to study this problem, antisera obtained from rabbits immunized against lipid A or poly I·C were used to determine if they would inhibit the mitogenicity of LPS and/or poly I·C.

When anti-lipid A antibody was incubated with varying concentrations of LPS and then ultracentrifuged, the supernatant fluids, when tested for mitogenicity, gave approximately 5,000 cpm of [^{3}H]TdR incorporation, as compared with 44,357 to 69,120 cpm for the NRS-treated controls (Fig. 5 *a*). Treatment of LPS with anti-poly I·C antibody did not alter its mitogenicity when compared with LPS treated with NRS. When anti-poly I·C antibody was used to treat poly I·C, however, there was a marked reduction in the mitogenic response (Fig. 5 *b*). Finally, and most important, anti-lipid A antibody treatment did not alter the mitogenicity of poly I·C.

Strain Differences in the Response to Poly I, Poly C, and Poly I·C.—Dean, Wallen, and Lucas (26) reported that while CD-1 and C3H mice strains responded to poly I·C, BALB/c mice did not. In order to further define the interstrain difference in the response to synthetic ribonucleotides, poly I, poly C, and poly I·C were incubated with spleen cells derived from male C57Bl/6J, C57Bl/10J, B10.D2/J, BALB/cJ, C3H/HeJ, and CBA/J mice. Spleen cells from four mice of each strain were cultured individually in triplicate for 24, 48, 72, and

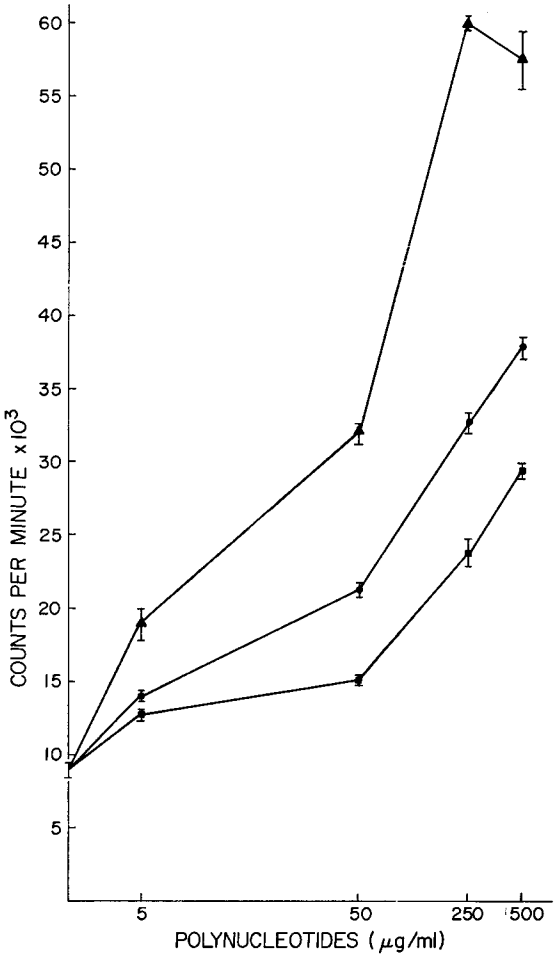

FIG. 3. The uptake of [^{3}H]TdR by DBA/2N spleen cells in response to varying concentrations of poly I (▲—▲), poly C (■—■), and poly I·C (●—●) at 48 h.

96 h. The mean of the four individual animals is expressed with the standard error of all 12 cultures as shown in Fig. 6. Poly I and poly C and poly I·C stimulated thymidine incorporation in spleen cells from all of the mouse strains studied, although there was considerable difference in the stimulation between strains. These studies support the interstrain differences noted by Dean et al. (25); however, contrary to their findings, we did see stimulation by poly I·C in spleen cells from the BALB/c strain. The very low variation in the response to these mitogens among different animals in the same strain is also shown in Fig. 6.

