Water-Soluble Adjuvant Obtained from *Bacterionemia matruchotii*

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The adjuvant effect of a butanol-extracted water-soluble adjuvant (Bu-WSA) obtained from *Bacterionemia matruchotii*, a gram-positive oral bacteria, was studied on the antibody response at the plaque-forming cell (PFC) level in murine spleens. Intraperitoneal injection of Bu-WSA caused significant increase in direct PFC numbers in spleens 1 to 3 days after the antigenic stimulation with sheep erythrocytes (SRBC). Injection of 100 to 800 μ g of Bu-WSA was effective, and 400 μ g of Bu-WSA seemed to be the optimum for induction of the adjuvant effect. The adjuvant effect was strongest when Bu-WSA was injected at the same time as the SRBC, but some effect was still observed when Bu-WSA was injected 7 days before or 1 day after the immunization. The adjuvant effect of Bu-WSA was greatest at high dose of antigen. The mice injected with Bu-WSA at the time of priming SRBC and then immunized with trinitrophenylated SRBC showed greater anti-trinitrophenyl PFC response than controls without the injection of Bu-WSA. Injections of Bu-WSA and SRBC together into congenitally athymic nude mice could elicit greater anti-SRBC PFC response than controls without Bu-WSA. These findings suggest that a part of the adjuvant effect of Bu-WSA depends on thymic cell function and another part does not.

Bacterionemia matruchotii is an indigenous gram-positive bacteria of dental plaque and plays a significant role in dental calculus formation through its calcifiability (6, 33). Dental calculus is generally considered to be one of the inflammatory agents in gingival tissues (30), but the precise role of *B. matruchotii* on the inflammatory and immunological processes during the dental calculus formation in hosts is not yet clear. In the previous paper (26), we described that *B. matruchotii* had some effects on macrophages, as the phagocytic function of the reticuloendothelial system in mice was significantly elevated for several days after the injection of this organism, and in vitro bactericidal activity of macrophages obtained from mice injected with *B. matruchotii* was stronger than that from normal mice. However, possible effects of *B. matruchotii* on lymphocytes have not been examined. In this paper, we describe experimental results in which a butanol-extracted water-soluble adjuvant (Bu-WSA) extracted from *B. matruchotii* caused a large increase in the number of antibody-producing cells in murine spleens when injected together with antigens, although whole cells of this organism had scarcely any adjuvant effect on the antibody response in mice. The Bu-WSA described here is a material chemically different from the chemically well-

defined water-soluble adjuvant of mycobacterium (1, 17).

MATERIALS AND METHODS

Bacterial strain. *B. matruchotii* L2 was cultured in brain heart infusion broth supplemented with 0.2% yeast extract at 37°C for 7 days and then harvested, washed with saline, and stored in 4°C.

Adjuvant. Bu-WSA was extracted from cells of *B. matruchotii* L2 in water-saturated *n*-butanol, digested by Pronase, and subsequently fractionated by gel filtration chromatography (15, 18). An aqueous suspension of bacteria (0.25 g/ml) was treated with an equal volume of water-saturated *n*-butanol at 4°C for 15 min. The aqueous phase of the biphasic system formed by centrifugation at 35,000 $\times g$ for 20 min was incubated with Pronase (20 μ g/ml; Kaken Chemicals Co., Ltd., Tokyo) overnight at 37°C and then concentrated by a rotary evaporator. The extract was fractionated by gel filtration on Sepharose 4B at 4°C with 0.15 M phosphate-buffered saline, pH 7.2, at a pressure of 20 to 30 mm of water (flow rate, 60 ml/h). The adjuvant-active fractions were pooled, dialyzed against distilled water, and lyophilized. For use, this purified adjuvant (Bu-WSA) was dissolved in saline and injected intraperitoneally (i.p.) into mice.

Animals. Male ICR strain mice were purchased from CLEA Japan Inc., Tokyo. Athymic nude mice (nu/nu) and haired heterologous mice (nu/+) bred on a BALB/c background were supplied from our animal colony. All mice were used at 8 to 10 weeks old.

Antigens and immunization. Sheep erythrocytes (SRBC) and 2,4,6-trinitrophenyl (TNP)-substituted SRBC were used as antigens for immunization. Mice were immunized i.p. with these antigens. TNP-substituted horse erythrocytes were used as indicator cells in the assay. The erythrocytes were coupled with 2,4,6-trinitrobenzene sulfate by modifications (29) of the procedure of Rittenberg and Pratt (28); a 100-mg portion of 2,4,6-trinitrobenzene sulfate dissolved in 7 ml of 0.02 M phosphate-buffered saline, pH 7.2, was added to 1 ml of washed, packed erythrocytes, and the mixture was agitated for 30 min at room temperature followed by four washings with phosphate-buffered saline.

