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STUDIES ON CELL WALLS OF GROUP A *STREPTOCOCCUS PYOGENES*, TYPE 12

II. PYROGENIC AND RELATED BIOLOGICAL ACTIVITIES OF THE HIGHER MOLECULAR WEIGHT FRACTION OF AN ENZYMATIC DIGEST OF THE CELL WALLS[1]

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SUMMARY Cell walls of group A *Streptococcus pyogenes*, type 12, strain S.F. 42 were solubilized by treatment with a purified preparation of the *Flavobacterium* L-11 enzyme which itself exhibited no pyrogenic activity. The higher molecular weight fraction (HMW) was obtained from the cell wall digest by Sephadex gel fractionation. This fraction was shown to contain rhamnose, glucosamine, muramic acid, glutamic acid, alanine and lysine as major constituents, and seemed to be a complex of glycopeptide and a group-specific C-polysaccharide.

On intravenous injection HMW rapidly provoked a high fever in rabbits. The fever was accompanied by a decrease in the number of leukocytes in the blood flow. This leukopenia was followed by leukocytosis. On a weight basis the pyrogenic potency of HMW was found to be greater than that of a finely dispersed preparation of cell walls obtained. The minimum pyrogenic dose of HMW in rabbits weighing about 2 kg was less than 0.1 mg. There was a definite, but partial, cross tolerance between the pyrogenicities of HMW and an endotoxic lipopolysaccharide (Difco) from *Escherichia coli*.

Intravenous injection of HMW sensitized the skin of rabbits to the injurious effects of epinephrine, injected intracutaneously. However, unlike the endotoxic lipopolysaccharide, HMW did not prepare or provoke the dermal Shwartzman reaction.

The significance of this first successful solubilization of a factor responsible for the pyrogenic and related biological activities of the cell walls of gram-positive bacteria using the cell wall lytic enzyme is discussed.

INTRODUCTION

Group A *Streptococcus pyogenes* produces a very wide variety of diseases in man. In connection with this, group A streptococci are charac-

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terized by production of a very large number of biologically active, extracellular products and their cell walls have pathogenic activities. The latter include induction of acute inflammation and prolonged remittent and intermittent multinodular lesions of dermal connective tissue, induction of carditis, which has many similar features to carditis in rheumatic fever in man, inhibition of phagocytosis, and pyrogenicity and related biological activities (Schwab and Ohanian, 1968).

The pathogenic properties of the cell walls of group A *S. pyogenes*, however, have been demonstrated exclusively by use of suspensions of finely dispersed cell wall fragments of an appropriate particle size prepared by sonication or other mechanical procedures. Thus it has been difficult to study the chemical entities in streptococcal cell walls responsible for the biological activities described above and the mechanism by which the active principles exhibit their pathogenic activities.

Previous work in this series (Hamada, Kotani and Kato, 1968) revealed that the cell walls of *S. pyogenes*, type 12, strain S.F.42 could be solubilized by a preparation of the *Flavobacterium* L-11 enzyme. A higher molecular weight fraction (HMW) was separated by gel filtration from a lower molecular weight fraction consisting of basal peptide subunits. HMW contained the bulk of the hexosamines and rhamnose of solubilized wall materials (corresponding to about 80 per cent of the cell walls used) together with some amino acids.

The present investigation was to see if the HMW could retain the pyrogenicity and related biological activities which Roberson and Schwab (1961) and Rotta and Bednář (1969) reported to be associated with the cell walls or their peptidoglycan (mucopeptide) of group A streptococci.

MATERIALS AND METHODS

1. *Test organism and its culture*

Strain S.F. 42 of group A *S. pyogenes*, type 12 was used as the test organism as the preceding study (Hamada et al., 1968). The streptococcal strain was cultivated at 37 C in Trypticase Soy Broth (BBL, Md., USA), which consisted exclusively of components of plant origin, instread of in Todd-Hewitt Broth as previously. After 18 hr incubation a final concentration of 0.2 per cent formalin was added and cultures were stored in the cold overnight to kill the organisms. The dead cells were then harvested in a refrigerated continuous centrifuge and were washed ten times with volume of pyrogen-free distilled water (see below) equivalent to 5 volumes of the cell paste.

In this and following procedures to prepare test materials, pyrogen-free water was used. This was obtained by distilling deionized water twice in an all glass apparatus and then immediately autoclaving it at 120 C for 20 min. The absence of pyrogenicity was proved by intravenous injection of two ml portions of the water into rabbits.

2. *Preparation of purified cell walls*

Washed cell paste (30 g wet weight) was mixed with water to a total volume of 120 ml and the mixture was stirred at 4 C overnight to obtain a homogeneous cell suspension. The cell suspension was submitted to mechanical disruption with a Braun cell homogenizer (Model MSK) as described in a preceding paper (Hamada et al., 1968). The disrupted cell suspension was buffered by addition of 30 ml of 0.2 M phosphate buffer, pH 8.0, and mixed with deoxyribonuclease 1 (No. DN-100, Sigma Chemical Co., Mo., USA) and ribonuclease A (type 1-A, Sigma), both at a final concentration of 10 μg/ml. After incubation at 37 C for 2 hr, the viscosity of the suspension had decreased markedly. The suspension was centrifuged at $450\times g$ for 20 min to remove undisrupted cells as a pellet. The pellet was again submitted to disruption in the Braun homogenizer and the resulting suspension was centrifuged at $450\times g$ for 20 min to separate undisrupted cells. The supernatant fluids of the first and second centrifugations were combined, and were examined by the gram stain in the presence of gram-positive cocci. The combined supernatant fluids which appeared virtually free from undisrupted cells were centrifuged at $4{,}000\times g$ for 60 min to separate the cell walls from the cytoplasmic membranes. The precipitated cell wall fraction was washed several times with pyrogen-free water (about 5 times volume of the precipitate) and then lyophilized. About two g of crude cell wall preparation were thus ob-

tained from 30 g (wet weight) of whole cells. The supernatant obtained by centrifugation at 4,500× *g* was recentrifuged at 20,000× *g* for one hr (Freimer, 1963). The precipitate, consisting of cytoplasmic membranes, was thoroughly washed with pyrogen-free water, lyophilized (90 mg dry weight) and reserved for use in a separate investigation.