Effect of Anti-Theta or Anti-MBLA Treatment of DBA/2N Spleen Cells on the

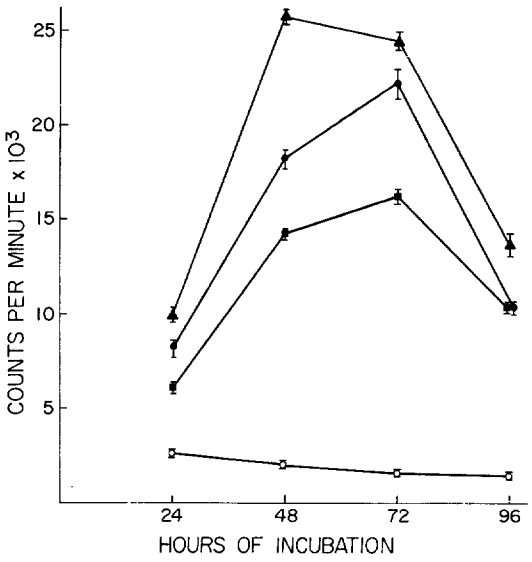

FIG. 4. The uptake of [³H]TdR by DBA/2N spleen cells in response to poly I (▲—▲), poly C (■—■), and poly I·C (●—●) at varying hours of incubation (control ○—○).

Mitogenicity of Poly I, Poly C, and Poly I·C.—In order to study the role of T and B cells on the mitogen response to poly I, poly C, and poly I·C, DBA/2N spleen cells were incubated with either mouse anti-θ or rabbit anti-MBLA and rabbit complement. Control incubations with complement and normal mouse and rabbit serum were run in parallel. Table I illustrates the actions of poly I, poly C, poly I·C, PHA-P, Con A, and LPS on 600,000 anti-θ- and complement-treated spleen cells. As expected, the response to PHA and Con A was reduced to less than control values by this treatment, while the response to LPS was only slightly diminished from 93,211 to 83,481 cpm. The mitogenic response to poly I, poly C, and poly I·C showed a significant increase from 17,314 to 26,328, from 10,512 to 15,226, and from 13,671 to 18,329 cpm, respectively, in the spleen cultures depleted of θ-bearing cells.

The effect of anti-MBLA treatment on responsiveness to the polynucleotides, PHA-P, Con A, and LPS, is shown in Table II. Anti-MBLA treatment decreased the response to LPS, poly I, poly C, and poly I·C to levels similar to that of the controls. The responses to PHA-P and Con A were only slightly decreased.

Effect of Thymectomy and Bone Marrow Reconstitution on the Response of C3H/HeJ Mouse Spleen Cells to Poly I, Poly C, and Poly I·C.—The ability of spleen cells from thymectomized, bone marrow-reconstituted (TxBM) mice to respond to poly I, poly C, poly I·C, PHA-P, Con A, and LPS were determined. There was no significant stimulation of spleen cells derived from these animals

