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Notes

Adjuvanticity of Mycobacterial Cell Walls[1]

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It is well known that complete Freund's adjuvant, which contains heat-killed mycobacterial cells in mineral oil, is one of the most potent adjuvants currently used, and *Mycobacterium tuberculosis* is the organism commonly utilized as a constituent in the adjuvant [4, 10]. Wax D from the human tubercle bacilli has been shown to be the adjuvant-active fraction [11–14, 16]. However, it has also been reported that completely lipid free tubercle bacilli [14] as well as the cell walls of tubercle bacilli [9] were found to be active as an adjuvant in the production of experimental allergic encephalomyelitis. A detailed investigation on the adjuvant activity of the cell wall of tubercle bacilli has not yet been carried out.

This communication describes the adjuvanticity of mycobacterial cell walls and a mucopeptide thereof on certain humoral and cellular antibody responses.

Cell walls were prepared from *Mycobacterium smegmatis, M. phlei, M. bovis* BCG, *M. kansasii* and *M. tuberculosis* Aoyama B by the method described previously [1, 3] using a French Press, a Ribi cell fractionator or ultrasonic oscillator. The supernatant fluid from the centrifugation of disrupted cells at 27 000×*g* for 60 min was dialysed against running water and lyophilized. This fraction was used as "cytoplasm" in this experiment. The crude cell walls obtained by fractional centrifugation were treated with trypsin, chymotrypsin and pronase, and delipidated by repeated extraction with ether–ethanol (1:1), chloroform and chloroform–methanol (2:1). The purity examination of cell walls by acid-fast staining, amino acid analysis and electron microscopical methods indicated that the cell wall preparations were not contaminated with the intact cells. "Acid-treated cell wall" was prepared from the cell walls of *M. bovis* BCG by the following procedures: The delipidated cell walls were treated with 0.1 N HCl solution at 60 C for 12 hr with shaking. After centrifugation, the residues were washed with water, and then extracted with acetone and ether to remove the bound lipid. The HCl-treated cell walls were further extracted with 5% trichloroacetic acid at 60 C for 24 hr. These cell walls were collected by centrifugation and then washed thoroughly with water,

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acetone and ether. Finally, the neutral polysaccharide moiety (arabinogalactan) of the cell wall was destroyed by treating the cell walls with 0.05 M metaperiodate at 4 C for 14 days in a dark room with continuous stirring. The "acid-treated cell wall" fraction was termed "MP fraction." The MP fraction thus obtained was found to contain a small amount of neutral sugar (3.5%), lipid (less than 3%), amino sugars (26.7%) and amino acids (58.3%) which are alanine, glutamic acid, diaminopimelic acid, in a molar-ratio of 1.6:1:1 as the major amino acids after hydrolysis. Bovine serum albumin (BSA), sheep red blood cells (SRBC) and sulfanylazo-bovine serum albumin (SA-BSA) were used as antigens. Female albino mice weighing 20–23 g of either ddO, ddK or ICR strain were injected with each of antigens in incomplete Freund's adjuvant (IFA) with and without either cell walls, MP fraction or cytoplasm. The antibody titers were determined by either direct or indirect hemagglutination method using microtiter equipment.

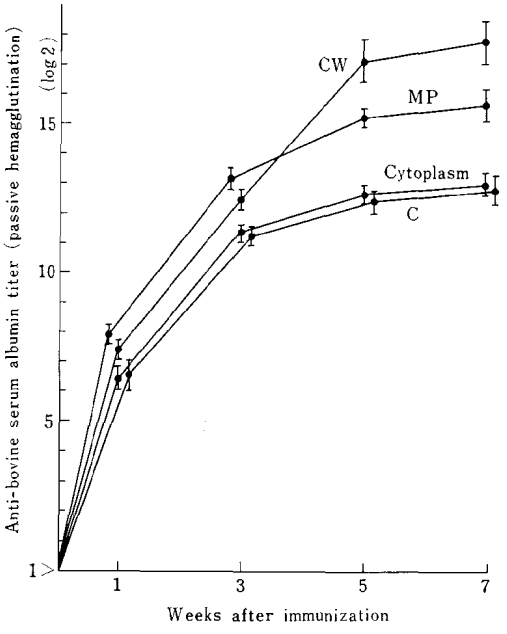

Fig. 1. Adjuvant activity of BCG cell wall, cytoplasm and "acid-treated cell wall" on the immune response to bovine serum albumin in ddO mice. Mice of ddO strain, were immunized subcutaneously with 150 μg of bovine serum albumin in 0.3 ml of incomplete Freund's adjuvant with or without (C) 250 μg of either cell walls (CW), "acid-treated cell wall" (MP) or cytoplasm. Each of mice was bled at 1, 3, 5, and 7 weeks after immunization and antibody titers were determined by passive hemagglutination method described previously [6] using microtiter. Each group consisted of 30 mice, and each point represents mean $\pm$ 1 standard error of the mean.

As shown in Fig. 1, the antibody titers of mice immunized with BSA in IFA with either cell walls or MP fraction of BCG strain were found to be significantly higher than those in IFA alone or with cytoplasm. This difference in antibody titers between these groups seemed more pronounced at later stages than at earlier stages. The MP fraction (acid-treated cell wall) appeared less potent than cell walls in adjuvanticity at the same doses, while the cytoplasm was shown not to have any adjuvanticity.

Similar results were obtained with the cell walls of *M. smegmatis, M. phlei, M. kansasii* or *M. tuberculosis* Aoyama B as indicated in Table 1. The cell wall of BCG strain was also found to possess adjuvanticity on the production of humoral antibody to sheep erythrocytes and haptenic or carrier determinants of SA-BSA in mice.

