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Arthritogenicity in Rats of Cell Walls from Several Streptococci Staphylococci and Two Other Bacteria[1] (39360)

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Adjuvant-induced arthritis (AA) in rats has been produced by a single injection of *Mycobacterium* in water-in-oil emulsion. It is known that *Nocardia* (1) and *Corynebacterium rubrum* (2) can replace *Mycobacterium* for AA production. Recently Koga *et al.* (3) have reported that the cell walls from *Streptomyces fradiae, Streptomyces lavendulae, Lactobacillus plantarum,* and *Staphylococcus aureus* were able to produce AA in rats, and all clinical features were identical to those produced by *Mycobacterium*. More recently it has been reported that infection with live *Salmonella enteriditis* was able to produce arthritis in the L-BN rats (4). Therefore, it should be reasonable to assume that a common principle(s) shared by various bacteria and probably located in the cell walls, is involved in the pathogenesis of AA. Based upon this reasoning we have undertaken to compare the arthritogenicity of various bacterial cell walls and to determine what components of the cell wall may be involved in production of the disease.

The following experiments are designed to test the arthritogenic potentiality of the cell walls from seven streptococcal and two staphylococcal strains and from two other bacteria.

Materials and methods. Animals. Female Lewis inbred rats (Microbiological Associates, Bethesda, Md.) were utilized. They weighed 150 to 174 g.

Cell wall preparations. The cell walls tested in the present study were generously supplied by Dr. S. Kotani (Dept. of Microbiology, Osaka University Dental School, Japan). The preparation of these cell walls is described in detail elsewhere (5).

Bacterial species tested were *Streptococcus lactis (Str. lactis* IFO 12546), *Str. faecalis* (IFO 12580), *Str. mutans* (BHT), *Str. salivarium* (HHT), *Str. salivarius* (IFO 3350), *Str. bovis* (IFO 12058), *Str. thermophilus* (IFO 3535), *Str. pyogens* (group A streptococcus, type 12, S.F. 42 strain), *Staphylococcus aureus (S. aureus* FDA 209P), *S. epidermidis* (ATCC 155), *Bacillus megaterium (B. megaterium* KM), *Micrococcus lysodeikticus (M. lysodeikticus* NTCC 2665), and *Mycobacterium smegmatis (M. smegmatis* ATCC 14468).

Preparation of the hydrosoluble peptidoglycans. Lysozyme-solubilized hydrosoluble products (peptidoglycans) were prepared from *M. smegmatis* and the human mixed strains of *Mycobacterium* (C, DT, PN), according to the method of Adam *et al.* (6) with some modifications (7). The preparation of the hydrosoluble peptidoglycans (PG) from *S. aureus* cell walls is described in detail elsewhere (5). These peptidoglycans were used as skin test antigens in the present study.

Production of AA. Rats were injected into both inguinal lymph nodes with a total volume of 0.01 ml of water-in-oil emulsion, containing 0.1 mg each of cell walls, according to the Newbould technique (8).

The cell walls were triturated well with mortar and pestle and thoroughly mixed in a mineral oil containing 15% of Arlacel A.

A water-in-oil emulsion was thus prepared by the dropwise addition of an equal volume of 0.01 *M* phosphate buffered saline (PBS), pH 7.2, in order to make a final concentration of the cell walls 0.1 mg per 0.01 ml of the water-in-oil emulsion.

Evaluation of AA. The rats were examined daily for 3 to 4 weeks after inoculation to evaluate the onset day of the disease and were graded from 0 to 4 points for each appendage, in accordance with a previous report (9). In general, the arthritogram score below 5 was evaluated as mild and

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transient arthritis; 6 to 10, moderate; 11 to 14, severe; and 15 to 20, extremely severe.

Skin testing. Skin tests were performed 21 days after inoculation on a shaved area of the flank by intradermal injection with 0.1 ml of 0.01 *M* PBS, pH 7.2, which contained 50 mg of test antigen. The diameter of the erythema and the induration of the skin test area was carefully recorded at 6 hr for immediate reaction, and 24 and 43 hr for delayed type hypersensitivity, according to the method of Flax *et al.* (10).

Results. Arthritogenicity of various different streptococcal cell walls. As shown in Table I, the cell walls from *Str. bovis, Str. lactis, Str. mutans, Str, salivarius* (HHT), *Str. pyogenes,* and *Str. thermophilus* were able to produce mild to severe disease with an incidence of 50, 70, 80, 25 (total for two species), 30, and 70%, respectively. The cell walls from *Str. salivarius* (IFO 3350) and *Str. faecalis* did not produce the disease.