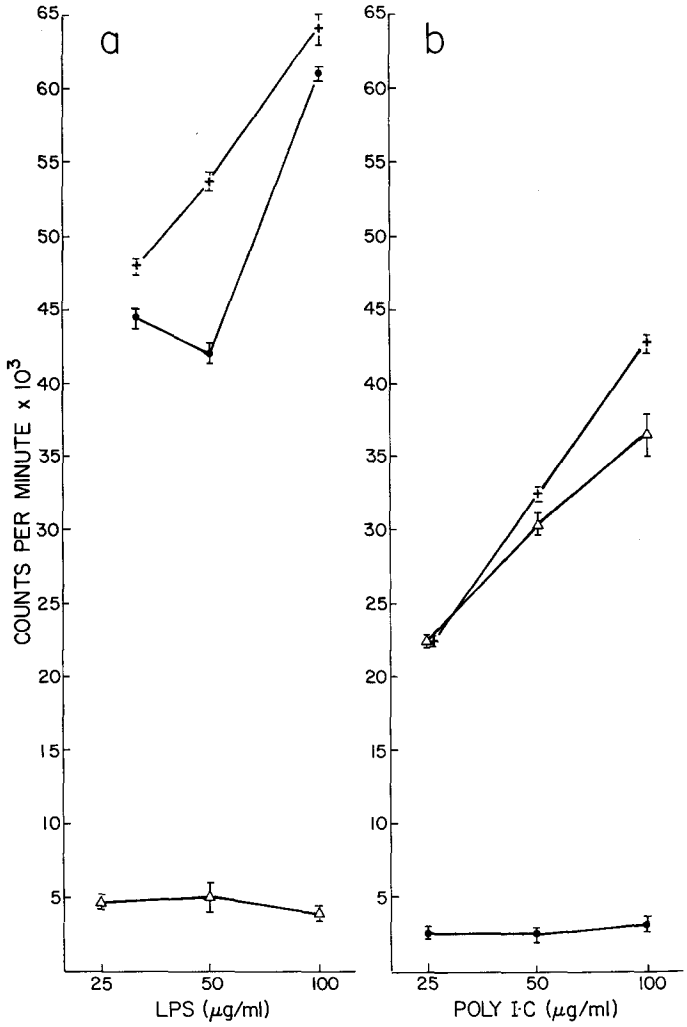

FIG. 5. The uptake of [^{3}H]TdR at 48 h by DBA/2N spleen cells in response to LPS and poly I·C treated with anti-lipid A or anti-poly I·C. (*a*) Anti-lipid A + LPS (△—△), anti-poly I·C + LPS (●—●), control NRS + LPS (+—+). (*b*) Anti-lipid A + poly I·C (△—△) anti-poly I·C + poly I·C (●—●), control NRS + poly I·C (+—+).

with PHA-P concentrations ranging from 0.001 to 1%. Thus, as seen in Table III, depletion of T cells in TxBM C3H/HeJ decreases the response to PHA-P and Con A to control levels and increases the LPS response. The responses to poly I, poly C, and poly I·C are also increased from 18,429 to 28,290, from 14,259 to 20,299, and from 16,582 to 23,698 cpm, respectively, in the TxBM animals when compared with sham-operated controls. When the spleens from

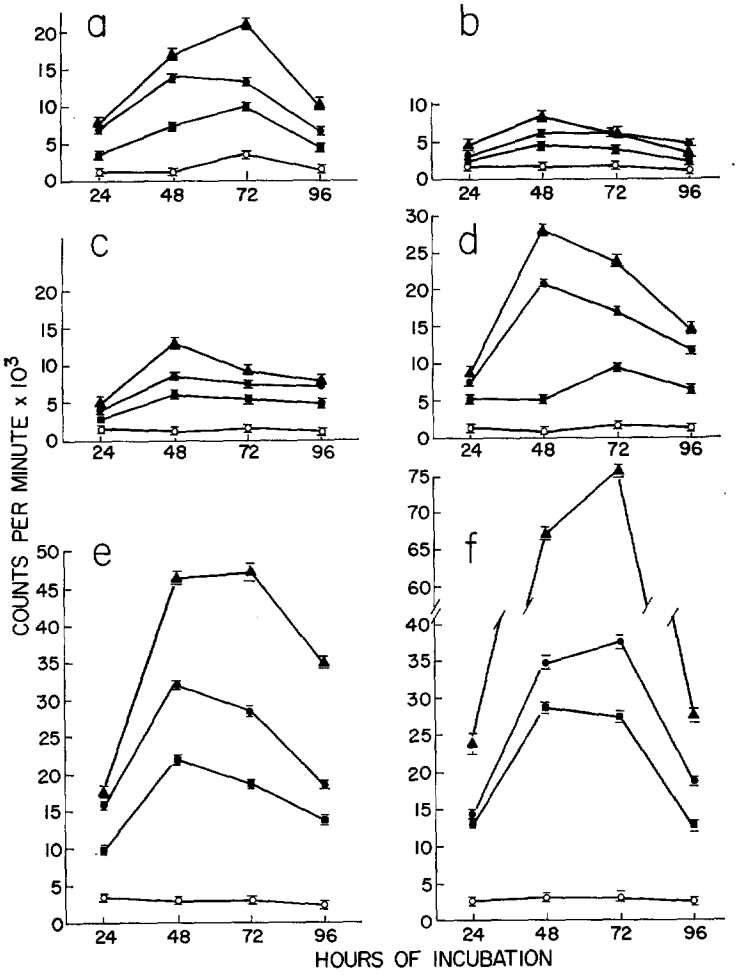

FIG. 6. The uptake of [^{3}H]TdR by (*a*) C57Bl/10J, (*b*) C57Bl/6J, (*c*) BALB/cJ, (*d*) B10.D2/J, (*e*) C3H/HeJ, and (*f*) CBA/J spleen cells in response to poly I ▲—▲, poly C ■—■, poly I·C ●—●, and media alone ○—○ at various hours of incubation.

the TxBM mice were treated with anti-θ and complement, there was no significant change in the response to poly I, poly C, or poly I·C when compared with the untreated TxBM cells. However, after anti-MBLA treatment of TxBM cells a marked decrease of thymidine incorporation with all mitogens tested was observed.

