NOTES

Species-Dependent Response to Streptococcal Lymphocyte Mitogens in Rabbits, Guinea Pigs, and Mice

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Extracellular mitogen preparations from NY5 and S84 strains of streptococci induced different responses among rabbit, guinea pig, and mouse lymphocytes. Evidence of T-cell mitogenicity is presented.

Nonspecific mitogenic activity of streptococcal origin has been found extensively among group A bacteria, both in cellular extracts (9) and in extracellular products (14). Many studies have been carried out with human lymphocytes, but conditions under which the response was observed might have been influenced somewhat by prior sensitization (2, 7, 13), because group A streptococci are ubiquitous pathogens in humans. Thus, to obtain responses under unsensitized conditions, laboratory animals should be used. Systematic study, however, has not been fully developed yet. We report here a comparative study on rabbit, guinea pig, and mouse lymphocyte responses to extracellular streptococcal mitogens from NY5 and S84 strains of bacteria with parallel tests on concanavalin A (ConA), pokeweed mitogen (PWM), and *Escherichia coli* lipopolysaccharide (LPS) stimulation.

Rabbit thymus, spleen, and lymph node cells responded well to streptococcal mitogens (Table 1). Response to NY5 strain mitogen was higher than that to S84 mitogen and almost comparable with that to ConA. Thymocyte response proved the T-lymphocyte mitogenicity but was lower than those of spleen and lymph node cells. This could be possibly due to a preferential activity for peripheral T-cells or additional B-cell mitogenicity or both. Spleen cells did not respond to the highest dose of S84 mitogen (100 $\mu\text{g/ml}$), whereas lymph node cells did. This suggests some toxicity relating to the cellular composition of the spleen, which may also explain the lack of response to PWM and the marked toxic effect of LPS. Blastogenic response to LPS was not found even when tested further at lower doses (0.5 to 0.005 $\mu\text{g/ml}$ in 10-fold dilution) on peripheral blood lymphocyte cultures of two rabbits (data not shown). Peripheral blood lymphocytes

also responded well to streptococcal mitogens and ConA, showing wide individual variation from rabbit to rabbit (Table 2).

Since purified erythrogenic toxins have been reported to exhibit mitogenic activity (4, 8, 12, 13), the difference between NY5 and S84 strain mitogens described herein might be attributed to type-specific characteristics of the toxin produced. To assess this possibility, S84 strain material obtained after precipitation by ethanol (S84 alc) was tested. Mitogenic response was evident, although a slight decrease in the optimal dose (1 $\mu\text{g/ml}$) and prominent decrease in a higher dose (10 $\mu\text{g/ml}$) were found (Table 3). On the other hand, skin reaction to S84 mitogen after ethanol treatment was almost nonexistent. Erythematous reaction was measured at 24 h after intracutaneous inoculation of 0.1 ml of mitogen solution in the back skin. Only doubtful reaction was elicited in two of four rabbits tested by inoculation of 20 μg of S84 alc, and no reaction at all was elicited to 2 μg . However, the reaction was positive for untreated S84 mitogen in the same doses, inducing erythema ranging between 30 by 28 mm in diameter and 16 by 15 mm in diameter, and for NY5 mitogen (100 and 10 μg) with erythema ranging between 22 by 17 mm and 4 by 3 mm in the same rabbits. No reaction was induced by inoculation of a purified S84 mitogen (S84 pur, 2 μg). Thus, the S84 strain preparation was found to lose skin-sensitizing activity after ethanol precipitation while retaining mitogenic activity. This suggests the existence of a mitogen or mitogens distinct from erythrogenic toxins among group A streptococcal products, as has been reported by others (6, 7, 13).

The guinea pig lymphocyte response was different from that of rabbit cells. Spleen and lymph node cells showed definite but lower re-

TABLE 1. *Stimulation of rabbit thymus, spleen, and lymph node cell cultures^a*

Mitogen ^b	Dose ^c ($\mu\text{g}/\text{ml}$)	Increase of tritiated uridine uptake ^d		
		Thymus	Spleen	Lymph node
NY5 ^e	50	47,863	89,742	128,824
	5	30,902	78,704	107,151
	0.5	11,220	34,355	67,608
	0.05	-891	8,053	14,791
S84 ^f	100	12,302	-9	53,703
	10	14,791	28,379	95,499
	1	15,848	43,251	87,096
	0.1	10,232	47,097	83,176
S84 pur ^g	1	17,782	41,304	91,201
	0.1	12,882	54,075	91,833
ConA ^h	50	38,904	62,517	194,984
	5	39,810	93,972	85,113
	0.5	23	11,040	13,489
PWM ⁱ	50	4,042	7	40,738
LPS ^j	50	3,715	-22,387	-6
	5	-52	-13,803	8
None ^k		7,244	35,974	9,772

