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Adjuvanticity (Immunity-Inducing Property) of Cord Factor in Mice and Rats

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Cord factor, a glycolipid in mycobacteria, was found to make a tolerogenic protein antigen immunogenic when injected 0 to 2 days before tolerogen injection in mice and rats. Cord factor was also found to increase phagocytic function of the reticuloendothelial system of mice, and the relationship between the immunity-inducing capacity and the phagocytic function is discussed.

Cord factor, a glycolipid regarded as a toxic component of *Mycobacterium tuberculosis*, has been proved to enhance antibody production in mice (2, 23) and induce delayed hypersensitivity in rats (23) and guinea pigs (14). Many immunological adjuvants such as bacterial endotoxin (4, 12), tubercle bacilli (7, 19), or pertussis vaccine (8) have been shown, in addition to augmenting immune responses, to prevent tolerance induction to aggregate-free protein antigens.

In the present study we examined the effect of cord factor injection on the process of tolerance induction to a tolerogenic protein antigen and found that cord factor given 0 to 2 days before tolerogen injection could interfere with the induction of tolerance in mice and rats. We examined also the effect of cord factor injection on the activation of the reticuloendothelial system (RES) in mice to determine whether the cord factor-mediated inhibition of the induction of tolerance was related to RES stimulation.

MATERIALS AND METHODS

Animals. CF1 female mice and Sprague-Dawley female rats of about 8 weeks of age were used.

Cord factor. Cord factor dissolved in Drakeol were injected intraperitoneally into mice (0.1 ml) and rats (0.2 ml) before tolerance induction or the carbon clearance test. Control animals were injected with Drakeol alone.

Tolerogen. The tolerogenic fraction of bovine gamma globulin (BGG) was prepared by centrifuging native BGG (nBGG) dissolved in saline (20 mg/ml) at 60,000 × *g* for 60 min. A 0.1-ml (mouse) or 0.2-ml (rat) portion of the upper half of the supernatant containing deaggregated BGG (dBGG) was injected intravenously.

Immunization. Immunization was performed 12 days after the injection of tolerogenic dBGG by injecting nBGG incorporated in complete Freund adjuvant (CFA) into the left hind footpads of animals. Circulating antibody titer was measured by passive hemagglutination, using sheep erythrocytes conjugated with nBGG by the carbodiimide method (16).

Skin reaction. For the skin reaction in rats, 0.1 ml of saline containing 50 μg of nBGG was injected intradermally into the flank, and the double skin thickness of the injected site was measured at 4, 24, and 48 h.

Phagocytic function. Phagocytic function of RES was measured in mice by the rate of clearance of colloidal carbon (C11/1431a, Günther Wagner, Hanover, Germany) by the method of Biozzi et al. (3). A 1-ml portion of carbon per 100 g of body weight at a concentration of 16 mg/ml was injected intravenously. Serial blood samples were removed at 3, 6, 9, 12, and 15 min after the injection and added to a 0.1% aqueous solution of sodium carbonate. Relative amounts of carbon were estimated by a Hitachi 181 spectrophotometer with a red filter. The phagocytic index K was calculated from the slope of the line obtained by plotting the density readings against time on a hemilogarithmic scale. The corrected phagocytic index α was calculated as $\alpha = \sqrt[3]{K}\ (Wb/Wsl)$, where Wb = body weight and Wsl = combined weight of spleen and liver.

RESULTS

Effect of cord factor on antibody production in mice injected with tolerogen. Intravenous injection of 3 mg of dBGG suppressed the antibody response to a subsequent injection of 1 mg of nBGG incorporated in CFA (Table 1), indicating that dBGG used was tolerogenic in this strain of mice (CF1). However, mice given 2 or 10 μg of cord factor along with tolerogenic dBGG showed significantly higher antibody titer than those given dBGG without cord factor. When cord factor was administered 2 days before tolerance induction, a similar effect was seen with a 10-μg amount, but only a slight effect was observed with a 2-μg amount (Table 2).

TABLE 1. *Effect of cord factor administered simultaneously with dBGG on the antibody response in mice*

Treatment[a]	No. of mice	Antibody titer (log 2 ± SD[b]) on:	
		Day 24[c]	Day 30[c]
dBGG + Drakeol	6	2.5 ± 0.6	4.8 ± 0.3
dBGG + cord factor (10 μg) in Drakeol	6	5.3 ± 1.5 ($P < 0.05$)	7.0 ± 0 ($P < 0.001$)
dBGG + cord factor (2 μg) in Drakeol	6	4.5 ± 0.6 ($P < 0.01$)	6.0 ± 0 ($P < 0.001$)
None	6	6.1 ± 0.3 ($P < 0.001$)	7.6 ± 0.5 ($P < 0.001$)

[a] Drakeol containing cord factor or Drakeol alone was injected intraperitoneally at the same time as an intravenous injection of 3 mg of dBGG. All mice were immunized 12 days later with 1 mg of nBGG in CFA containing 50 μg of *M. tuberculosis*.