PFC assay and estimation of adjuvant effect. Direct 19S hemolytic anti-SRBC or anti-TNP plaque-forming cells (PFC) in the spleens of mice were assayed by the technique of Jerne et al. (11) or its modification (28). Numbers of 7S hemolytic plaques were estimated by the indirect technique using hyper-immune rabbit anti-mouse γ -globulin serum (32). Five mice were used for each determination of the PFC response in one group, and the arithmetic mean of the number of the PFC in the spleens of five mice and the standard error were calculated. The adjuvant effect of Bu-WSA was estimated by the comparison between PFC numbers of Bu-WSA-treated mice and those of the controls.

RESULTS

Preparation of Bu-WSA. Chromatographic profiles of the butanol extract of *B. matruchotii*

after the passage over a Sepharose 4B column are shown in Fig. 1. There were two prominent carbohydrate-rich peaks. A preliminary experiment revealed that the first peak contained some adjuvant substance, but other fractions did not. Therefore, the fractions around the first peak (tube numbers 55 to 75) were pooled together, dialyzed against distilled water, lyophilized, and stocked as a Bu-WSA.

Elementary analysis of Bu-WSA was as follows: C = 25.96%, H = 4.47%, N = 8.35%, P = 9.79%. Bu-WSA contained 25.5% neutral sugars. Amino sugars were not detected by the Elson-Morgan test, and uronic acid was not detected by the carbazole- H_2SO_4 test.

Adjuvant effect of Bu-WSA on the primary PFC response. An i.p. injection of whole cells (1 mg) of *B. matruchotii* with 10^8 SRBC into mice provoked a weak adjuvant effect on anti-SRBC PFC responses in their spleens. The PFC numbers in their spleens did not exceed by two or three times those of immune controls during the period of the primary response (data not shown). However, the water-soluble extract (Bu-WSA) of the organism had strong adjuvant effect. After a simultaneous injection with SRBC and Bu-WSA (400 μ g), the kinetics of the primary direct and indirect PFC responses in the spleens of mice were examined during a 10-day

FIG. 1. Chromatographic profiles of butanol extract of *B. matruchotii* after passage through a Sepharose 4B column. Six-milliliter fractions were collected and analyzed for protein by optical density at 280 nm (●) and for nucleic acid by optical density at 254 nm (▲). Carbohydrate content was determined on 1 ml of each fraction by absorbance at 490 nm (■) according to the method of Dubois et al. (5).

period (Fig. 2). As controls, the mice receiving only SRBC were included. Bu-WSA showed an effect during the period of days 1 to 3 after the immunization. On day 2, the PFC number of Bu-WSA-injected mice was 10 times greater than that of controls. The direct PFC response reached a peak on day 4, but at that time and thereafter, no adjuvant effect of Bu-WSA could be observed. In addition, no adjuvant effect of Bu-WSA was seen on the primary indirect PFC response on days 7 and 10 after immunization.

Dose effect of Bu-WSA. Mice were i.p. injected with 10^8 SRBC and various doses (25 to 800 μ g) of Bu-WSA together, and 2 days later, anti-SRBC PFC responses in their spleens were examined. The maximum response was observed in the mice injected with 400 μ g of Bu-WSA, and

the numbers of direct PFC in these mice were up to 13-fold higher than those of immune controls (Fig. 3).

Relation between timing of Bu-WSA injection and its adjuvant effect on anti-SRBC PFC response. The relation between timing of the injection of Bu-WSA and that of SRBC was found to be important for the adjuvant effect of Bu-WSA on the primary response. Groups of mice were injected with 400 μ g of Bu-WSA at varying times either before or after the immunization with 10^8 SRBC. The PFC numbers in the spleens of these mice were determined 48 h after the immunization and compared with those in control mice which received SRBC alone. As shown in Fig. 4, differences in the times of injection of Bu-WSA affected significantly its adjuvant effect. Simultaneous injection with SRBC and Bu-WSA resulted in the greatest effect. The adjuvant effect of Bu-WSA decreased as the time between SRBC and Bu-WSA injections was increased. The anomalous result when Bu-WSA was injected 7 days before SRBC injection could not be explained, but very similar effects after the injection of bacterial polysaccharide adjuvants were described by Behling and Nowotny (4).