^a Male JW-NIBS strain rabbits (The Nippon Institute for Biological Sciences, Tachikawa, Tokyo) were used at 9 months of age. Thymus, spleen, and mesenteric lymph nodes were squeezed manually in a glass homogenizer containing Dulbecco divalent cation-free phosphate-buffered saline, pH 7.2. After removing gross clumps by 1G sedimentation and washing once in phosphate-buffered saline, cells were suspended in TC 199 medium supplemented by autologous inactivated plasma (5%) and distributed 1 ml each into small test tubes. Cell concentration was adjusted to $2 \times 10^6/\text{ml}$. After 2 days of culture, blastogenic response was assayed by [^3H]uridine uptake.

^b Mitogen (50 $\mu\text{g}/\text{ml}$) was added at the start of culture.

^c Doses of streptococcal mitogens from NY5 and S84 strain bacteria are shown in terms of protein concentration, those of ConA and LPS are shown in weight, and that of PWM is shown in volume (microliters). Protein content was determined by the Lowry method with bovine serum albumin as the standard (11).

^d [^3H]uridine (specific activity, 28 Ci/mmol, Radiochemical Centre, Amersham) was added, 1 $\mu\text{Ci}/\text{ml}$, for 17 h after 46-h culture. Incorporation of isotopes was measured by the liquid scintillation method. Geometric mean of Δ disintegrations per minute (stimulated - unstimulated cultures) in experiments on three rabbits is presented.

^e NY5 strain streptococci were grown in a diffusate medium (10) prepared from a digested peptone (Mikuni Chemicals, Tokyo). A 24-h culture supernatant was precipitated by ammonium sulfate at 2/3 saturation and successively by cold ethanol (3). Phosphate-buffered saline was used as the diluent.

^f S84 strain streptococci were grown to stationary phase for 48 h in Todd-Hewitt broth. The culture supernatant was first precipitated by ZnCl_2 added to 1% and followed by ammonium sulfate at 56% saturation. Streptolysin O was partially absorbed by calcium phosphate gel (1).

TABLE 1. *Continued*

^g A preparation obtained from growing phase culture supernatant and purified through Sephadex G50 filtration (1).

^h Grade III, Sigma Chemical Co.

ⁱ GIBCO Laboratories, Grand Island, N.Y.

^j *E. coli* O127:B8, Difco.

^k Geometric mean of the disintegrations per minute of unstimulated cultures.

TABLE 2. *Stimulation of rabbit blood lymphocyte cultures^a*

Mitogen	Dose ($\mu\text{g}/\text{ml}$)	No. of rabbits tested	Increase of tritiated uridine uptake ^b	
			Range	Geometric mean
NY5	50	16	319,615-17,714	81,283
	5	11	91,347-6,749	40,747
S84	10	16	221,681-9,207	52,480
	1	16	214,579-6,809	38,913
ConA	50	9	556,086-5,921	89,125
	5	5	130,683-53,412	75,857
None ^c		16	14,757-2,084	4,683

^a Male JW-NIBS strain rabbits were used at 6 to 9 months of age. Peripheral blood was drawn from the ear artery with heparin as the anticoagulant. Blood leukocytes were collected after erythrocyte sedimentation by polyvinyl alcohol, and granulocytes were deprived by adherence to nylon cloth as described previously (1). Culture was prepared in the same way as that of lymphoid organs, except that the cell number was adjusted to $10^6/\text{ml}$. Culture periods were 63 to 65 h in total, 46 to 48 h before 17-h incorporation of [^3H]uridine.

^b Δ Disintegrations per minute (stimulated - unstimulated cultures).