[b] SD, Standard deviation.

[c] Day after immunization. The difference of each group in antibody titer from the group given dBGG and Drakeol was evaluated by using Student's *t* test.

TABLE 2. *Effect of cord factor administered 2 days before dBGG on the antibody response in mice*

Treatment[a]	No. of mice	Antibody titer (log 2 ± SD[b]) on:	
		Day 24[c]	Day 30[c]
dBGG + Drakeol	6	3.1 ± 0.3	4.8 ± 0.6
dBGG + cord factor (10 μg) in Drakeol	6	6.0 ± 0 ($P < 0.001$)	7.0 ± 1.2 ($P < 0.02$)
dBGG + cord factor (2 μg) in Drakeol	6	3.9 ± 0.3 ($P < 0.02$)	5.5 ± 0.6
None	6	6.1 ± 0.3 ($P < 0.001$)	7.6 ± 0.5 ($P < 0.001$)

[a] Drakeol containing cord factor or Drakeol alone was injected intraperitoneally 2 days before intravenous injection of 3 mg of dBGG. All mice were immunized 12 days after injection of dBGG with 1 mg of nBGG incorporated in CFA containing 50 μg of *M. tuberculosis*.

[b] SD, Standard deviation.

[c] Same as footnote *c* in Table 1.

Effect of cord factor on the skin reaction and antibody production in rats injected with tolerogen. As shown in Fig. 1, the pretreatment of rats with an intravenous injection of 4 mg of tolerogenic dBGG before immunization with 3 mg of nBGG in CFA suppressed completely the immediate as well as delayed-type skin reactions. However, when rats were given 40 μg of cord factor simultaneously with dBGG, they showed reactions comparable with those of normal untreated rats. Rats pretreated with dBGG and cord factor in the same way showed, however, suppressed skin reaction when immunized with 100 μg, instead of 3 mg, of nBGG in CFA (Fig. 2). Effects of pretreatment with dBGG and cord factor on antibody production were similar to those on the skin reaction (Table 3).

Effect of cord factor on the clearance of carbon particles in mice. The effect of cord factor injection on phagocytic activity of RES in mice was studied. Table 4 shows that 2 and 10

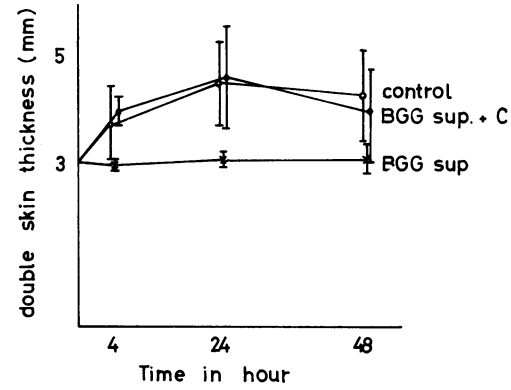

FIG. 1. *Effect of cord factor administered simultaneously with dBGG on the skin reaction in rats immunized with 3 mg of nBGG incorporated in CFA. A total of 40 μg of cord factor dissolved in Drakeol was injected intraperitoneally, and 4 mg of tolerogenic dBGG was injected intravenously. Symbols: ○, Drakeol; ×, dBGG + Drakeol; ●, dBGG + cord factor in Drakeol. Immunization was performed 12 days later with 3 mg of nBGG incorporated in CFA containing 200 μg of M. tuberculosis. The skin reaction was tested 25 days after immunization with 50 μg of nBGG in saline. The points and vertical lines indicate the mean and standard deviations from five rats.*