Antigenic doses affecting adjuvant effect of Bu-WSA on anti-SRBC PFC response. Groups of mice were injected with 2×10^5 , 2×10^7 , or 2×10^9 SRBC with or without 400 μ g of Bu-WSA, and 2 days later, PFC numbers in their spleens were assessed. As shown in Table 1, the greater the dose of antigen that was administered, the better the adjuvant effect of the Bu-WSA.

Effect of Bu-WSA on carrier-specific T cells helping anti-TNP PFC response. To examine whether the adjuvant effect of Bu-WSA is involved in the generation or activation of helper thymus-derived (T) lymphocytes, the following experiment was carried out. Groups of mice were primed with 10^8 SRBC alone or 10^8 SRBC and 400 μ g of Bu-WSA together. As controls, unprimed mice and Bu-WSA-injected mice were included. Ten days later, all the mice were immunized with 10^8 TNP SRBC. Forty-eight hours after the immunization, they were sacrificed, and anti-TNP PFC responses in their spleens were assessed. As shown in Table 2, anti-TNP PFC responses in the mice which received Bu-WSA at the time of priming with SRBC were significantly higher than those of others. This result suggests that Bu-WSA has some effect on the generation of carrier-specific helper T cells after the priming with the carrier, and the generated T cells may enhance their anti-TNP PFC response after the immunization.

FIG. 2. Adjuvant effect of Bu-WSA on the development of immunoglobulin M (—) and indirect immunoglobulin G (---) PFC in the spleen after the primary immunization of mice with SRBC. Symbols: (●) average number ($\pm$ standard error) of PFC/spleen of mice injected with 400 μ g of Bu-WSA and 10^8 SRBC simultaneously; (○) average number ($\pm$ standard error) of PFC/spleen of mice injected with 10^8 SRBC (control).

FIG. 3. Dose effect of Bu-WSA on anti-SRBC PFC response in the spleens of mice. Ratio = number of PFC in the experiment/number of PFC in control.

FIG. 4. Effect of time between Bu-WSA and SRBC injections on anti-SRBC PFC response. Mice were immunized with 10^8 SRBC (day 0) and injected with 400 µg of Bu-WSA at varying times as indicated. Forty-eight hours after the immunization, the number of direct PFC in the spleens of mice was determined. Ratio = number of PFC in the experiment/number of PFC in control.

Adjuvant effect of Bu-WSA on anti-SRBC PFC response in nu/nu mice. Congenitally athymic nude mice are poorly capable of re-

TABLE 1. Relationship between doses of SRBC and adjuvant effect of Bu-WSA on direct anti-SRBC PFC response

Injection dose of SRBC	Treatment of Bu-WSA (400 µg)	Direct PFC/spleen ^a (Mean ± SE ^b)	Ratio ^c
2×10^9	Yes	13,200 ± 200	14.5
	No	912 ± 30	1.0
2×10^7	Yes	4,290 ± 790	8.7
	No	490 ± 59	1.0
2×10^5	Yes	1,140 ± 300	4.5
	No	252 ± 176	1.0

^a PFC was determined 48 h after immunization.

^b SE, Standard error.

^c Ratio = number of PFC in Bu-WSA-treated mice/number of PFC in Bu-WSA-untreated control.

sponding to thymus-dependent antigens in antibody responses, as they lack mature T lymphocytes cooperating with antibody-producing bone marrow-derived (B) lymphocytes. The PFC numbers in the spleens of nu/nu mice 4 days after the immunization with 10^8 SRBC are very few compared with those of heterozygous nu/+ mice (Table 3). However, simultaneous injections with SRBC and Bu-WSA into the nu/nu mice elicited a 4.6-fold increase in the PFC in their spleens, although the number of PFC in those mice was still less than that of nu/+ mice. Bu-WSA is capable of substituting the helper function of antigen-specific T lymphocytes to some extent, and the adjuvant effect of

TABLE 2. Effect of Bu-WSA on carrier-specific T cells helping anti-TNP PFC response

Group	Priming (day 0)	Immunization (day 10)	Direct anti-TNP PFC/spleen (Mean $\pm$ SE ^a) (day 12)	Ratio ^b
1	None (control)	TNP SRBC ^c	6,120 $\pm$ 2,130	1.0
2	SRBC ^d	TNP SRBC	10,600 $\pm$ 1,700 ^e	1.7
3	SRBC + Bu-WSA ^f	TNP SRBC	18,200 $\pm$ 1,500 ^e	3.0
4	Bu-WSA	TNP SRBC	5,640 $\pm$ 1,060	0.9

^a SE, Standard error.^b Ratio = number of PFC in the experiment/number of PFC in control.^c Total of 10⁸ TNP-coated SRBC, i.p.^d Total of 10⁸ SRBC, i.p.^e $P < 0.01$ when compared with group 1 by Student's t test.^f 400 μ g, i.p.^g $P < 0.025$ when compared with group 2 by Student's t test.