Ability of Spleen Cells from Nu/Nu Mice to Respond to Poly I, Poly C, and Poly I·C.—Spleen cells derived from Nu/Nu mice were unable to respond to PHA-P in concentrations ranging from 0.001 to 1%. The response of these cells to the other mitogens and PHA-P at 0.05% is shown in Table IV. No mitogenic

TABLE I

[^{3}H]TdR Uptake of Anti-θ-Treated DBA/2n Spleen Cells in Response to Mitogens at 48 h

Mitogen	Concentration	Uptake of [^{3}H]thymidine		*P* value
		NMS treated*	Anti-θ treated*	
		cpm ‡ (% SE)		
Control		2,596 (4.8)	2,466 (9.0)	—
PHA-P	0.05%	75,065 (3.2)	1,092 (8.9)	—
LPS	50 μg/ml	93,211 (1.6)	83,481 (1.5)	<0.01
Con A	1.25 μg/ml	113,853 (8.5)	573 (15.8)	—
Poly I	250 μg/ml	17,314 (3.4)	26,328 (5.5)	<0.001
Poly C	250 μg/ml	10,512 (8.1)	15,526 (6.3)	<0.01
Poly I·C	250 μg/ml	13,671 (3.3)	18,329 (2.6)	<0.001

* DBA/2N spleen cells treated with anti-θ or NMS and complement and then purified by Ficoll-Hypaque gradient centrifugation.

‡ Percent standard error of triplicate cultures.

TABLE II

[^{3}H]TdR Uptake of Anti-MBLA-Treated DBA/2n Spleen Cells in Response to Mitogens at 48 h

Mitogen	Concentration	Uptake of [^{3}H]thymidine	
		NRS treated*	Anti-MBLA treated*
		cpm‡(% SE)	
Control		2,596 (4.8)	3,374 (6.5)
PHA-P	0.05%	78,035 (1.4)	65,698 (3.6)
LPS	50 μg/ml	78,850 (1.7)	2,725 (11.2)
Con A	1.25 μg/ml	94,500 (2.7)	88,283 (2.2)
Poly I	250 μg/ml	13,857 (6.9)	4,072 (1.7)
Poly C	250 μg/ml	7,446 (4.8)	2,144 (6.0)
Poly I·C	250 μg/ml	12,791 (2.9)	2,462 (7.6)

* DBA/2N spleen cells treated with anti-MBLA or NRS and complement and purified by Ficoll-Hypaque gradient centrifugation.

‡ Percent standard error of triplicate cultures.

activity was seen with either PHA-P or Con A, whereas there was a good response to LPS, poly I, poly C, and poly I·C. Treatment of the Nu/Nu spleen cells with anti-θ and complement had little effect on the response to these mitogens, while anti-MBLA and complement treatment reduced the responses of all the mitogens tested to control levels.

Ability of Poly I, Poly C, Poly I·C, LPS, PHA-P, and Con A to Stimulate Lymphotoxin Production.—Table V shows the levels of LT produced by DBA/2N, Nu/Nu, and TxBM with PHA-P, Con A, poly I·C, and LPS. PHA-P and Con A stimulated DBA/2N spleen cells to produce approximately equivalent amounts of LT with titers of 105 and 100, respectively. However, neither LPS nor poly I·C stimulated any detectable levels of LT in these mice. Spleen cells

TABLE III

[^{3}H]TdR Uptake of TxBM, TxBM + Anti-Theta-Treated, TxBM + Anti-MBLA-Treated, and Control C3H/HeJ Spleen Cells in Response to Mitogens at 48 h