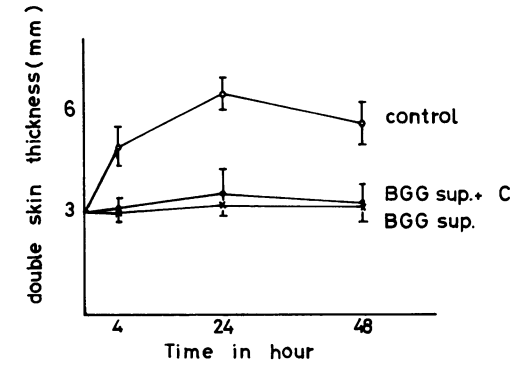

FIG. 2. *Effect of cord factor administered simultaneously with dBGG on the skin reaction in rats immunized with 100 μg of nBGG incorporated in CFA. A total of 40 μg of cord factor was injected intraperitoneally, and 4 mg of dBGG was injected intravenously. Symbols: ○, Drakeol; ×, dBGG + Drakeol; ●, dBGG + cord factor in Drakeol. Immunization was performed 12 days later with 100 μg of nBGG incorporated in CFA containing 200 μg of M. tuberculosis. The skin reaction was performed 15 days after immunization, using 50 μg of nBGG. Points and vertical lines indicate the mean and standard deviations from five rats.*

μg of cord factor given 30 min before the carbon injection elevated the phagocytic index slightly, but the difference between cord factor-treated and control groups was not statistically significant (experiments 1 and 2). However, 2 or 10 μg of cord factor given 1 day (experiments 3 and 4) and 2 μg of cord factor given 2 days (experiment 5) before the carbon injection caused a significant increase in the phagocytic index. In contrast, a larger amount (30 μg) of cord factor given 1 day before the carbon injection suppressed the phagocytic activity.

TABLE 3. *Effect of cord factor administered simultaneously with dBGG on the antibody response in rats*

Treatment[a]	No. of rats	Antibody titer (log 2 ± SD)[b]	
		Expt 1	Expt 2
dBGG + Drakeol	5	3.9 ± 1.1	0.6 ± 0.1
dBGG + cord factor (40 μg) in Drakeol	5	5.3 ± 1.0	1.0 ± 0.7
Drakeol	5	5.2 ± 0.7	5.2 ± 0.3 ($P < 0.001$)

[a] Drakeol containing cord factor or Drakeol alone was injected intraperitoneally at the same time as an intravenous injection of 4 mg of dBGG in saline. Immunization was performed 12 days later with 3 mg (experiment 1) or 100 μg (experiment 2) of nBGG incorporated in CFA containing 200 μg of *M. tuberculosis*.

[b] Antibody titer was measured 7 days after the skin reaction. (The skin reaction of experiment 1 is shown in Fig. 1 and that of experiment 2 is shown in Fig. 2.) SD, Standard deviation.

DISCUSSION

The property of a substance that modulates a tolerogenic stimulus into an immunogenic signal is considered to be an expression of adjuvant activity. Dresser called this modulating property an extrinsic adjuvanticity and viewed it as a very stringent test of adjuvant effect (7). In fact, many adjuvants caused antibody production when injected along with tolerogens (8, 12, 19).

Thus, our previous observation that cord factor was an adjuvant in mice and rats in that it enhanced antibody production and caused delayed hypersensitivity (23) was confirmed in the present study. Under the appropriate conditions the dBGG, an otherwise tolerance-inducing antigen, immunized animals when injected along with cord factor.

This effect of cord factor was not always complete, however. Mean antibody titers of the animals treated with dBGG and cord factor were generally lower than those of normal untreated animals. Also, no significant immune response could be elicited in the rats treated with dBGG and cord factor, when immunization was performed with the small, instead of large, dose of native antigen in CFA (Fig. 2).

Among many factors regulating immune responses, macrophages play a vital role (5, 11, 13, 19): when the function of RES was suppressed, tolerance was induced more easily (5,

TABLE 4. *Effect of cord factor on the clearance of carbon from the blood of mice*

Expt no.	Interval[a]	Pretreatment	Phagocytic index K[b]	Corrected phagocytic index α[b]
1	30 min	Drakeol alone	0.042 ± 0.012 (12)	5.29 ± 0.66 (12)
		Cord factor (2 μg) in Drakeol	0.048 ± 0.021 (12)	5.86 ± 0.77 (12)
2	30 min	Drakeol alone	0.039 ± 0.015 (12)	5.55 ± 0.61 (12)
		Cord factor (10 μg) in Drakeol	0.047 ± 0.014 (12)	6.03 ± 0.65 (11)
3	24 h	Drakeol alone	0.045 ± 0.013 (11)	5.53 ± 0.68 (11)
		Cord factor (2 μg) in Drakeol	0.083 ± 0.044 (12) ($P < 0.02$)	6.67 ± 1.28 (12) ($P < 0.05$)
		Cord factor (10 μg) in Drakeol	0.075 ± 0.026 (11) ($P < 0.005$)	6.66 ± 0.77 (11) ($P < 0.005$)
4	24 h	Drakeol alone	0.050 ± 0.017 (6)	5.54 ± 0.37 (6)
		Cord factor (1 μg) in Drakeol	0.070 ± 0.009 (7) ($P < 0.05$)	5.93 ± 0.55 (7)
		Cord factor (30 μg) in Drakeol	0.032 ± 0.006 (7) ($P < 0.05$)	4.64 ± 0.48 (7) ($P < 0.01$)
5	48 h	Drakeol alone	0.051 ± 0.021 (12)	5.39 ± 0.77 (12)
		Cord factor (2 μg) in Drakeol	0.059 ± 0.025 (10)	6.28 ± 0.56 (10) ($P < 0.01$)
		Cord factor (10 μg) in Drakeol	0.049 ± 0.021 (11)	5.98 ± 0.80 (11)