^c Disintegrations per minute of unstimulated cultures.

sponse than rabbit lymphocytes (Table 4). No response to S84 mitogen was found even when tested in a wide range of dosages (100 to 0.01 μg for S84 and 1 to 0.001 μg for S84 pur in 10-fold dilution, respectively) in either spleen or lymph node cells obtained from four animals whose response to NY5 mitogen was evident (data not shown). Spleen cell response to PWM, in contrast to rabbits, was in a degree comparable to that of lymph node cells. Response to ConA was as marked as that of rabbits. No response, but some toxic effect, was found for LPS in spleen and lymph node cells from two guinea pigs (data not shown). Since guinea pig thymus became involuted very early in this strain, even in neonates, the T-cell mitogenicity of NY5 product was tested by the peripheral T-cell depletion effect. This was performed by following the rabbit erythrocyte (RE) rosette method (16). Rosette-forming cells (RE-RFC) in the lymphoid cell suspension before culture ranged between

TABLE 3. Activity of S84 strain mitogen before and after ethanol treatment^a

Mitogen	Dose (µg/ml)	Increase of uridine uptake ^b
S84	10	33,884
	1	25,118
S84 alc ^c	10	13,803
	1	28,840
	0.1	14,791
	0.01	4,677
None ^d		4,897

^a Four experiments of rabbit blood lymphocyte culture from three rabbits are summarized. Culture period was 65 h in total, 48 h before 17-h incorporation of [³H]uridine. Ethanol precipitation of S84 strain mitogen was performed similarly as for the NY5 mitogen (3).

^b Geometric mean of Δ disintegrations per minute (stimulated - unstimulated cultures).

^c Ethanol-treated S84 strain mitogen; doses shown in terms of protein concentration.

^d Disintegrations per minute of unstimulated cultures.

TABLE 4. Stimulation of guinea pig spleen and lymph node cell cultures^a

Mitogen	Dose (µg/ml)	Increase of tritiated uridine uptake ^b	
		Spleen	Lymph node
NY5	500	18,620	21,379
	50	31,622	16,982
	5	30,902	15,488
	0.5	4,897	3,630
	0.05	1	4
ConA	50	114,815	144,543
	5	100,000	63,095
	0.5	6,165	12,882
PWM	50	14,125	16,982
None ^c		21,433	12,589

^a Strain 13 guinea pigs of both sexes were used at the ages of 2 to 4 months. Spleen and mesenteric lymph node cell culture was prepared in the same way as that of rabbits. TC 199 medium was supplemented by autologous plasma. Cell number in culture was 2×10^6 per ml. Experiments on five guinea pigs, three female and two male, are summarized. Culture period was 63 h in total, 46 h before 17-h incorporation of [³H]uridine.

^b Geometric mean of Δ disintegrations per minute (stimulated - unstimulated cultures).

^c Geometric mean of disintegrations per minute of unstimulated cultures.

11 and 37%. RE-RFC were completely removed after centrifugation through Ficoll-Paque solution (Pharmacia). Response to NY5 mitogen and PWM was totally abolished after RE-RFC depletion, whereas that to ConA remained to a slight degree (Table 5). This might have been

due to ConA stimulation of T-cells belonging to low affinity RE-RFC not detected in the present experiment. RE-rosette formation of blast cells generated by stimulation with NY5 mitogen and ConA was also tested. Most of the blast cells showed the presence of the T-cell marker (Table 6 and Fig. 1). T-lymphocyte stimulation by

TABLE 5. Decrease of guinea pig lymphocyte response after depletion of RE-RFC^a

Mitogen ^b	No. of tests		Increase of tritiated uridine uptake ^c	
	Spleen	Lymph node	Undepleted	Depleted
NY5	3	3	23,988	-12
ConA	3	2	107,646	5,272
PWM	2	1	9,272	-10

^a For removal of RE-RFC, 0.1 volume of 50% RE suspension in phosphate-buffered saline was added to 3 to 5 ml of lymphoid cell suspension. The mixture was stirred intermittently by hand during incubation at room temperature for 30 min or more, placed on 10 ml of Ficoll-Paque solution (Pharmacia) in a 40-ml centrifuge tube, and centrifuged at 1,000 rpm for 10 min. The supernatant, including the Ficoll-Paque layer, was poured into another tube, washed twice in phosphate-buffered saline, and suspended in TC 199 medium containing plasma. Cell number of the RE-RFC-depleted cultures was adjusted to the same density as that of untreated cultures (2×10^6 /ml). Experiments on three female guinea pigs are summarized. Culture period was 63 h in total, 46 h before 17-h incorporation of [³H]uridine.

^b Doses of mitogens were 50 µg for NY5 and ConA and 50 µl for PWM per ml of culture.

^c Geometric mean of Δ disintegrations per minute (stimulated - unstimulated cultures).