Mitogen	Concentration	Uptake of [^{3}H]thymidine			
		Normals*	TxBM*	TxBM* + anti-θ treated	TxBM* + anti-MBLA treated
		cpm‡ (% SE)			
Control		1,697 (5.0)	705 (4.7)	1,076 (6.8)	1,183 (6.6)
PHA-P	0.05%	58,881 (3.3)	1,247 (8.0)	1,212 (13.8)	1,478 (1.6)
LPS	50 μg/ml	68,239 (2.2)	90,775 (1.6)	128,064 (3.1)	3,036 (15.3)
Con A	1.25 μg/ml	64,543 (2.3)	896 (2.8)	889 (7.2)	2,116 (29.1)
Poly I	250 μg/ml	18,429 (3.2)	28,290 (1.9)	29,349 (1.3)	1,613 (46.3)
Poly C	250 μg/ml	14,295 (3.9)	20,229 (2.6)	16,775 (5.8)	1,978 (11.4)
Poly I·C	250 μg/ml	16,582 (3.6)	23,689 (3.8)	22,864 (3.0)	1,354 (29.1)

* C3H/HeJ TxBM spleen cells were used untreated, treated with anti-θ, or anti-MBLA and complement and purified by Ficoll-Hypaque gradient centrifugation.

‡ Percent standard error of triplicate cultures.

TABLE IV

[^{3}H]TdR Uptake of Nu/Nu, Nu/Nu Anti-θ-Treated and Nu/Nu Anti-MBLA-Treated Spleen Cells in Response to Mitogens at 48 h

Mitogen	Concentration	Uptake of [^{3}H]thymidine		
		Nu/Nu*	Nu/Nu* + anti-theta treated	Nu/Nu* + anti-MBLA treated
			cpm‡ (% SE)	
Control		1,228 (5.2)	961 (2.6)	584 (20.3)
PHA-P	0.05%	1,736 (2.9)	874 (3.5)	336 (6.3)
LPS	50 μg/ml	105,811 (3.4)	125,152 (3.0)	448 (4.9)
Con A	1.25 μg/ml	1,564 (5.8)	744 (1.7)	346 (5.3)
Poly I	250 μg/ml	30,630 (2.4)	21,117 (4.4)	741 (3.4)
Poly C	250 μg/ml	18,290 (3.1)	16,372 (4.4)	433 (3.6)
Poly I·C	250 μg/ml	24,711 (3.8)	18,408 (2.7)	687 (2.3)

* Nu/Nu spleen cells were used untreated, treated with anti-θ, or anti-MBLA serum and complement and purified by Ficoll-Hypaque gradient centrifugation.

‡ Percent standard error of triplicate cultures.

from nude mice (Nu/Nu) and TxBM mice failed to produce detectable levels of LT when incubated with any of the mitogens noted in Table V.

DISCUSSION

The initial reaction of lymphocytes with mitogens takes place via cell-surface receptors (4–6, 31). The density and distribution of binding sites on T and B cells for mitogens appear to be similar (4–6), and recent evidence suggests that lymphocyte activation requires that the stimulants are at least divalent (14). Thus monomeric

TABLE V

Lymphotoxin Production by DBA/2N Spleen Cells Stimulated with Mitogens

Mitogen	Concentration	Reciprocal of ID_{50}*		
		DBA/2N	Nu/Nu	TxBM
Control		0	0	0
PHA-P	0.05%	105	0	0
LPS	50 μg/ml	0	0	0
Con A	1.25 μg/ml	100	0	0
Poly I·C	250 μg/ml	0	0	0

* The reciprocal of the dilution which shows 50% inhibition of [^{3}H]thymidine incorporation when compared with media control.

(Fab) anti-immunoglobulin does not alter receptor distribution (32), neither does it trigger a proliferative response (33, 34). These monomers do, however, bind to the cell surface; and, if they are bridged with antiglobulin, a response is induced. These results suggest that a critical degree of cross-linkage of receptors is obligatory for initiation of lymphocyte responses. It is highly probable that the same requirements are made of antigens since free haptens uniformly fail to activate and mono-substituted hapten-carrier protein conjugates bind but fail to alter receptor distribution on antihapten-reactive cells (35). This requirement is also reflected in the observation that the size and density of antigenic determinants (14) has a profound influence on immunogenicity in vivo and in vitro and may provide the rationale of cell interactions in immunity. This interpretation focuses attention on the early cell-surface events in lymphocyte triggering and carries with it the implication that an effective response may relate to the pattern of determinant-receptor interaction (e.g. extent of cross-linkage) (14, 36).

The well-known ability of the thymic-independent antigen LPS to activate B cells (1–3) raised the question if this trait were shared by other thymic-independent antigens. It is of note that in this regard the thymic-independent antigens, SSS III and POL, are able to activate B cells in preliminary experiments conducted in our laboratory. However, at least one thymic-independent antigen, PVP, has been suggested not to be a mitogenic agent (2) and this has been confirmed by us.