Guinea pigs immunized with BSA in IFA with either BCG cell walls or MP fraction developed Arthus and delayed type of reaction, when skin-tested with an intradermal injection of 10 μg of BSA at 4 weeks after immunization as shown in Fig. 2. In contrast, there occurred only Arthus type of skin reaction in guinea pigs immunized with BSA in IFA alone. Sensitized lymphocytes, obtained from guinea pigs immunized with BSA in IFA containing BCG cell wall

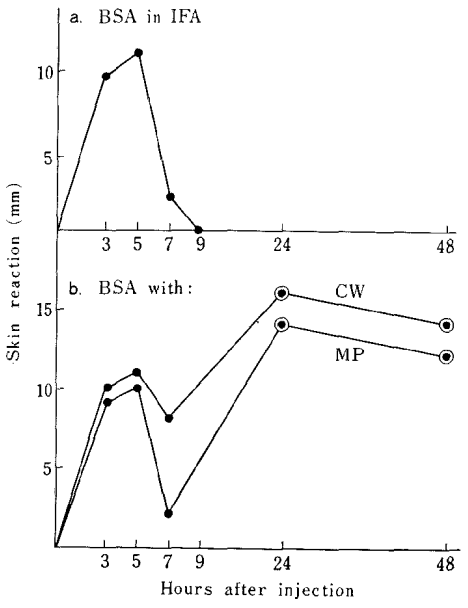

Fig. 2. Skin reaction with bovine serum albumin (10 μg) in sensitized guinea pigs. **a.** Guinea pigs were immunized with 750 μg of bovine serum albumin in incomplete Freund's adjuvant. **b.** Guinea pigs were immunized with 750 μg of bovine serum albumin in incomplete Freund's adjuvant with 750 μg of BCG cell wall (CW) or BCG "acid-treated cell wall" (MP). The reaction was carried out at 4 weeks after immunization and expressed by the mean diameters of erythema (●) or induration (◉).

or MP fraction were cultured in the presence of BSA and then the culture supernatant was purified to obtain migration-inhibitory factor-containing fraction by gel filtration on Sephadex G-100 devised by Bloom et al. Thus, purified fraction was shown to inhibit the migration of the peritoneal exudate macrophage derived from normal guinea pigs using David's method.

It was previously shown that "mycolic acid-arabinogalactan-mucopeptide" is the common structure of wax D and cell wall of mycobacteria [1–3, 5, 15]. More recently, we have reported on the detailed chemical structures of the mucopeptide moiety of cell walls obtained from mycobacteria, nocardia and corynebacteria [1, 3, 15]. The chemical structure of the cell walls of these bacteria are almost identical, however, the molecular weight of mycolic acid of the cell walls differ [3], and while mycobacteria and nocardia have *N*-glycolylmuramic acid, corynebacteria have *N*-acetylmuramic acid as components of the cell wall mucopeptide [1, 3].

The results described in this note provide clear evidences that cell wall and MP fraction of *M. bovis* BCG possess the potent adjuvanticity not only on the circulating antibody production to BSA, SRBC and SA-BSA in mice, but also on the development of delayed type hypersensitivity to BSA in guinea pigs. Similarly, the cell walls of *M. smegmatis, M. phlei, M. kansasii* and *M. tuberculosis* Aoyama B showed potent adjuvanticity on the circulating antibody production to BSA as shown in Table 1. Previously, Kotani and his coworkers [8] have shown that the adjuvant activity of "bound wax D" was not lost, even if bound wax D obtained from BCG cell wall lysed with L_{11} enzyme was treated with trichloroacetic acid. In our experiments, it was also shown that the MP fraction prepared by treating BCG cell wall with hydrochloric acid, trichloroacetic acid and metaperiodate, was still effective in enhancing the humoral and cellular antibody responses. From these results, it is assumed that the mucopeptide moiety of mycobacterial cell wall plays the important role on the development of adjuvanticity of mycobacterial cell wall. As was pointed out previously by Koga et al. [7] and White et al. [13], wax D fractions prepared from either *M. bovis* BCG, *M. smegmatis, M. avium, M. phlei,* or *M. kansasii* were found to have no adjuvanticity on the immune response. From above results, it can be concluded that cell walls are the important active constituent

Table 1. Adjuvant activity of various mycobacterial cell walls on the immune response to bovine serum albumin in ddK mice

Cell wall prepared from	No. of mice	Anti-bovine serum albumin titers (log_2) (passive hemagglutination)		
		3 weeks[a]	5 weeks[a]	7 weeks[a]
M. bovis (BCG)	30	8.4±0.3[b]	11.5±0.5[b]	12.9±0.4[b]
M. kansasii	30	8.5±0.5	11.2±0.3	12.4±0.4
M. phlei	30	8.2±0.3	11.2±0.2	12.1±0.3
M. smegmatis	30	7.2±0.5	11.1±0.6	11.4±0.5
M. tuberculosis (Aoyama B)	30	8.1±0.4	11.4±0.4	12.6±0.4
Control	30	7.5±0.5	8.4±0.3	8.6±0.4

[a] Weeks after immunization.

[b] Standard error.

Mice of ddK strain, were immunized subcutaneously with 150 μg of bovine serum albumin in 0.3 ml of incomplete Freund's adjuvant with or without 250 μg of mycobacterial cell walls. Each of the mice was bled at 3, 5, and 7 weeks after immunization and antibody titers were determined by the methods of Kishimoto et al. [6] using microtiter methods.

in adjuvanticity of mycobacterial cells. The adjuvanticity of cell walls of nocardia and corynebacteria, as well as the mode of adjuvanticity of these cell walls are being investigated in our laboratory.

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