The crude cell walls were then digested successively with pronase, trypsin and pepsin. Pronase digestion was carried out by the method of Heymann, Manniello and Barkulis (1963): one g of crude cell wall preparation was suspended in 100 ml of 0.05 M phosphate buffer, pH 7.0 and incubated with 10 mg of pronase P (Kaken Chemical Co., Tokyo) at 37 C for 24 hr in the presence of one ml of chloroform and three ml of toluene. The digested walls were sedimented by centrifugation at 8,000× *g* for 30 min, and washed six times with 100 ml portions of pyrogen-free water, and then once with 100 ml of 0.05 M phosphate buffer, pH 7.0. Then the precipitated walls were resuspended in 100 ml of the latter buffer containing 10 mg of crystalline trypsin (Trypsilin, Mochida Pharmaceutical Co., Tokyo), and the suspension was incubated at 37 C for two hr. The incubation mixture was centrifuged at 8,000× *g* for 30 min, and the precipitated cell walls were thoroughly washed with pyrogen-free water as described above. Then they were washed once with 100 ml of 0.2 M glycine-HCl buffer, pH 2.2, and digested with 10 mg of pepsin (Sigma) in 100 ml of the glycine-HCl buffer at 37 C for 2 hr. Then the cell walls were washed eight times with 100 ml portions of pyrogen-free water, and lyophilized. The yield of purified cell wall preparation from one g of crude cell walls was 387 mg.

3. *Preparation of Flavobacterium L-11 enzyme*

The L-11 enzyme preparation used in the previous study which had been partially purified by Sephadex G-75 gel filtration, was unsuitable for the present work because it has strong pyrogenic activity. Therefore, the L-11 enzyme was purified by CM-cellulose column chromatography to obtain a preparation which was not contaminated with pyrogenic substances. One g of a lyophilized, crude L-11 enzyme preparation of the same lot as that used previously was dissolved in 10 ml of pyrogen-free 0.01 M phosphate buffer, pH 6.0 and the solution was centrifuged at 10,000× *g* for 30 min at 4 C. The insoluble precipitate was redissolved in 10 ml of the above buffer and the solution was centrifuged. The second precipitate was discarded. The crude enzyme solution obtained by combination of the first and second supernatants was applied to a CM-cellulose column (2.2 cmϕ× 15 cm) equilibrated with 0.01 M phosphate buffer, pH 6.0. The washing fluid from the column had been shown to be nonpyrogenic on intravenous injection into rabbits. The column was eluted successively with 0.01 M buffer, pH 6.0, 0.05 M buffer, pH 8.0 and 0.1 M buffer, pH. 8.0. Fractions of 10 ml of effluent were collected. Their extinctions at 340 mμ (due to brown-colored substances in the crude enzyme specimen) and at 280 mμ (due to protein) and their lytic activities against whole cells of group A *S. pyogenes*, strain S.F. 42, and *Staphylococcus aureus*, strain Copenhagen were measured.

Fig. 1 shows that lytic activity was eluted in two broad peaks. The first peak (effluent volume, 450–650 ml) showed strong lytic activity against *S. pyogenes* whole cells, but the activity against *S. aureus* whole cells was weak and the solution was heavily contaminated with brown colored materials. The second peak (effluent volume, 800–1,000 ml), on the other hand, exhibited strong lytic activities against whole cells of both *S. pyogenes* and *S. aureus* despite of its very low protein content. It did not contain significant amounts of colored materials. The fractions comprising the second peak, therefore, were pooled and dialyzed twice against one liter portions of 0.005 M phosphate buffer, pH 8.0, for 12 hr each. The lytic activity of the resultant enzyme preparation was assayed against the cell walls of *S. pyogenes*, strain S.F. 42 by the method described previously (Kato et al., 1962), and found to be 1.68 units/ml. The protein content of the preparation was estimated to be 0.17 mg/ml using bovine serum albumin (Crystalline, Armour Pharmaceutical Co., USA) as a standard. The pyrogenicity of this purified enzyme preparation was examined by intravenous injection into rabbits, and it was found that 0.5 ml aliquots of the enzyme solution, at least, produced no significant febrile response.

4. *Chemical analyses*

Amino acids and amino sugars in test materials were determined with a Hitachi amino acid analyzer (Model KLA-3B, Hitachi Ltd., Tokyo) on specimens hydrolyzed by heating in 6N HCl at 100 C for 12 hr in sealed ampoules. Uronic acid was assayed by the method of Dische (1947) without prior hydrolysis. Analyses of fatty acid by gas chromatography was