[a] Interval between pretreatment and carbon injection.

[b] Mean ± standard deviation. Numbers in parentheses indicate the numbers of mice in each group.

19, 21); when the function of RES was enhanced, the induction of tolerance was prevented (8, 19); and strain difference of mice in ease of tolerance induction was reported to depend upon the activity of macrophages (6, 19). Experiments in vitro revealed that the presence of macrophages led to immunity, whereas their absence resulted in tolerance (10), and that antigen-bearing macrophages generated helper T cells, whereas a soluble antigen without macrophages generated suppressor T cells (15). It was also suggested that an antigen has, in general, tolerogenic as well as immunogenic components (17, 19), and the efficiency and rate with which the immunogenic component is handled by macrophages will thus determine whether immunization or tolerance may be induced (13, 19).

These facts and considerations led us to test whether cord factor may activate the function of RES. In the present study, cord factor was found to increase the uptake of carbon particles by RES when it was given to mice in the amounts and at the time interval that converted tolerance into immunity. The many reports mentioned above suggest that cord factor might activate macrophages to take up and/or process antigen, to which the mechanism of the modulating effect (adjuvanticity) of cord factor could, at least partly, be attributable. The increase in phagocytosis of aggregated material remaining in the deaggregated protein solution might be sufficient to induce immunity. Alternatively, the pinocytosis of tolerogenic soluble dBGG might be increased by cord factor, which could in turn contribute to the induction of immunity.

Contrary to this inference, the ability of endotoxin, another well-known bacterial adjuvant, to modulate a tolerogenic stimulus into an immunogenic one was found not to be related to its ability to enhance RES activity (12), but to the ability to bypass the T cell helper function (18).

There seems to be, however, a difference in the mechanism(s) operating in the action of these two bacterial substances. The interference of endotoxin with the induction of tolerance was observed only when it was injected either at the same time or up to 2 days after the injection of tolerogen, and no such interference occurred when it was given before the injection of tolerogen (12), whereas the injection of cord factor prevented the induction of tolerance when it was injected before the injection of tolerogen, as shown in the present study. Adjuvant activity of cord factor as measured by the enhancement of antibody production was also exerted when it was injected 5 days before the injection of antigen (22), whereas the prior injection of endotoxin proved to be suppressive for antibody production (20).

The injection of endotoxin 3 days before the injection of tolerogen could not interfere with the induction of tolerance, although the RES activity was enhanced (12). Since endotoxin is a very complex, large molecular substance having a variety of biological activities, its injection prior to tolerogen might interfere with the initial step of immune response through some suppressing phenomena, such as antigenic competition or cytotoxicity. For example, a large amount of a mycobacterial adjuvant, wax D, was found not to be able to exert its adjuvant effect through antigenic competition (24). An active constituent of immunosuppressive effect of endotoxin injected prior to antigen appeared to be in the lipid A component that is related to its toxic activity (20). Also, as revealed in the present study, a large amount of cord factor (30 μg) suppressed the RES activity, possibly through its cytotoxic property. In this regard investigations by the use of simple, chemically defined synthetic adjuvants such as *N*-acyl-D-glucosamine (1) or *N*-acetyl muramyl-L-alanyl-D-isoglutamine (9) would be interesting. Recently, it was found in our laboratory that *N*-acetyl muramyl-L-alanyl-D-isoglutamine, which is active as adjuvant, enhanced definitely the RES activity, whereas its stereoisomer, *N*-acetyl muramyl-L-alanyl-L-isoglutamine, which is inactive as adjuvant, did not enhance the activity (unpublished data).

At any rate, we feel, however, that many studies should still be done before the relationship between activation of macrophage and adjuvant activity of cord factor or of other adjuvants is finally established. Although possibilities of the involvement of other types of cells, including B or suppressor T cells, can not be excluded, it is beyond the scope of the present study to discuss such possibilities.

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