Arthritogenicity of two staphylococcus and miscellaneous bacterial cell walls. As shown in Table II, *M. smegmatis* cell walls and human mixed strains of *Mycobacterium* (C, DT, PN) produced extremely severe disease with 100% of incidence. The clinical signs appeared 6 to 9 days after inoculation and reached a mean high score of 20 and 19.5, respectively.

S. aureus cell walls produced severe disease with an incidence of 100% in one experiment and of 90% in another experiment, while *S. epidermidis* cell walls were unable to produce disease. The cell walls from *B. megaterium* and *M. lysodeikticus* were also found to be nonarthritogenic.

Delayed skin hypersensitivity in rats immunized with various cell walls. The results are shown in Table III. The rats immunized with *M. smegmatis* elicited strong delayed hypersensitivity to peptidoglycans of either *M. smegmatis* or *S. aureus* but not to PPD, while the rats immunized with human mixed strains of *Mycobacterium* (C, DT, PN) elicited strong delayed hypersensitivity to peptidoglycans and PPD. The *S. aureus* cell walls also induced strong delayed hypersensitivity to all peptidoglycans but not to PPD. The cell walls of *S. epidermidis, B. megaterium,* and *M. lysodeikticus* did not induce any skin reactions to PPD or peptidoglycans.

Immune response of the rats immunized with various streptococcal cell walls. As can be seen in Table IV, there is a very good correlation between development of the disease and delayed hypersensitivity reactions to the peptidoglycan of human mixed strains of *Mycobacterium* (C, DT, PN). All the rats which developed the disease after inoculation of the cell walls from *Str. thermophilus, Str. pyogenes,* and *Str. salivarius* (HHT) elicited a positive delayed hypersensitivity to the peptidoglycan of *Mycobacterium* (C, DT, PN). However, the rats which did not develop the disease after inoculation of the above cell walls showed no skin response except one which was immunized with *Str. salivarius* (HHT). The rats immunized with

TABLE I. ARTHRITIS-INDUCING ABILITY OF VARIOUS STREPTOCOCCAL CELL WALLS.

Source of cell wall	Dose[a] (mg/rat)	Adjuvant Arthritis			
		Incidence	Onset day	(Average)	Mean high score
Str. bovis	0.1	5/10	10–15	(12.0)	14.6
Str. lactis	0.1	7/10	12–14	(13.4)	11.0
Str. mutans	0.1	8/10	12–15	(14.1)	11.5
Str. salivarius[b]	0.1	0/10	–		–
Str. salivarius[c]	0.1	1/10	13	(13.0)	12.0
Str. salivarius[c]	0.1	4/10	12–15	(13.4)	10.5
Str. pyogenes	0.1	3/10	11	(11.0)	12.0
Str. thermophilus	0.1	7/10	12–15	(12.6)	7.9
Str. faecalis	0.1	0/10	–		–
Control[d]		0/10	–		–

[a] Female Lewis rats were immunized in both inguinal lymph nodes with 0.01 ml of a water-in-oil emulsion which contained of 0.1 mg each of bacterial cell walls.

[b] *Str. salivarius* (IFO-3350).

[c] *Str. salivarius* (HHT).

[d] Lewis rats were immunized with a water-in-oil emulsion without any bacteria.

TABLE II. ARTHRITIS-INDUCING ABILITY OF TWO STAPHYLOCOCCAL CELL WALLS AND MISCELLANEOUS BACTERIAL CELL WALLS.

Source of cell wall	Dose[a] (mg/rat)	Adjuvant arthritis			
		Incidence	Onset day	(Average)	Mean high[b] score
Mycobacterium					
M. smegmatis	0.1	10/10	6-8	(7.2)	20.0
C, DT, PN[c]	0.1	10/10	7-9	(7.7)	19.5
Staphylococcus					
S. aureus	0.1	10/10	9-16	(12.0)	14.4
	0.1	9/10	8-10	(11.7)	13.1
S. epidermidis	0.1	0/10	–		–
	0.1	0/10	–		–
Miscellaneous or others					
B. megaterium	0.1	0/10	–		–
M. lysodeikticus	0.1	0/10	–		–

[a] Female Lewis rats were immunized in both inguinal lymph nodes with 0.01 ml of a water-in-oil emulsion which contains 0.1 mg each of material.