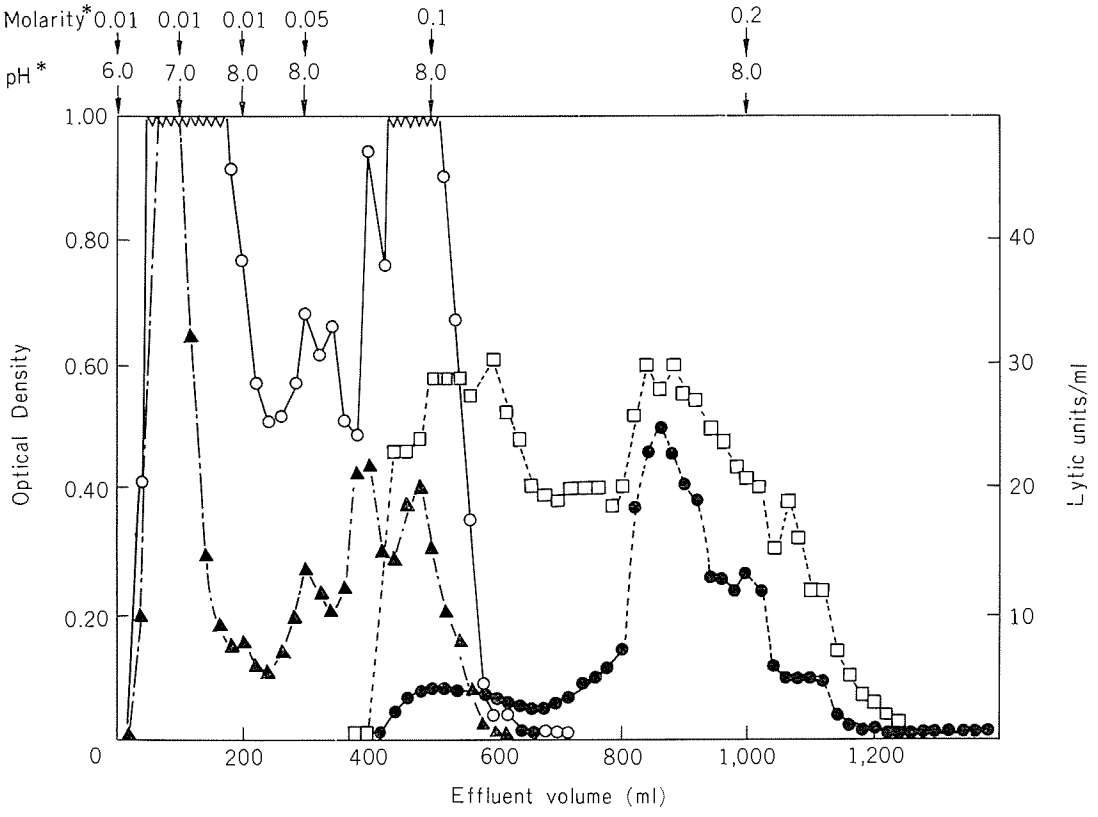

FIGURE 1. *Purification of crude L-11 enzyme by CM-cellulose column chromatography.*
□-------□ : *Lytic activity against S. pyogenes whole cells.*
●-------● : *Lytic activity against S. aureus whole cells.*
○———○ : *OD at 280 mμ.*
▲- - - -▲ : *OD at 340 mμ.*
* *Molarity and pH values of the phos-phate buffers used for elution.*

kindly carried out on methylated test specimens by Dr. O. Kanemasa, Department of Bacteriology, Okayama University Medical School. Other analyses were performed as described previously (Hamada et al., 1968).

5. *Rabbits*

Random-bred male white rabbits, weighing about 2 kg, purchased from several dealers were used throughout the present study. They were kept in an air-conditioned room and fed on standard diet without antibiotic supplement.

6. *Assay of the febrile response and leukocyte count*

Rabbits were fixed in steel boxes with openings for the head and rectum. On the day of an experiment they were kept in boxes for at least two hr before injection of test materials, and no food or water was given during the test period. Rectal temperatures were measured with a clinical thermometer at appropriate intervals after the test materials had been injected into the marginal ear vein. Before injection two readings were taken to establish a base line.

The pyrogenicity of each test material was examined in at least three rabbits. Thus each point in

the fever response curves in the figures is an average value. A rise of 0.5 C or more in rectal temperature was regarded as being significant, since fluctuations of rectal temperature in test rabbits were less than 0.3 C under the experimental conditions. Rabbits were only used in one pyrogenicity test to avoid the complication of an immunologic mechanism or tolerance.

In some experiments, a free flowing blood sample from the marginal ear vein was taken immediately after temperature measurements to determine the leukocyte response to test materials. Leukocyte counts were made in the conventional manner.

7. *General*

Strict precautions were taken to prevent contamination of test materials with exogenous pyrogens. All equipment, including glassware, and Sephadex and CM-cellulose were thoroughly rinsed with pyrogen-free water, and all reagents used in preparation of test materials were dissolved in pyrogen-free water. Glassware was further heated at 170 C for several hr to make sure that it was free from pyrogen. Pyrogen-free needles (Terumo Sterile-Disposable, Jintan-Terumo Co., Tokyo) were used for injections.

RESULTS

1. *Isolation and chemical properties of HMW from digests of purified cell walls with the L-11 enzyme*

A portion (350 mg) of the purified cell wall preparation was suspended in 70 ml of 0.05 M phosphate buffer, pH 8.0, containing 35 streptolytic units of the L-11 enzyme. Sodium azide (7 mg) was added as a preservative and the suspension was incubated at 37 C for 96 hr. During the incubation the turbidity of the reaction mixture decreased by about 60 percent. Then the mixture was centrifuged at $40,000 \times g$ for 60 min. The precipitate was washed with pyrogen-free water by centrifugation and the washing fluid was combined with the first supernatant. The washed residue was lyophilized (185 mg dry weight), and redigested with the L-11 enzyme in the same way as before. This resulted in about 55 percent reduction in turbidity. The solubilized material was obtained as described above.

The solubilized material obtained by the first treatment with enzyme was concentrated to about 2 ml in a rotary evaporator in a water bath at 37 C. It was then applied to a column of Sephadex G-50 (bead form, coarse) connected by a polyethylene capillary tube to a column of Sephadex G-25 (bead form, coarse), and the column were eluted with pyrogen-free water. The elution profile on gel filtration was similar to that obtained previously (*cf.* Fig. 3 in the previous paper; Hamada et al., 1968). The fractions eluted immediately after the V_0 were pooled, concentrated and lyophilized (HMW,

TABLE 1. *Chemical analyses of HMW isolated by digestion of the cell walls of group A S. pyogenes, type 12, strain S.F. 42 with the L-11 enzyme*

Component	mμmoles/mg	Molar ratio to glutamic acid	Per cent in the cell walls
Rhamnose	1,900	9.0	31.2
Total hexosamine	1,500	7.1	26.8
(Glucosamine)	(940)	(4.5)	(16.8)
(Muramic acid)	(188)	(0.9)	(4.7)
Glutamic acid	210	1.0	3.1
Alanine	756	3.6	6.7
Lysine	254	1.2	3.7
Ammonia	114	0.5	0.2
Total phosphorus	114	0.5	0.4

110 mg dry weight). The soluble material from the second enzyme digestion was similarly fractionated and yielded about 50 mg of HMW. These two HMW fractions were found to have essentially the same chemical and biological properties.