Poly I·C has been shown to be a thymic-independent antigen by its ability to induce antibody in TxBM mice. In addition, treatment with rabbit antimouse thymocyte serum results in an augmented antibody response to poly I·C (17). In the immune deficient CBA/HN mouse strain (37), a presumed B-cell defect is associated with an inability to form antibody to poly I·C (38). In this strain antibody to poly I·C can be induced by complexing the polynucleotide with methylated bovine gamma globulin. These studies suggest that poly I·C can interact with B cells directly, inducing antibody formation without T-cell cooperation. However, T cells appear to be able to influence the formation of antibody against poly I·C either by acting as suppressor cells (17), or by cooperating with B cells when the poly I·C is complexed to a protein carrier (28, 38).

Experiments designed to determine the role of B and T cells in the mitogenic

activity induced by poly I, poly C, and poly I·C indicated that T cells were not stimulated directly by these polynucleotides and that T cells were not necessary for B-cell activation. This conclusion was based on the response of Nu/Nu and C3H/HeJ TxBM mouse spleen cells and anti-theta-treated DBA/2N spleen cells to the three polynucleotides. The failure of PHA and Con A to stimulate these cultures confirmed the relative depletion of T cells. It is unlikely that small numbers of residual T cells could have influenced the response to poly I, poly C, or poly I·C in view of the inability of anti-θ and complement treatment of the TxBM or Nu/Nu cells to change these responses (Tables III and IV).

Bacterial LPS contamination was not considered in the previously reported studies of the mitogenicity of poly I·C (25, 26). However, its presence as a contaminant was a possibility in view of the bacterial source of the polyribonucleotide phosphorylase used in the preparation of the synthetic polynucleotides. The ability of poly I·C to act as a B-cell mitogen is central to the main theme of interest of this paper, namely, that thymic-independent antigens other than LPS might also act as B-cell mitogens. The evaluation of LPS contamination of poly I·C was initially approached by examining poly I and poly C for mitogenic activity. It was reasoned that if both the poly I and poly C from which the poly I·C was complexed were not mitogenic, then the activity of poly I·C could not be secondary to LPS. That this was not the case was clearly demonstrated in Fig. 3, where it is shown that both poly I and poly C are mitogenic and that poly I is more stimulatory than the double-stranded poly I·C. Thus, this approach did not answer the question of LPS contamination. Therefore, in order to study this problem by a different method, rabbit antibody directed against poly I·C was incubated with LPS and poly I·C. When the supernatant fluid of these mixtures was assayed for mitogenicity, the activity of poly I·C was abolished, while the activity of the LPS was slightly decreased (Fig. 5). This experiment does not conclusively exclude the possibility that the poly I·C was contaminated with LPS of a different antigenic specificity than the LPS used as a mitogen in our experiments. However, reports have recently demonstrated that the mitogenic moiety of LPS was the lipid A portion of the molecule (9, 39, 40), and it has been shown that rabbit antiserum against lipid A cross-reacts with lipid A from 13 different gram-negative bacteria (41). The experience with the antiserum used in our studies was similar (unpublished data). It is likely, therefore, that if the mitogenic activity of poly I·C was secondary to a contaminating bacterial LPS, then anti-lipid A antibody would abolish this activity. Fig. 5 demonstrates that anti-lipid A has little effect on the activity of poly I·C at concentrations of 25, 50, and 100 μg/ml. As expected, it abolished the activity of LPS. The inability of the multispecific anti-lipid A to abolish the mitogenic activity of poly I·C is strong evidence against its contamination with LPS.

Examination of the data derived from experiments with TxBM and anti-θ-treated spleen cells reveals a significant increase in the response to all three poly-

nucleotides in these spleen cell cultures when they were compared with normal controls (Tables III and VI). These results could be explained by the increased percentage of B cells in these experiments or alternatively by the presence of a suppressor T cell in the untreated control mice. If is of interest to note that augmentation of the antibody response to the thymic-independent antigens PVP, keyhole limpet hemocyanin (KLH) (42), SSS III (43), and poly I·C (17) by treatment of mice with antilymphocyte serum has been shown. These studies have been interpreted as demonstrating the presence of T cells that acted to suppress the antibody response of B cells to these antigens.

