[Chem. Pharm. Bull. 29(12)3731—3734(1981)]

Mitogenic Effect of Lactobacilli on Murine Lymphocytes

TADAYORI SHIMIZU,*,[a] ICHIJI MIFUCHI,[a] and TERUO YOKOKURA[b]

Department of Microbiology, Shizuoka College of Pharmacy,[a] 2-2-1 Oshika, Shizuoka 422, Japan, and Yakult Institute for Microbiological Research,[b] 1796 Yaho, Kunitachi, Tokyo 186, Japan

(Received June 4, 1981)

The mitogenic activity of whole cells of 18 strains of lactobacilli toward splenocytes from C57BL/6 mice was studied. Although most of the strains showed no mitogenicity, *L. fermentum* and *L. plantarum* were capable of increasing the incorporation of ^{3}H-thymidine (^{3}H-TdR) into splenocytes. Both strains were also able to stimulate the incorporation of ^{3}H-TdR into splenocytes that had been treated with rabbit antithymocyte serum in the presence of complement in order to kill T-lymphocytes, but did not stimulate the incorporation into thymocytes. These results indicate that *L. fermentum* and *L. plantarum* cells act on the B-lymphocyte population of splenocytes.

Keywords——lactobacillus; mitogenic effect; ^{3}H-thymidine; C57BL/6; splenocyte; thymocyte

Various gram negative microorganisms and their components, particularly bacterial lipopolysaccharide (LPS), have been reported to have a mitogenic effect on lymphocytes in animals.[1] Grampositive bacteria such as mycobacteria, nocardia and corynebacteria, are also able to elicit the mitogenic effect.[2] Although lactobacilli are commonly found in the mammalian intestine and have been shown experimentally to stimulate a humoral immune response,[3] little attention has been paid to their mitogenic activity.[4]

Therefore, it seemed worthwhile to examine the mitogenic effect of the gram positive bacteria, lactobacilli, on lymphocytes of mice. In this paper, we describe the mitogenic effect of 18 strains representing 12 species of lactobacilli.

Experimental

Organisms——Bacterial strains obtained from the American Type Culture Collection or newly isolated and identified according to Bergey's Manual[5] were used. They are listed in Table I. The bacteria were cultured at 37°C for 48 h in Rogosa's medium.[6] The cells were harvested by centrifugation (8000 rpm, 15 min, 4°C), washed several times with distilled water and lyophilized.

Animals——Six- to 12-week-old C57BL/6 male mice (Shizuoka Agricultural Cooperative for Laboratory Animals, Hamamatsu) were used throughout the experiments.

Mitogens——As standard mitogenic substances, concanavalin A (Con A) and LPS were used. Con A was purchased from Boehringer Mannheim, West Germany, and LPS was extracted from *Salmonella typhimurium* LT-2 by the phenol-water method.[7]

Mitogenicity Assay——Spleen and thymus were removed and teased with forceps in cold Eagle's minimal essential medium (Nissui Seiyaku Co., Ltd., Tokyo). After brief sedimentation, the cells in the supernatant were collected, washed with Eagle's medium and then suspended in 0.83% NH_4Cl buffer for 1 to 2 min until the erythrocytes were lysed. The residual lymphocytes were washed with Eagle's medium and their viability was determined by trypan blue dye exclusion. To adjust the cell number, the lymphocytes were suspended in RPMI-1640 medium (GIBCO, Grand Island Biological Co., U.S.A.) supplemented with 10% fetal calf serum (GIBCO). The cell suspension (2×10^6/0.25 ml) and 0.15 ml of a suspension of heat-killed lactobacilli were placed in a sterile culture tube (16 × 125 mm) and the final total volume was adjusted with RPMI-1640 medium to 3 ml per tube. The suspension was incubated 37°C in a humidified CO_2 incubator (Type CO-MINI A-1, Tokiwa Co., Ltd., Tokyo) with a flow of 5% CO_2 in air. Each culture was pulsed with 1 μCi of ^{3}H-thymidine (^{3}H-TdR; thymidine-6-^{3}H, Radiochemical Centre, England) for the final 16 h of incubation. After incubation for 64 h, the cells were harvested on a glass fiber filter (Whatman, GF/C). The filters were washed with Hanks' solution and 5% trichloroacetic acid, and dried. The radioactivity taken up by the cells was measured with a scintillation counter (Aloka, LSC-661, Aloka Co., Tokyo). Triplicate tubes were

used for each test. The results are expressed as the mean of counts per min (CPM) per culture with the standard error.