Table 1 shows that HMW contained glutamic acid, alanine, lysine, muramic acid, glucosamine and rhamnose in molar ratios of 1.0: 3.6: 1.2: 0.9: 4.5: 9.0. Other amino acids which had been found as minor components of the original cell wall preparation were not detected in HMW. Chemical analyses suggest that the HMW was probably a complex of soluble glycopeptide and group specific C-polysaccharide. Considerable amounts of phosphorus and ammonia were also detected. The former may be involved in the linkage between the C-polysaccharide and glycopeptide, since group A streptococcal cell walls are reported to have a high content of muramic acid-6-phosphate (Liu and Gotschlich, 1967; Heymann, Manniello and Barkulis, 1967). However, Muñoz, Ghuysen and Heymann (1967) suggested that there is another type of linkage (see discussion). Ammonia may have been underestimated due to limitations inherent in analysis using an amino acid analyzer. The ammonia is assumed to be involved in amidation of the (α-)carboxyl group of glutamic acid residues.

The sum of the components shown in Table 1 only accounts for 72 per cent by weight of the HMW specimen. Accordingly the fatty acid and uronic acid contents of HMW were examined. Traces of fatty acids ($C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, and $C_{18:1}$: and two other unidentified acids) were found but no uronic acid. The ash content could not be determined because only a limited amount of material was available.

2. *Fever response and change in leukocyte count following intravenous injection of purified cell walls*

The pyrogenicity of the purified cell wall preparation and its effect on the leukocyte count were examined. Aliquots of a homogenous suspension of cell wall fragments (1 mg/ml) prepared by treatment of the purified cell wall preparation in a Super-Sonic Vibrator (UR-150P, Tominaga Works, Ltd., Tokyo) for 30 min, were injected intravenously into rabbits. The change in temperature and leukocyte count in rabbits after injections of one or two mg of the finely dispersed cell wall preparation are shown in Fig. 2. In agreement with the results of Roberson and Schwab (1961), rapid elevation of temperature and leukopenia in the circulation were observed within an hr. The temperature remained high for a few hours, and

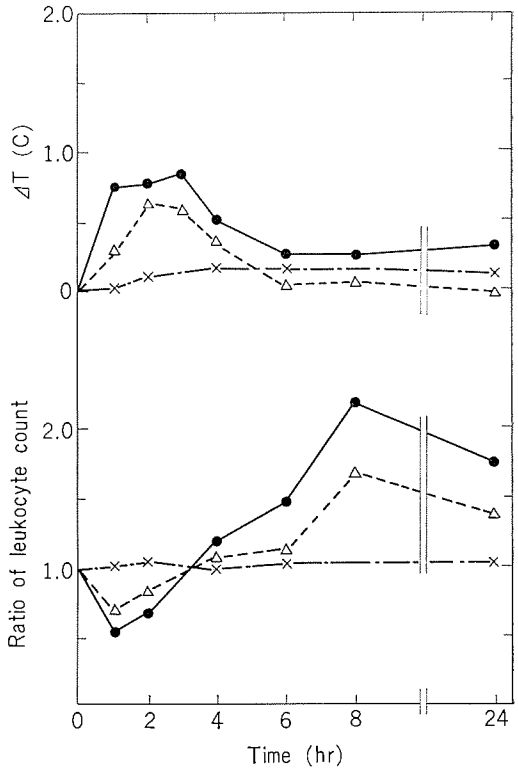

FIGURE 2. *Changes in body temperature and leukocyte count following intravenous injection of sonicated cell walls.*

×———×: *Control receiving no cell walls.*
△-------△: *1 mg cell walls.*
●———●: *2 mg cell walls.*

Each point represents the average of values in 3 rabbits.

then gradually returned to normal. The time of change from leukopenia to marked leukocytosis coincided with the time of decrease in temperature. No significant changes in temperature or leukocyte count were observed in control rabbits after intravenous injection of 2 ml of pyrogen-free water.

3. *Fever response and change in leukocyte count following intravenous injection of HMW*

The changes in rectal temperature and leukocyte count after intravenous injection of 0.05 to 1.0 mg HMW are shown in Fig. 3. The changes were qualitatively similar to those after injection of finely dispersed cell wall fragments (Fig. 2). Rapid and marked increase in temperature occurred within one hr and the leukocyte count firstly decreased, then increased and finally gradually reverted to the initial level. From the dose-fever or dose-leukocyte count response curves, it is concluded that the minimum effective dose is about 0.1 mg, although the response with HMW seems to be greater than that with cell wall fragments, while the reverse was true for the extents of leukopenia and leukocytosis.

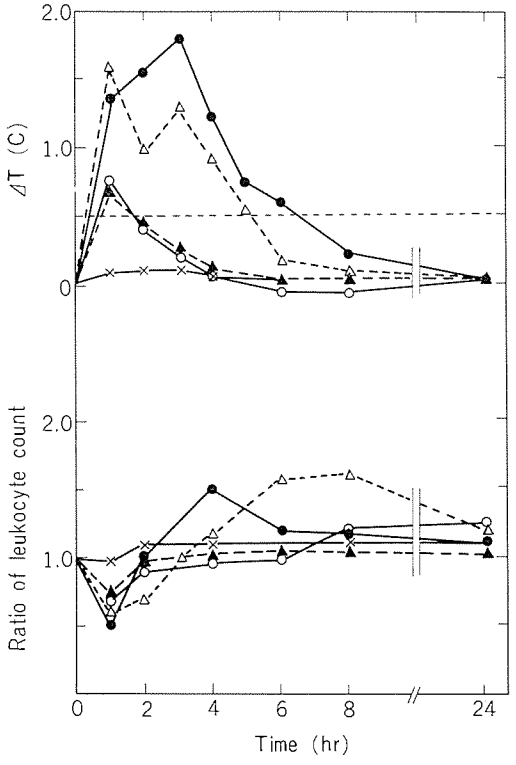

FIGURE 3. *Changes in temperature and leukocyte count following intravenous injection of HMW isolated by digestion of cell walls with the L-11 enzyme.*
×——×: *0.05 mg of HMW.*
○——○: *0.1 mg of HMW.*
▲------▲: *0.2 mg of HMW.*
●——●: *0.5 mg of HMW.*
△-------△: *1.0 mg of HMW.*
Each point represents the average of values in 3 rabbits.