TABLE 3. Effect of Bu-WSA on direct anti-SRBC PFC responses in the spleens of nu/nu and nu/+ mice

Group	Mice	Treatment	Direct PFC/spleen ^a (Mean $\pm$ SE ^b)
1	nu/nu	SRBC ^c	6,230 $\pm$ 1,100
2	nu/nu	Bu-WSA ^d + SRBC	28,500 $\pm$ 3,800 ^e
3	nu/+	SRBC	176,000 $\pm$ 16,000
4	nu/+	Bu-WSA + SRBC	382,000 $\pm$ 109,000 ^f
5	nu/+	Bu-WSA	952 $\pm$ 188

^a PFC was determined 4 days after the treatment.^b SE, Standard error.^c Total of 10⁸ SRBC, i.p.^d 400 μ g, i.p.^e $P < 0.01$ when compared with group 1 by Student's t test.^f $0.05 < P < 0.1$ when compared with group 3 by Student's t test.

Bu-WSA may be caused partially via the direct activation of antibody-producing B lymphocytes without the help of T lymphocytes.

DISCUSSION

Many kinds of microorganisms and their components (19), including *Mycobacterium* (1, 7, 14, 17), *Corynebacterium parvum* (*Propionibacterium acnes*) (9, 10, 21, 34, 35), *Bordetella* (12, 20), *Klebsiella* (24), and other gram-negative bacteria, have adjuvant effects. Some kinds of oral microorganisms, such as *Veillonella*, *Fusobacterium*, *Bacteroides*, and *Actinomycetes*, are also known to have adjuvant effects on antibody responses (27), and the adjuvant effects of these oral microorganisms are presumed to play some role in the pathogenesis of periodontal diseases (8).

The microorganism *B. matruchotii* is capable of stimulating phagocytic function of the reticuloendothelial system and increasing bactericidal activity of macrophages in mice (26). However, previous or simultaneous injections of these organisms with SRBC as an antigen into

mice elicit hardly any adjuvant effect on the antibody-forming cell response in their spleens. This finding does not indicate, however, that these organisms contain no immunologically active substances. Indeed, we demonstrated that a water-soluble extract (Bu-WSA) prepared from the organism according to the method of Morrison and Leive (18) had a significant adjuvant effect when injected with SRBC to mice. The effect of Bu-WSA was observed only on early events in the direct anti-SRBC PFC response in the spleen of mice (Fig. 2). Small amounts (10 or 20 μ g) of bacterial lipopolysaccharide (22, 25) and capsular polysaccharide of *Klebsiella pneumoniae* (24) are effective enough to induce in vivo adjuvant action in mice, but a relatively large amount (over 100 μ g) of Bu-WSA was necessary to evoke the prominent effect (Fig. 3). Adjuvant effect of Bu-WSA was more sustained in the mice immunized with higher doses of the antigen (Table 1). Simultaneous injection of the antigen and Bu-WSA was the most effective, but administration of Bu-WSA before or after the immunization could still elicit the adjuvant effect to some extent (Fig. 4). Significant amounts of both antigenic and adjuvant stimuli must be necessary for the target to initiate adjuvant effect in mice. The reason why the whole cells of *B. matruchotii* showed little adjuvant effect might be explained if in vivo degradation of the whole cells could not provide the target with enough of the adjuvant.

When microbial adjuvants are injected before immunization with an antigen, one sometimes finds lowered antibody responses (31) or oscillation of the antibody responses (4). However, Bu-WSA did not have such suppressive effects on antibody response (Fig. 4). Bu-WSA seems to be unable to activate suppressor T cells regulating the antibody response (16) or to exhaust antibody-producing cell precursors as lipopolysaccharide does (23).