ATS Treatment of Spleen Cells——Rabbit antithymocyte serum (ATS) was prepared according to the method of Gray *et al.*[8] The prepared ATS showed 2^9 and 2^3 cytotoxic titer for mouse thymocytes and bone marrow cells, respectively, when it was examined by the trypan blue dye exclusion method.[9] The splenocytes from C57BL/6 mice were incubated with suitably diluted ATS for 45 min at 37°C in the presence of complement (guinea pig serum) in order to kill T-lymphocytes in the splenocyte population.

Statistical Analysis——The significance of the results was analyzed by the use of Student's t test. P-values greater than 0.05 were considered not significant.

Results and Discussion

The results of mitogenic assays are summarized in Table I. Whole cells of some lactobacilli were definitely mitogenic for splenocytes, but most of the strains showed no mitogenicity at 100 μg/ml (Table I) and 10 μg/ml (data not shown).

Table I. Mitogenic Effect of Lactobacilli on Splenocytes of C57BL/6 Mice

Experiment No.	Bacterial strain		Dose (μg/ml)[a]	Mean±S.E. (cpm)	Stimulation rate[b]	p
7	*L. delbrueckii*	YIT 0080 (ATCC 9649)	100	212±13	0.7	
7	*L. leichmannii*	YIT 0087 (ATCC 4797)	100	445±45	1.5	$0.05<p<0.1$
5	*L. lactis*	YIT 0086 (ATCC 12315)	100	221±8	0.9	
4	*L. bulgaricus*	YIT 0046	100	361±31	1.5	$0.1<p<0.2$
5	*L. helveticus*	YIT 0083 (ATCC 15009)	100	181±63	0.7	
6	*L. acidophilus*	YIT 0163	100	281±14	1.5	$0.1<p<0.2$
2	*L. acidophilus*	YIT 0168	100	308±15	1.0	
7	*L. salivarius*	YIT 0153	100	306±64	1.0	
3	*L. salivarius*	YIT 0155	100	291±24	1.4	
2	*L. casei*	YIT 0078 (ATCC 393)	100	254±19	0.8	
2	*L. casei*	YIT 0105 (ATCC 7469)	100	278±85	0.9	
6	*L. casei*	YIT 0151	100	323±24	1.2	
1	*L. casei*	YIT 9018	100	431±16	1.2	
3	*L. plantarum*	YIT 0102 (ATCC 14917)	100	575±172	2.8	$0.02<p<0.05$
6	*L. plantarum*	YIT 0158	100	328±35	1.8	$0.05<p<0.1$
1	*L. fermentum*	YIT 0159	100	1617±25	4.6	$p<0.001$
1			50	1463±90	4.0	$p<0.001$
1			10	1429±21	3.9	$p<0.001$
6	*L. brevis*	YIT 0076 (ATCC 14869)	100	204±5	1.1	
4	*L. jugurti*	YIT 0085	100	309±19	1.3	
1	None (control)			361±41		
2	None (control)			305±5		
3	None (control)			206±8		
4	None (control)			235±58		
5	None (control)			251±39		
6	None (control)			185±55		
7	None (control)			297±38		

Salmonella typhimurium LPS (10 μg/ml) and Con A (1 μg/ml) were added to the splenocytes.
The mean incorporations (cpm) of ^{3}H-TdR by these mitogens in the seven different experiments were as follows; LPS: (1) 3687, (2) 2665, (3) 5071, (4) 2147, (5) 911, (6) 752, (7) 1727. Con A: (1) 27293, (2) 38556, (3) 26122 (4) 23574, (5) 7490, (6) 7130, (7) 18910.
a) μg of heat-killed lactobacilli per ml.
b) Stimulation rate: mean cpm of the experimental group divided by that of the appropriate control group.