The purified L-11 enzyme specimen used in the present study did not influence the temperature or leukocyte count even at the dose calculated to be present in 1 mg of HMW preparation, assuming that all the enzyme employed in solubilization of the cell walls was recovered in the HMW preparation. Therefore, the responses to injection of HMW were not due to extraneous pyrogens derived from the L-11 enzyme preparation. Thus the fever and leukocyte responses shown in Fig. 3 are due to enzymatically solubilized cell wall components themselves.

4. *Change in fever response on repeated injections of HMW and cross-tolerance between HMW and E. coli lipopolysaccharide*

When rabbits received repeated injections of HMW, their febrile response gradually decreased, as shown in Fig. 4, indicating that they became tolerant to the pyrogenic effect of HMW. A first injection of either 0.5 or 1.0 mg of HMW caused a rise in temperature of 1.5 C or more within one hr, and the temperature remained high for several hr. When the same rabbits were given two injections every other day, they showed much smaller fever responses. These rabbits which had become partially tolerant to HMW were given an intravenous injection of 1 μg of *Escherichia coli* lipopolysaccharide (*E. coli* 0128: B12, Difco, Mich., USA) two days after the last injection of HMW. They showed a fever response after the lipo-

polysaccharide injection, but significantly less than that of control animals which had not received HMW. When rabbits which had become partially tolerant to HMW were kept for 2 weeks, and then injected with either HMW or lipopolysaccharide their febrile responses were the same as those of normal animals.

In the converse experiment, rabbits were made tolerant to the lipopolysaccharide from *E. coli* by daily injections of 1 μg for the first 3 days and then 2 μg for the following 4 days, as shown in Fig. 5. Two days later they were challenged with 0.5 mg of HMW. The fever response elicited was significant (ca. 0.5 C), but was transient and weak, compared with that of control animals, which had not been treated with lipopolysaccharide. These experiments suggest that reciprocal cross-tolerance between

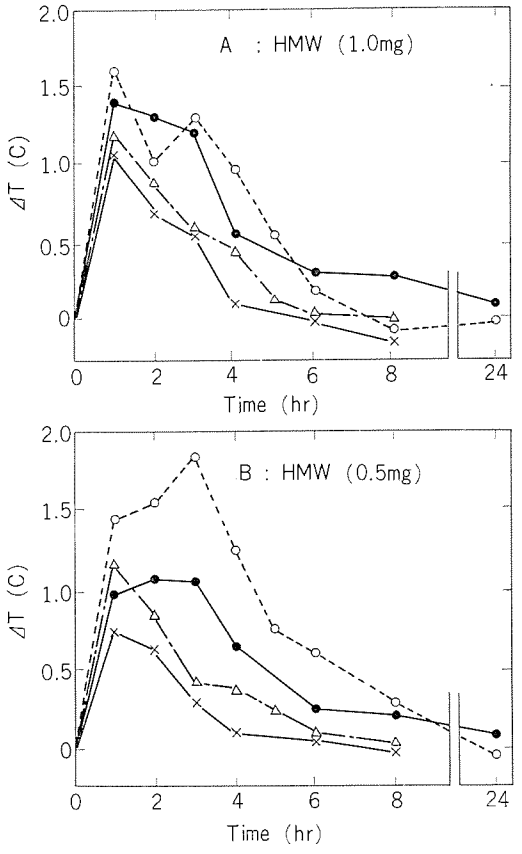

FIGURE 4. *Febrile responses of HMW-tolerant rabbits to lipopolysaccharide (LPS) from E. coli (Difco). Rabbits received 3 intravenous injections of HMW (0.1 mg each in A and 0.5 mg each in B) every other day, and an intravenous injection of LPS (1 μg) on the 7th day.*

○-------○ : *Febrile response on the first injection of HMW.*

△-------△ : *Febrile response on the second injection of HMW.*

×———× : *Febrile response on the third injection of HMW.*

●———● : *Febrile response on injection of LPS.*

Each point represents the average of values in 3 rabbits.

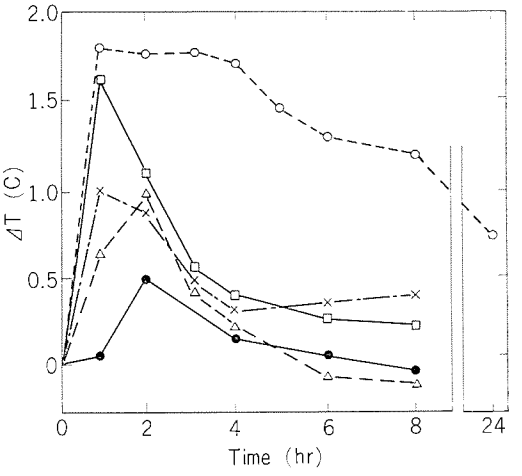

FIGURE 5. *Febrile responses of LPS-tolerant rabbits to HMW.*

Rabbits were given 7 daily intravenous injections of LPS (1 μg each for the first 3 days and 2 μg each for the following 4 days), and then an intravenous injection of HMW (0.5 mg) on the 9th day.

○-------○ : *Febrile response to the first injection of LPS.*

□———□ : *Febrile response to the third injection of LPS.*

×-----× : *Febrile response to the fifth injection of LPS.*

△----△ : *Febrile response to the seventh injection of LPS.*

●———● : *Febrile response to injection of HMW.*

LPS: A lipopolysaccharide specimen from E. coli (Difco).

Each point represents the average of values in 3 rabbits.

HMW from group A *S. pyogenes* and the lipopolysaccharide from *E. coli* develops in these animals against the induction of fever, though in both cases tolerance is incomplete.