TABLE 6. Frequency of RE-RFC among blast cells in guinea pig lymphocyte cultures stimulated by NY5 strain mitogen or ConA^a

Mitogen ^b	No. of animals	No. of tests	% RE-RFC			
			Spleen	Lymph node	Range	Mean
NY5	4	6	82, 88, 86	78, 91, 72	72-91	83
ConA	4	5	88, 85, 94	72, 94	72-94	87

^a Percentage of RE-RFC among blast cells generated in a 3-day culture is given. RE were washed and suspended in phosphate-buffered saline. One-tenth volume of 5% RE suspension was added to washed blast cell suspension in phosphate-buffered saline. After centrifugation at 800 rpm for 3 min, the mixture was left at room temperature for 30 min or more, stirred gently by hand, and examined in wet preparation under a phase-contrast microscope. Cells attached to three or more erythrocytes were assessed as positive. Cells having prominent nucleolus and being at least twice as large in diameter as erythrocytes were assessed as blasts. One hundred or more blasts were counted in a single culture. Blasts centrally located in the large aggregate occasionally found in ConA-stimulated cultures were not counted.

^b Dose of mitogens was 50 µg/ml of culture.

FIG. 1. A guinea pig blast cell actively forming RE-rosette seen in 3-day culture stimulated by NY5 strain streptococcal mitogen. Note nuclear size, prominent nucleolus, and surface process attached to erythrocytes. (Phase-contrast microscopy; original magnification, 400 $\times$).

streptococcal mitogens has been reported from several laboratories (4-6, 15). Our findings in connection with rabbit thymocyte response to both NY5 and S84 mitogens and guinea pig peripheral T-cell response to the former also confirmed the T-cell mitogenicity.

Mouse lymphocyte response to streptococcal mitogens was similar to that of guinea pigs. Thymus and spleen cells showed a low but definite response to NY5 mitogen, but a doubtful response to S84 mitogen (Table 7). ConA produced marked response in thymus and spleen cells. LPS induced definite response in spleen cells, in contrast to rabbit and guinea pig cells. Apparent variation between the strains of mice tested, both in mitogen-stimulated and unstimulated cultures, was noted.

The studies described here show species-dependent response to streptococcal mitogens among rabbits, guinea pigs, and mice. Our previous report showed human lymphocyte response comparable with that of rabbit lymphocytes to S84 strain mitogen (2). Species-dependency is a characteristic feature of nonspecific bacterial mitogens. Phytomitogens, such as ConA and PWM, have no qualitative distinction among responding species, as also seen in the present study. The lymphoid system of a given species may be characterized by its responsiveness to bacterial mitogens.

TABLE 7. Stimulation of mouse thymus and spleen cell cultures^a

Mito-gen	Dose ^b (μ g/ml)	Increase of tritiated uridine uptake ^c			
		BALB/c thymus ^d	BALB/c spleen ^e	DBA/2 spleen ^f	BDF ₁ spleen ^g
NY5	500	15628	21424	85	NT ^h
	50	2972	20073	7011	87581
	5	NT	8	NT	5602
S84	100	NT	6754	191	NT
	10	609	8619	4224	9134
S84 pur	1	2733	1799	NT	3205
ConA	50	177322	34105	47426	42882
	5	NT	60344	NT	170723
PWM	50	12987	12168	NT	NT
LPS	50	1365	30937	NT	101502
	5	NT	NT	50990	90849
None		5500	7200	2373	44066

^a Each experiment was performed with thymus or spleen cells collected from two mice. RPMI 1640 medium supplemented with an inactivated rabbit serum (5%) was used. Otherwise, cultures were prepared similarly as those of rabbit and guinea pig lymphocyte cultures. Cell number was 2×10^6 /ml. Culture period was 63 to 65 h in total, 46 to 48 h before 3 H incorporation of [3 H]uridine.

^b Doses of NY5 and S84 strain mitogen are shown in terms of protein concentration; those of ConA and LPS in weight and that of PWM are shown in terms of volume (μ l).

^c Difference between average disintegrations per minute of stimulated and unstimulated cultures in duplicate or triplicate.

^d Male BALB/cJCL mice (The Central Laboratory for Experimental Animals, Kawasaki, Japan), 4 weeks old.

^e Male, 7 weeks old.

^f Male DBA/2NCrj mice (The Japan Charles River Co., Ltd., Atsugi, Japan), 12 weeks old.

^g Male F₁ hybrid mice by crossing female BALB/c and male DBA/2 mice, 12 weeks old, raised at the Laboratory Animal Center, Teikyo University School of Medicine.

^h NT, Not tested.

ⁱ Average disintegrations per minute of unstimulated cultures.

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