L. fermentum showed the strongest activity, when tested at three doses, 100, 50 and 10 μg/ml. The two strains of *L. plantarum* were also capable of increasing the incorporation of ^{3}H-TdR. The mitogenic effect of these organisms was observed consistently in several experiments. The results with *L. plantarum* agree with the findings of others that the cell wall of *L. plantarum* has a mitogenic effect on murine splenocytes.[4]

TABLE II. Mitogenic Effect of Lactobacilli on B-lymphocytes in Murine Splenocytes

Bacterial strain or mitogen		Dose (μg/ml)[a]	Untreated splenocytes Mean±S.E. (cpm)	Untreated splenocytes Stimulation rate[b]	ATS-treated splenocytes[c] Mean±S.E. (cpm)	ATS-treated splenocytes[c] Stimulation rate
L. fermentum	YIT 0159	100	387±57	2.8*	957±46	6.3**
L. plantarum	YIT 0102	100	395±19	2.9*	724±21	4.7**
L. lactis	YIT 0168	100	131±21	1.0	75±12	0.5
L. acidophilus	YIT 0168	100	122±10	0.9	90±10	0.6
L. casei	YIT 0086	100	171±18	1.2	78±21	0.5
LPS		10	2110±802	15.4	5675±932	37.2
Con A		1	8546±535	62.4	187±4	1.2
None (control)			137±8		153±25	

* $0.02>p>0.05$, ** $p<0.001$.
a) See footnote *a*) in Table I.
b) See footnote *b*) in Table I.
c) Splenocytes were treated with rabbit antithymocyte serum in the presence of guinea pig complement.

Next, to determine whether *L. fermentum* and *L. plantarum* are T- or B-lymphocyte mitogens, T-lymphocytes in a splenocyte population were killed by ATS in the presence of complement (Table II). After the treatment with ATS, the mitogenic response of splenocytes to Con A, a T-lymphocyte mitogen,[10] was markedly reduced, whereas the incorporation of ^{3}H-TdR into ATS-treated splenocytes incubated with *S. typhimurium* LPS, a B-lymphocyte mitogen,[11] increased. *L. fermentum* and *L. plantarum* were also able to stimulate the incorporation of ^{3}H-TdR into ATS-treated splenocytes, but *L. lactis*, *L. acidophilus* and *L. casei* did not affect the incorporation of ^{3}H-TdR into normal or ATS-treated splenocytes.

Furthermore, *L. fermentum* and *L. plantarum* did not increase the ^{3}H-TdR incorporation into the thymocytes (Table III).

TABLE III. Mitogenic Effect of Lactobacilli on Murine Thymocytes

Bacterial strain or mitogen		Dose (μg/ml)[a]	Mean±S.E. (cpm)	Stimulation rate[b]
L. fermentum	YIT 0159	100	163±19	1.0
L. plantarum	YIT 0102	100	162±52	1.0
L. casei	YIT 0078	100	175±22	1.1
LPS		10	162±54	1.0
Con A		1	842±73	5.3
None (control)			158±20	

a) See footnote *b*) in Table I.
a) See footnote *b*) in Table I.

Thus, it is likely that *L. fermentum* and *L. plantarum* are B-lymphocyte mitogens.

We have shown that lactobacilli are to some extent mitogenic for splennocytes of mice. However, the exact nature of the chemical differences that cause the differences in the mitogenicity of different species of lactobacilli is unknown.

Acknowledgement We thank Miss Kazuyo Terada for skillful technical assistance in this work.

References and Notes

1) P.A. Campbell, *Bacteriol. Rev.*, **40**, 284 (1976).
2) I. Azuma, T. Taniyama, K. Sugimura, A.A. Aladin, and Y. Yamamura, *Jpn. J. Microbiol.*, **20**, 263 (1976).