5. *Fever response of rabbits to HMW after chlorpromazine administration*

Rabbits were injected intravenously with 2 mg of chlorpromazine (Contomin, Yoshitomi Pharmaceutical Co., Osaka) per kg body weight (Haan and Meyer, 1968). Thirty min later, they were given 0.5 mg of HMW intravenously. Fig. 6 shows that in control rabbits receiving chlorpromazine without HMW the temperature decreased appreciably (ca. 2 C) and remained low for 8 hr while in receiving both chlorpromazine and HMW it decreased less than 1 C and returned to normal within 4 to 6 hr. The above results indicate that HMW has a pyrogenic effect on rabbits even when their temperature is lowered by premedication with chlorpromazine. However, it is uncertain from our experiments whether chlorpromazine administration influences the febrile response to HMW at all.

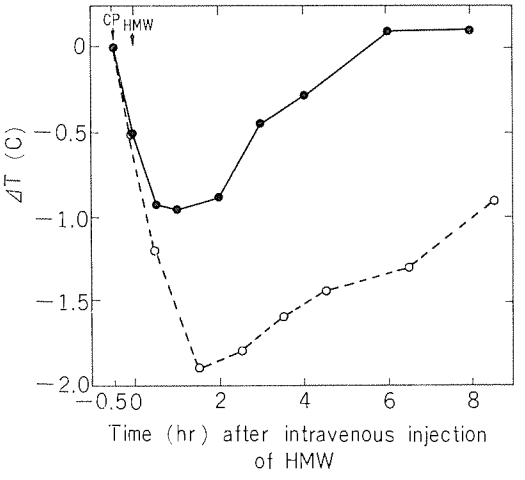

FIGURE 6. *Effect of chlorpromazine on the febrile response of rabbits to HMW.*

●——●: *Febrile response to HMW (0.5 mg) after administration of chlorpromazine (CP, 2 mg/kg).*

○-------○: *Febrile response of control rabbits administered with chlorpromazine (2 mg/kg) but not HMW.*

Each point represents the average of values in 3 rabbits.

6. *Effect of heating HMW on its pyrogenic activity*

The heat stability of the pyrogenic activity of HMW was determined by heating a solution of HMW (1 mg/ml) in pyrogen-free water at 100 C for 10 min, and then testing its pyrogenicity. The heated solution of HMW retained full pyrogenicity. It should be mentioned that the cell wall preparation from which HMW was derived, had been heated at 60 C for 16 hr in the purification procedure during digestion with pronase.

7. *Abilities of HMW to enhance the reactivity of rabbit skin to epinephrine and to prepare or provoke the local Shwartzman reaction*

Lipopolysaccharides from gram-negative bacteria, and streptococcal cell constituents enhance the skin reaction to epinephrine and prepare or provoke the local Shwartzman reaction (see Discussion), so these biological activities were examined with HMW. Rabbits were given an intravenous injection of HMW (2 mg), and one hr later, they were injected intradermally with 10, 50 or 100 μg of epinephrine in 0.2 ml of pyrogen-free water. As seen in Fig. 7, rabbits treated with HMW developed indurate areas with petechiae within several hr after injection of epinephrine. During the following 24 hr, these lesions gradually became hemorrhagic and then necrotic. Epinephrine did not produce any significant lesion in control animals which had not received HMW. In these lesions, scattered foci of cell infiltration were recognized in the dermal connective tissue but infiltration did not reach the muscular layer. The nuclei of the infiltrating cells were badly damaged, and their origin could not be identified with certainty, though they appeared to be derived from lymphocytes and/or neutrophil leukocytes.

The ability of HMW to prepare or provoke the dermal Shwartzman reaction in rabbits was

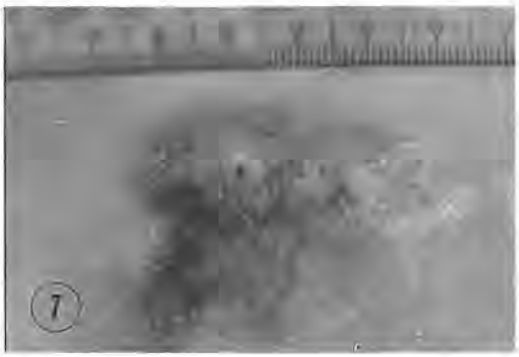

FIGURE 7. *Macroscopic appearance of the epinephrine test on rabbit skin (24 hr after intradermal injection of epinephrine).*

Intravenous injection of HMW (2.0 mg) was followed one hr later by intracuteneous injection of epinephrine (100 μg/0.2 ml).

also examined. The experimental conditions (kinds and doses of test materials in preparative and provocative injections) are shown in Table 2. No discernible reaction at the intradermal injection sites occurred with any of the combination tested in which either or both the preparative and provocative injection was HMW. Thus HMW did not either prepare or provoke the dermal Shwartzman reaction. On the other hand, a typical dermal Shwartzman reaction was seen in a control experiment, where a preparation of *E. coli* lipopolysaccharide was used as both the preparative and provocative agent.

DISCUSSION

Cell wall lipopolysaccharides (endotoxins) from gram-negative bacteria are pyrogens and they are ubiquitous in nature. They are so pyrogenic that they have been widely used as agents to induce a febrile response in studies on the pathogenesis of fever. On the other hand, some constituents of gram-positive bacteria cause fever injected intravenously into rabbits (Atkins and Freedman, 1963). However, the pyrogenicity of gram-positive bacteria is less than that of the lipopolysaccharides of gram-negative bacteria, and the nature of the principle(s) responsible for fever production is unknown. Among the pyrogens derived from gram-positive bacteria, those of group A *S.*

TABLE 2. *Inability of HMW to prepare or provoke the local Shwartzman reaction in rabbits*

Preparatory injection (intradermal, mg/0.2 ml)		Provocative injection (intravenous, mg/2 ml)		Shwartzman reaction
HMW	2.0	*E. coli* LPS	0.2	Negative
〃	1.0	〃	0.2	Negative
E. coli LPS	0.2	〃	0.2	Positive
〃	0.1	〃	0.2	Positive
〃	0.05	〃	0.2	Positive
〃	0	〃	0.2	Negative
HMW	2.0	HMW	2.0	Negative
〃	1.0	〃	2.0	Negative
E. coli LPS	0.2	〃	2.0	Negative
〃	0.1	〃	2.0	Negative
〃	0.05	〃	2.0	Negative
〃	0	〃	2.0	Negative