[b] This score was calculated by averaging the highest score of each rat.

[c] Human mixed strains of *Mycobacterium*. The delipidated cells of these Mycobacteria were used instead of cell wall.

TABLE III. IMMUNE RESPONSE OF THE RATS IMMUNIZED WITH VARIOUS BACTERIA.

Immunization[a]	Adjuvant arthritis	48-hr skin reaction[c] Average diameter ± SE (mm)			
		PPD	Hydrosoluble product (peptidoglycan) of		
			C, DT, PN	*M. smegmatis*	*S. aureus*
M. smegmatis	+[d]	Neg.	Neg.	16.0 ± 0.6	10.8 ± 0.5
C, DT, PN[b]	+	15.5 ± 1.0	16.0 ± 0.6	12.0 ± 1.2	9.9 ± 0.7[f]
S. aureus	+	Neg.	12.8 ± 0.7	15.2 ± 0.9	11.2 ± 0.6
S. epidermidis	–[e]	Neg.	Neg.	Neg.	
M. lysodeikticus	–	Neg.	Neg.	Neg.	
B. megaterium	–	Neg.	Neg.	Neg.	

[a] All rats were immunized in both inguinal lymph nodes with 0.01 ml of water-in-oil emulsion containing 0.1 mg each of bacterial cell wall except human mixed strains of Mycobacteria (C, DT, PN).

[b] Delipidated cells were used instead of cell wall.

[c] Skin tests were performed 21 days after inoculation with 0.1 ml of PBS containing 50 μg each of test antigens.

[d] All of the rats developed the disease.

[e] None of the rats developed the disease.

[f] The test antigen utilized here was a different lot of peptidoglycan of *S. aureus* from that used in the other two groups.

nonarthritogenic cell walls such as *Str. salivarius* (KFO 3350) and *Str. faecalis*, and the control normal rats did not elicit any skin reaction. None of the rats in this experiment demonstrated an immediate type skin reaction.

Discussion. The present data indicate that the cell walls of *Str. thermophilus, Str. bovis, Str. lactis, Str. mutans, Str. pyogenes,* and *Str. salivarius* (HHT) were able to produce the disease while the cell walls of *Str. faecalis* and *Str. salivarius* (IFO 3350) were unable to do so. The arthritis and other features were identical to those produced by *Mycobacterium* (e.g., *M. smegmatis* and human mixed strains of *Mycobacterium).* It was shown in a previous report (3) that the cell walls of *S. aureus* and *Str. pyogenes* produced little or no sign of arthritis. However, we found that cell walls of *S. aureus* did produce severe disease with more than 90% of incidence and *Str. pyogenes* cell walls also produced the disease with over 30% of the animals tested. These discrepancies could be the result of the composition of mineral oil employed. In our present study we used mineral oil containing 15% of Arlacel A, while in the previous study (3) mineral oil containing 5% of Arlacel A was used. In the case of *Mycobacterium* and its

TABLE IV. IMMUNE RESPONSE OF THE RATS IMMUNIZED WITH VARIOUS STREPTOCOCCAL CELL WALLS.

Immunization[a]	Adjuvant[b] arthritis	48-Hr skin reaction Average diameter ± SE (mm) PPD	Hydrosoluble product (peptidoglycan) of C, DT, and PN	
Str. thermophilus	+	Neg.	10.6 ± 0.9	(7/7)[c]
	−	Neg.	Neg.	(3/3)[d]
Str. pyogenes	+	Neg.	10.3 ± 0.6	(3/3)[c]
	−	Neg.	Neg.	(7/7)[d]
Str. salivarius (HHT)	+	Neg.	13.5 ± 1.2	(4/4)[c]
	−	Neg.	13.0 ± 0.0	(1/6)[e]
Str. salivarius (IFO-3350)	−	Neg.	Neg.	(10/10)
Str. faecalis	−	Neg.	Neg.	(10/10)
Control[f]	−	Neg.	Neg.	(10/10)

[a] All the rats were immunized in both inguinal lymph nodes with 0.01 ml of water-in-oil emulsion containing 0.1 mg each of bacterial cell wall and 21 days later skin tested with 50 μg each of test antigen.
[b] +, Arthritic rat; −, nonarthritic rat.
[c] Number of rats with positive skin tests/number of arthritic rats.
[d] Number of rats with negative skin tests/number of nonarthritic rats.
[e] Number of rats with positive skin tests/number of nonarthritic rats.
[f] Control rats were immunized with incomplete adjuvant.

wax D, we could not find any difference in their arthritogenicity when the two mineral oil preparations were used. However, the mineral oil containing 15% of Arlacel A seems to give rise to a better water-in-oil emulsion (thick emulsion), at least with the cell walls described above. The higher Arlacel A concentration may be necessary because of the much lower lipid content and a much higher polysaccharide content in these bacterial cell walls as compared to that in the Mycobacterial cell walls (11).