3) *a*) S. Kotani, Y. Watanabe, T. Shimono, F. Kinoshita, T. Narita, K. Kato, D.E.S. Stewart-Tull, I. Morisaki, K. Yokogawa, and S. Kawata, *Biken J.*, **18**, 93 (1975); *b*) N. Bloksma, H. Van Dijk, P. Korest, and J.M. Willer, *Immunobiol.*, **156**, 309 (1979); *c*) N. Bloksma, E. De Heer, H. Van Dijk, and J.M. Willer, *Clin. Exp. Immunol.*, **37**, 367 (1979).
4) *a*) T. Saito-Taki, M.J. Tanabe, H. Mochizuki, M. Nakano, M. Tsujimoto, S. Kotani, K. Yokogawa, and S. Kawata, *Microbiol. Immunol.*, **24**, 249 (1980); *b*) H. Takada, S. Nagao, S. Kotani, S. Kawata, K. Yokogawa, S. Kusumoto, T. Shiba, and I. Yano, *Biken J.*, **23**, 61 (1980).
5) R.E. Buchanan and N.E. Gibbons (eds.). "Bergey's Manual of Determinative Bacteriology," 3rd ed. Williams and Wilkins Co., Baltimore, 1974.
6) C. Efthymiou and P.A. Hansen, *J. Infect. Dis.*, **110**, 258 (1962).
7) O. Westphal, O. Lüderitz, and F. Bister, *Z. Naturforsch.*, **7b** 148 (1952).
8) J.G. Gray, A.P. Monaco, M.L. Wood, and P.S. Russell, *J. Immunol.*, **96**, 217 (1966).
9) E.A. Boyse, L.J. Old, and G. Thomas, *Transplant. Bull.*, **29**, 63 (1962).
10) J.D. Stobo, *Transplant. Rev.*, **11**, 60 (1972).
11) J. Andersson, O. Sjöberg, and G. Möller, *Transplant. Rev.*, **11**, 131 (1972).

Chem. Pharm. Bull. 29(12)3734—3737(1981)

Synthesis of Des-Pro^2-Bradykinin and Its Behavior in Chromatography[1)]

Masao Naruse,*,[a] Kumiko Yoshizawa,[b] Terutoshi Kimura,[b] and Shumpei Sakakibara[b]

Medical Service Department, Pharmaceuticals Division, Kyowa Hakko Kogyo Co. Ltd.,[a] Tokyo 100, Japan and Peptide Institute, Protein Research Foundation,[b] Minoh, Osaka 562, Japan

(Received June 16, 1981)

Des-Pro^2-Bradykinin was synthesized by the classical solution method as a reference compound for a possible contaminant in synthetic bradykinins, and its behavior in various chromatographic analyses was examined. It showed almost the same *Rf* values as bradykinin under several different conditions in cellulose thin layer chromatography and in paper electrophoresis, but was clearly separable from bradykinin by reversed phase high performance liquid chromatography under specific conditions. Since des-Pro^2-bradykinin was found to have a potent bradykinin-potentiating activity, contamination by this material should be carefully avoided in bradykinin synthesis.

Keywords——des-Pro^2-bradykinin; bradykinin; synthesis; HPLC of bradykinin; ileum contraction; hypotension

Bradykinin is a hypotensive peptide with nine amino acid residues[2)] and has been synthesized by many workers.[3)] However, careful measurement of the biological potency of different products does not always give the same value.[4)] We considered that this discrepancy might be due to the presence of by-products which are difficult to remove from the desired product. Such by-products might contaminate synthetic bradykinin and modify its biological properties. Recently, Arakawa isolated a peptide from a tryptic digest of human sera, and showed that it had hypotensive activity in the rat; the peptide had an amino acid composition corresponding to that of des-proline-bradykinin, but the amino acid sequence has not been determined yet.[5)] Independently, we synthesized des-Pro^2-bradykinin as a possible contaminant of synthetic bradykinins and examined its behavior in chromatographic analyses. Its biological properties have also been studied in our laboratory and will be reported elsewhere.[6)]

Des-Pro^2-bradykinin was synthesized by a classical solution procedure as shown in Fig. 1. The fully protected octapeptide was deprotected by the HF/anisole procedure,[7)] and then purified by ion-exchange chromatography on CM-cellulose followed by column chromato-