The interval between the preparatory and provocative injections was 18 hr. A lipopolysaccharide (LPS) preparation from E. coli (Difco) was used as a reference. The results of the dermal Shwartzman reaction were read 2 and 6 hr after the intravenous challenge.

pyogenes have been the most extensively studied. Stetson (1955) reported that when a suspension of group A streptococci was disrupted with glass beads in a Mickle disintegrator and then centrifuged, the resulting supernatant (" lysate ") caused systemic reactions including fever, similar to those produced by endotoxic lipopolysaccharides, when injected intravenously into rabbits. However it was unknown what component of the cells was responsible for the reactions produced. Subsequently, Roberson and Schwab (1961) obtained highly purified cell wall preparations from group A streptococci by differential centrifugation of a disrupted cell suspension, digestion of the cell wall fraction with papain and purification by sedimentation through sucrose gradients. These preparations evoked various reactions in rabbits, similar to those caused by the " lysate " of Stetson (1955). More recently, streptococcal cell wall mucopeptide which had been " solubilized " by ultrasonication was shown by Rotta and Bednář (1969) to elicit a reproducible febrile response when injected intravenously into rabbits. Their studies showed that the mucopeptide obtained as an insoluble residue by extracting the cell walls of group A *S. pyogenes* with formamide, was pyrogenic when " solubilized " by sonication, but that the C-polysaccharide extracted was not. " Solubilized " cell wall mucopeptides from groups B and L streptococci were also found to have pyrogenic activity. Both groups of investigators pointed out that the particle size of test materials was a critical factor in determining their pyrogenicities or other related biological activities and that the minimum effective doses were significantly reduced by " solubilization " of the test materials by sonic vibration. Apart from the " endotoxic " pyrogens cited above, Kim and Watson (1970) reported the presence of pyrogenic exotoxin, a complex of protein with hyaluronic acid in the culture filtrate of group A streptococci.

The present investigation clearly showed that the higher molecular weight fraction (HMW) isolated from the cell walls of group A *S. pyogenes*, type 12, strain S.F. 42 using the L-11 enzyme, possessed biological activities similar to those exhibited by the cell walls or their peptidoglycans. It should be emphasized that HMW was not a preparation of cell wall fragments randomly divided by mechanical means like the test materials so far used, but consisted of macromolecules obtained by enzymatic hydrolysis of streptococcal cell wall peptidoglycan. This seems to be the first soluble preparation of the pyrogenic principle from the cell walls of gram-positive bacteria. Atkins and Morse (1967) attempted to solubilize the pyrogenic agent in *S. aurues* cell walls by extraction with hot trichloroacetic acid and digestion with egg white lysozyme, but without success. The minimum pyrogenic dose of HMW was found to be between 0.05 and 0.1 mg (Fig. 3). This is in the same order as the value reported for a highly purified preparation of cell walls by Roberson and Schwab (1961). The pyrogenicity of HMW seemed to be greater than that of the sonicated cell wall fragments used as a reference in the present study. Rotta and Bednář (1969) claimed that a reproducible fever response was provoked by intravenous injection of as little as one μg of " solubilized " peptidoglycan. This minimum effective dose seems to be extraordinarily low compared with that reported by Roberson and Schwab (1961) and that found in the present work. The reason why the cell wall peptidoglycan specimen prepared by Rotta and Bednář (1969) had such high pyrogenicity is not known, but might be attributable to pecularities of the streptococcal strain used in their study. The minimum pyrogenic dose of the heat-labile exotoxin obtained from group A streptococcal filtrates was 0.07 μg/kg (Kim and Watson, 1970).

The possibility that the observed pyrogenicity and related biological activities of HMW were due to contaminating substances other than cell wall constituents should be considered. The effects of heat-labile exotoxic pyrogens can be excluded because the pyrogenicity of HMW

did not diminish on heating at 100 C for 10 min, though the heat stability of exotoxic pyrogen might be increased by adsorption of the pyrogen onto cell walls or cell wall components. A more important factor to be excluded is endotoxic lipopolysaccharides of gram-negative bacteria which are widely distributed in nature, and are extremely pyrogenic. The minimum effective dose of the lipopolysaccharides for rabbits is reported to be in the order of 10^{-3} μg/kg body weight (Westphal et al., 1952; Landy and Johnson, 1955; Westphal and Lüderitz, 1954). Thus, as in the previous investigations by others, meticulous care had to be taken to avoid contamination with exogenous bacterial lipopolysaccharides, as described in Materials and Methods. In addition, differences between the profiles of the febrile responses induced by HMW and endotoxic lipopolysaccharides (monophasic fever of short duration *vs.* biphasic fever of long duration) and the inability of HMW to prepare or provoke the dermal Shwartzman reaction serve as circumstantial evidence to indicate that the pyrogenicity of HMW is not due to contaminating exogenous endotoxic lipopolysaccharides.