On the other hand, we repeatedly confirmed the observations of Koga *et al.* (3) in which the cell walls from *S. epidermidis, B. megaterium,* and *M. lysodeikticus* were nonarthritogenic. The failure of these cell walls to produce the disease may be relevant to the fact that they were unable to replace *Mycobacterium* in complete Freund adjuvant in guinea pigs (5). All of the arthritogenic cell walls used here have been shown to function as adjuvants in guinea pigs both by increasing antibody synthesis and inducing delayed hypersensitivity (cell-mediated immunity) to ovalbumin (5). However, the cell walls of *Str. salivarius* (IFO 3350) and *Str. faecalis* having an adjuvant activity (5) could not produce the disease, as is shown in Table I. Thus the present results provide the view that adjuvanticity is needed for bacterial cell walls to produce the disease, while arthritogenicity requires an additional component which must be a specific antigen responsible for the disease production. One must also consider the possibility that the arthritogenicity of certain bacterial cell walls as described here may be attributed to the amount of this antigen available in the cell walls.

The present results also indicate that a very good correlation exists between development of arthritis and the positive delayed hypersensitivity to the peptidoglycans, with the exception of one rat who was immunized with *Str. salivarius* (HHT) and thereafter did not develope arthritis but exhibited positive skin test (Table III). Thus these results strongly suggest that this delayed hypersensitivity may be involved in the pathogenesis of the disease. Furthermore, it is of interest that the rats immunized with either *S. aureus* cell wall or *M. smegmatis* cell wall elicited strong delayed hypersensitivity to the peptidoglycans from both cell walls. A similar cross-reaction was also observed between the peptidoglycans from the cell walls of *S. aureus* and human mixed strains of Mycobacteria (C, DT, and PN). These cross-reactions among peptidoglycans may be related to the similarity of peptidoglycan structure among bacterial cell walls (11,

12). The results in Table IV provide an additional evidence of this cross-reaction.

It is also clear from the present results that PPD and its hypersensitivity *was not involved* in the pathogenesis of the disease, because Streptococcal cell walls do not contain PPD-like substances which are specific for *Mycobacterium* and did not induce PPD hypersensitivity in the rats which developed severe disease.

In view of the fact that many bacterial infections can cause acute infectious arthritis in man (13), our present study supports the view that a common structure among bacterial cell walls may exert an important etiological role in the development of infectious arthritis in man. It is also of interest to know that mycoplasma (14) and diphtheroid organisms (15, 16) can be isolated from the rheumatoid arthritic joints in man and that these organisms can also produce polyarthritis in rats (3, 17). Furthermore, it has been shown that there is an increased incidence of septic arthritis in patients with rheumatoid arthritis (18). Therefore, the present results, taken together with above reports (3, 14–18), provide further support to the hypothesis that bacterial infection may be partly involved in the pathogenesis of rheumatoid arthritis and may have some influence on the course and development of this disease, possibly in these disorders serving either as direct antigenic stimulants or more likely as adjuvants to assist in the invasion and/or propagation of another infectious agent, such as a virus, mycoplasma, fungus, etc. In this connection, the use of well-characterized cell walls as used in this rat model system may also be helpful to elucidate the pathogenesis of joint disease in man. Work is currently in progress in our laboratories along these lines.

Summary. Bacterial cell walls from *Str. bovis, Str. lactis, Str. mutans, Str. thermophilus, Str. salivarius,* and *Str. pyogenes* were able to produce polyarthritis in rats but *Str. faecalis* cell walls were nonarthritogenic. *S. aureus* cell walls produced extremely severe disease. It was also shown that cell walls from *S. epidermidis, B. megaterium,* and *M. lysodeikticus* were nonarthritogenic. A close correlation was observed between development of arthritis and the delayed hypersensitivity to bacterial peptidoglycans but *not with the PPD* hypersensitivity. It was suggested that the adjuvanticity of bacterial cell walls is needed to induce the disease and that arthritogenicity requires a specific antigen in addition to the presence of an adjuvant-inducing agent.

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