The synergistic involvement of B and T cells in the antibody responses to hapten-carrier con-

jugates and thymus-dependent xenogeneic erythrocyte antigens has been well established. The mice injected with Bu-WSA at the time of priming with the carrier and then immunized with TNP carrier showed significantly greater anti-TNP PFC response in their spleens than controls without the injection of Bu-WSA (Table 2). These experiments suggest that Bu-WSA may act directly on carrier-specific T cells, and consequently, these activated helper T cells accelerate the generation of anti-TNP antibody-producing cells in the spleen. On the other hand, the injections of Bu-WSA and SRBC together into congenitally athymic nu/nu mice also elicited significantly greater anti-SRBC PFC responses than controls without Bu-WSA, although the response was lower than that of their heterologous zygotes nu/+ mice injected with SRBC (Table 3). This finding that Bu-WSA can partially substitute for the function of T cells on the anti-SRBC PFC response in the absence of T cells demonstrates that some part of the adjuvant effect of Bu-WSA is not dependent on T cells. The adjuvant activity of Bu-WSA is apparently not mediated by a direct nonspecific stimulation of antibody-forming B cells, which seems to be one of the possible mechanisms for the adjuvant activity of endotoxin (2, 3), since in none of the experiments did we observe any increase in the background number of antibody-forming cells against either SRBC or TNP horse erythrocytes after the injection of Bu-WSA (Table 3 and unpublished observations). These results indicate the possible existence of multiple targets for Bu-WSA to induce its adjuvant effect, but further investigation will be necessary to clarify the cellular mechanisms involved.

Water-soluble adjuvants have been obtained from the cell wall of various gram-positive bacteria (13) and cells of *Mycobacterium* (1, 7, 14, 17). As Bu-WSA does not contain detectable amino sugars, our substance seems to be different from others. Further characterization of the active components in Bu-WSA is in progress.

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LITERATURE CITED

- Adam, A., R. Ciorbaru, J.-F. Petit, and E. Lederer. 1972. Isolation and properties of a macromolecular, water-soluble, immuno-adjuvant fraction from the cell wall of *Mycobacterium smegmatis*. Proc. Natl. Acad. Sci. U.S.A. 69:851-854.
- Andersson, J., F. Melchers, C. Galanos, and O. Lüderitz. 1973. The mitogenic effect of lipopolysaccharide on bone marrow-derived mouse lymphocytes. J. Exp. Med. 137:943-953.
- Andersson, J., O. Sjöberg, and G. Möller. 1972. Induction of immunoglobulin and antibody synthesis *in vitro* by lipopolysaccharides. Eur. J. Immunol. 2:349-353.
- Behling, U. H., and A. Nowotny. 1977. Immune adjuvancy of lipopolysaccharide and a nontoxic hydrolytic product demonstrating oscillating effects with time. J. Immunol. 118:1905-1907.
- Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350-356.
- Ennever, J. 1960. Intracellular calcification by oral filamentous microorganisms. J. Periodontol. 31:304-307.
- Hiu, I. J. 1977. The adjuvant active fraction of delipidated mycobacteria. Nature (London) 267:708-709.
- Horton, J., J. J. Oppenheim, and S. E. Mergenhagen. 1974. A role for cell-mediated immunity in the pathogenesis of periodontal disease. J. Periodontol. 45:351-360.
- Howard, J. G., G. H. Christie, and M. T. Scott. 1973. Biological effects of *Corynebacterium parvum*. IV. Adjuvant and inhibitory activities on B lymphocytes. Cell. Immunol. 7:290-301.
- Howard, J. G., M. T. Scott, and G. H. Christie. 1973. Cellular mechanisms underlying the adjuvant activity of *Corynebacterium parvum*: interactions of activated macrophages with T and B lymphocytes. Ciba Found. Symp. 18:101-120.
- Jerne, N. K., A. Nordin, and C. Henry. 1963. The agar plaque technique for recognizing antibody-producing cells, p. 109-122. In B. Amos and H. Koprowski (ed.), Cell-bound antibodies. Wistar Institute Press, Philadelphia.
- Kind, L. S. 1957. Relationship of the anaphylaxis sensitizing and adjuvant properties of *Hemophilus pertussis* vaccine. J. Immunol. 79:238-242.
- Kotani, S., T. Narita, D. E. S. Stewart-Tull, T. Shimonono, Y. Watanabe, K. Kato, and S. Iwata. 1975. Immunoadjuvant activities of cell walls and their water-soluble fractions prepared from various Gram-positive bacteria. Biken J. 18:77-79.
- Kumazawa, Y., A. Shibusawa, T. Suzuki, and K. Mizunoe. 1976. Separation of an adjuvant-active glycolipid lacking peptide moiety from wax D preparation of *Mycobacterium tuberculosis* strain Aoyama B. Immunochemistry 13:173-177.
- Maddy, A. H. 1964. The solubilisation of the protein of the ox-erythrocyte ghost. Biochim. Biophys. Acta 88:448-449.
- Marx, J. L. 1975. Suppressor T cells: role in immune regulation. Science 188:245-247.
- Modolell, M., G. A. Luckenbach, M. Parant, and P. G. Munder. 1974. The adjuvant activity of a mycobacterial water soluble adjuvant (WSA) *in vitro*. I. The requirement of macrophages. J. Immunol. 113:395-403.
- Morrison, D. C., and L. Leive. 1975. Fractions of lipopolysaccharide from *Escherichia coli* O111:B4 prepared by two extraction procedures. J. Biol. Chem. 250:2911-2919.
- Munoz, J. 1964. Effect of bacteria and bacterial products on antibody response. Adv. Immunol. 4:397-440.
- Murgo, A. J., and T. J. Athanassiades. 1975. Adjuvant effect of *Bordetella pertussis* vaccine to sheep erythrocytes: enhancement of antibody formation by using subcutaneous administration of adjuvant and antigen. Infect. Immun. 12:969-977.
- Nagoya, T., F. Kobayashi, and K. Nomoto. 1977. Immunological properties of *Propionibacterium acnes*. I. Potentiation and suppression on antibody response to sheep and hamster erythrocytes in mice. Microbiol. Immunol. 21:33-44.
- Nakano, M., T. Shimamura, and K. Saito. 1971. Cellular mechanisms of adjuvant action of bacterial lipopolysaccharide in anti-sheep red blood cell antibody response. Jpn. J. Microbiol. 15:149-158.
- Nakano, M., M. J. Tanabe, T. Saito, and T. Shima-