What is the chemical nature of the principle responsible for pyrogenicity or related biological activities in group A streptococcal cell walls? From analytical data on HMW together with the results reported in the previous paper (Hamada et al., 1968), and information on the chemical structure of group A *S. pyogenes* cell walls reported by others (Muñoz et al., 1967; Heymann et al., 1963), we tentatively propose that HMW may be a complex of glycopeptide and a group specific C-polysaccharide. The glycan portion of HMW may be composed of alternate repeating units of *N*-acetylmuramic acid and *N*-acetylglucosamine residues arranged in linear strands as in the cell wall peptidoglycan of other bacterial species. Analyses showed that HMW contained 0.9 mole muramic acid per mole of glutamic acid residue. From the unusual lability of the muramic acid residues in streptococcal cell walls on acid hydrolysis and the fact that the L-11 enzyme has *N*-acetylmuramyl-L-alanine amidase activity as well as D-alanyl-L-alanine endopeptidase activity against group A streptococcal cell walls, many of the muramic acid residues in the glycan portion of HMW do not seem to be substituted with basal peptide subunits. It should be pointed out that not all the *N*-acetylmuramic acid-L-alanine linkages or D-alanyl-L-alanine bonds were susceptible to the amidase or endopeptidase of L-11 enzyme, and many of them were not hydrolyzed enzymatically for some reason such as steric hindrance. The nature of the linkage between the peptidoglycan and C-polysaccharide moieties is unknown. The high content of muramic acid-6-phosphate in group A streptococci, reported by Liu and Gotschlich (1967) suggests that C-polysaccharide may be linked by phosphodiester bonds to C-6 of the muramic acid residues in the glycan. On the other hand, Muñoz et al. (1967) studied group A *S. pyogenes* cell walls (Type 14) and concluded that G-polysaccharide is linked by phosphodiester bonds to C-6 of muramic acid residues and C-polysaccharide combines through a trirhamnosyl fragment with C-4 of the muramic acid residue. However, previous study (Hamada et al., 1968) showed that the cell wall preparation used in the present study contained no glucose, but Muñoz et al. (1967) reported that glucose is a major constituent of G-polysaccharide. This observation and the fact that a ninhydrin-positive peak, which was probably muramic acid-6-phosphate, was detected by amino acid analysis of a hydrolysate of the cell walls used in the present study, seem to indicate the presence of phosphodiester linkages involving C-6 of muramic acid residues between glycopeptide and C-polysaccharide in HMW. What is the minimum structure (components or linkages) responsible for the pyrogenicity or related biological activities exhibited by HMW? According to Rotta and Bednář (1969), the C-polysaccharide portion of group A streptococcal cell walls does not seem to be associated with pyrogenic activity. If so, the structure re-

sponsible for the pyrogenicity of HMW should reside in the glycopeptide portion. In the present investigation we attempted to destroy the pyrogenic activity of HMW by further degradation with the enzyme from *Chalaropsis sp.* or egg white lysozyme, but were unsuccessful. Another way to elucidate the structure of cell wall peptidoglycan responsible for its biological activities is to see whether random polypeptide synthesized from glutamic acid (L or D isomers), lysine and alanine (L or D isomers) residues have the same biological activities as cell wall peptidoglycan. Karakawa et al. (1970) used these polypeptides in studies on the antigenic specificity of cell wall peptidoglycans. In the present study, the pathogenesis of the fever induced by HMW was not examined. Atkins and Snell (1965) and Snell and Atkins (1968), showing that there were similarities between the profiles of the fever response induced by intravenous injections of whole cells of gram-positive bacteria and those elicited by organic or inorganic colloidal particles such as dextran, methylcellulose, calcium phosphate, sulfur, kaolin, quartz, thorium dioxide etc. They pointed out that the pyrogenicity of gram-positive bacterial cells might depend on some physicochemical properties such as particle sizes or surface charges common to both bacterial cells and colloidal particles. In support of this possibility, Atkins and Morse (1967) cited the fact that disruption of the mucopeptide structure of *S. aureus* cell walls by lysozyme treatment abolished ability of the cells to cause fever. Roberson and Schwab (1961) also considered that the pyrogenic activity in streptococcal cell walls is very similar to that of a class of colloidal substances of diverse chemical composition. The fact that in the present study HMW in the form of a true solution elicited a remarkable febrile response in rabbits does not seem to be compatible with the view of Atkins and his coworkers, and suggests that the chemical structure of streptococcal cell walls may be a more important factor in determining the manifestation of their pyrogenicity or other biological activities.

The fever response elicited by injection of HMW was much more rapid than that caused by whole cells or cell walls of gram-positive bacteria (Stetson, 1955; Roberson and Schwab, 1961; Atkins and Freedman, 1963; Rotta and Bednář, 1969). The fever elicited by endotoxic lipopolysaccharides may be due to liberation of an endogenous pyrogenic substance from the host tissue (Atkins and Snell, 1965). If the fever elicited by whole cells or cell walls of gram-positive bacteria is also caused in this way, then it is understandable that HMW, which is soluble and does not require phagocytosis and solubilization or degradation by leukocytes before inducing a response, unlike particulate material, should induce a more prompt fever response than particulate material. The fever induced by HMW may also be a manifestation of hypersensitivity to related antigens of bacteria to which the test rabbits were sensitized either endogenously or exogenously. In this connection it has been reported that fever was elicited by intravenous administration of old tuberculin to BCG-infected rabbits (Hall and Atkins, 1959). Moreover rabbits which had been sensitized by repeated injections of bovine serum albumin (Farr, 1959) or human serum albumin (Root and Wolff, 1968) developed fever when challenged intravenously with specific antigens. Streptococcal C-polysaccharide and glycopeptide which are major components of HMW are both reported to be antigenic (Karakawa and Krause, 1966; Karakawa, Lackland and Krause, 1966; Karakawa et al., 1967). Consequently, the above possibility cannot be excluded, although the pattern of the fever response induced by HMW was definitely different from that induced by hypersensitivity.

Severe dermal necrotic reaction (Stetson, 1955; Thomas, 1956) developed in the rabbits on injection of epinephrine after intravenous injection of HMW but not an injection of epinephrine only. However, HMW did not prepare or provoke the local Shwartzman reaction. Colloidal particles of group A streptococcal cell walls (Roberson and Schwab, 1961) or of cell

wall peptidoglycans (Rotta and Bednář, 1969) are reported to induce positive epinephrine and Shwartzman reactions in which they serve both as preparatory and provocative factors. The findings that HMW, unlike cell walls or their peptidoglycans, was active in enhancing the susceptibility of rabbits to intradermal injection of epinephrine but not in development of dermal Shwartzman reactivity may reflect a difference in the sensitivities of these two reactions or a difference between HMW and cell walls or their peptidoglycans.

Reports on other biological activities of HMW such as its ability to induce cardiac lesions in mice on intraperitoneal injection will be present in the following paper in this series (Narita et al., 1971).

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