The difference in the mitogenicity of these polynucleotides among different strains is most interesting. At least four factors could explain the differences between the inbred strains: (*a*) differences in the absolute numbers of B cells per spleen, (*b*) maturational differences in B-cell function, (*c*) differences in the numbers or activity of suppressor cells, (*d*) differences in the ability of B cells to be activated or to recruit other B cells. Reported percentages of immunoglobulin-bearing spleen cells in various mouse strains have shown only small differences between "normal" strains (44, 45). The number of B cells per spleen could probably not account for the strain differences noted for the mitogenicity of these polynucleotides. If B-cell maturation is not complete at birth and the rate of maturation varies between strains, this could explain the differences in response to poly I, poly C, and poly I·C in these strains. In this context, it is interesting to note that the ability of T cells to react to Con A (46) and to respond in mixed lymphocyte cultures (47) does not reach maturity until 6–8 wk and 14–20 wk, respectively. Studies of the ability of antithymocyte serum to enhance the immune response to SSS III in mice have shown differences between strains (48). These experiments could be explained by interstrain differences in the activity or numbers of suppressor T cells. Data regarding the ability of B cells to be activated or to recruit other B cells are not available.

The data in Table V demonstrate that both PHA-P and Con A stimulate spleen cells to produce LT. Nude and TxBM mice did not produce LT in response to any of the mitogens. This strongly suggests that LT production is a property of T cells, and the inability of LPS or poly I·C to stimulate LT production by these cells is further evidence that poly I·C is a B-cell mitogen. These data are in general agreement with those obtained by Shacks et al. (49).

The mechanism of direct B-cell stimulation by antigens is unknown, but studies of flagellin by Feldmann and Basten (14) suggest that the ability of thymic-independent antigens to induce antibody is related to the multivalent character of their antigenic determinants. In a T-cell depleted culture of mouse spleen cells, antibody response to polymerized flagellin (POL) was noted, but there was no response to monomeric flagellin or to flagellin-coated red cells. In non-T cell-depleted cultures, the response to POL and monomeric flagellin were comparable. It has also been shown that POL, when mixed with a thymic-dependent antigen and cultured with spleen cells derived from Nu/Nu mice, influenced these T-deprived cultures to form antibody to the

normally thymic dependent antigen (50). This ability of POL to influence B cells to be directly stimulated by thymic-dependent antigens has been also noted for LPS (51).

The ability of the thymic-independent antigens poly I·C, POL, and SSS III to mimic the B-cell mitogenicity of LPS may be explained by the similar polymeric multivalent nature of the determinants of these materials. This suggests that the mechanism of B-cell triggering of antibody formation to these antigens and their mitogenic properties might both be related to their unusual molecular structure. The multivalent nature of their determinants could act to trigger specific B-cell antibody formation without T-cell cooperation under certain conditions, while under different circumstances B-cell mitogenesis could occur. Evidence for this has been demonstrated by Möller et al. (52) who showed that in order for LPS to influence T cell-deprived animals to make antibody against a thymic-dependent antigen (sheep red cells), the LPS had to be coated on the red cell. This property of LPS has been attributed to its ability to induce B-cell mitogenesis. In addition, Andersson et al. (7) have shown that the concentration of LPS needed to augment the B-cell response to an unrelated antigen and to induce B-cell mitogenesis was 10–100 times greater than that required for specific antibody formation. These studies and the inability of the thymic-independent antigen PVP to induce B-cell mitogenesis indicate that although the molecular structure of thymic-independent antigens are important for their antigenicity, other factors are also necessary for B-cell mitogenesis.

SUMMARY

The synthetic single- and double-stranded polynucleotides, poly I, poly C, and poly I·C, were shown to induce thymidine incorporation in six inbred strains of murine spleen cells. This stimulation was shown to be secondary to B-cell activation and not due to contamination of the polynucleotides with bacterial lipopolysaccharide (LPS). The ability of poly I·C to act as a B-cell mitogen, in addition to its behavior as a thymic-independent antigen, suggested that these two phenomena may be related. The similarity of the molecular structure of poly I·C to LPS, a material which also acts as a thymic-independent antigen and a B-cell mitogen, supports the hypothesis that the polyvalent nature of these materials accounts for their functional interaction with murine B cells.

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