- mura. 1976. Immunosuppressive effect of bacterial lipopolysaccharide on antibody response. *Jpn. J. Microbiol.* **20**:53-58.
24. Nakashima, I., T. Kobayashi, and N. Kato. 1971. Alterations in the antibody response to bovine serum albumin by capsular polysaccharide of *Klebsiella pneumoniae*. *J. immunol.* **107**:1112-1121.
25. Neter, E. 1969. Endotoxins and the immune response. *Curr. Top. Microbiol. Immunol.* **47**:82-124.
26. Nitta, T., S. Okumura, and M. Nakano. 1977. Effect of *Bacterionema matruchotii* on immune response. I. Enhancement of the phagocytic and bactericidal functions. *Jpn. J. Bacteriol.* **32**:691-696.
27. Okada, H., M. Inui, K. Kawagoe, H. Nakata, and M. Aono. 1974. Adjuvant effects of oral microorganism on the immunological responses. *Arch. Oral Biol.* **19**:761-766.
28. Rittenberg, M. B., and K. L. Pratt. 1969. Anti-trinitrophenyl (TNP) plaque assay. Primary response of Balb/c mice to soluble and particulated immunogen. *Proc. Soc. Exp. Biol. Med.* **132**:575-581.
29. Saito, T., and M. Nakano. 1975. Effect of bacterial lipopolysaccharides on anti-trinitrophenyl antibody-producing cells. Nonspecific modification of the affinity at the cellular level. *Jpn. J. Microbiol.* **19**:419-425.
30. Scherp, H. W. 1962. Discussion of bacterial factors in periodontal disease. *J. Dent. Res.* **41**:327-330.
31. Schwab, J. H. 1975. Suppression of the immune response by microorganisms. *Bacteriol. Rev.* **39**:121-143.
32. Šterzl, J., and I. Riha. 1965. Detection of cells producing 7S antibodies by the plaque technique. *Nature (London)* **208**:858-859.
33. Takazoe, I. 1961. Study on the intracellular calcification of oral aerobic leptotrichia. *Shika Gakuho* **61**:394-401.
34. Warr, G. W., and K. James. 1975. Effect of *Corynebacterium parvum* on the class and subclass of antibody produced in the response of different strains of mice to sheep erythrocytes. *Immunology* **28**:431-442.
35. Warr, G. W., and V. S. Šljivic. 1974. Enhancement and depression of the antibody response in mice caused by *Corynebacterium parvum*. *Clin. Exp. Immunol.* **17**:519-532.