Production and partial characterization of anti-cord factor (trehalose-6,6‘-dimycolate) IgG antibody in rabbits recognizing mycolic acid subclasses of Mycobacterium tuberculosis or Mycobacterium avium

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Abstract

An ELISA with cord factor (trehalose-6,6′-dimycolate) is useful for the serodiagnosis of tuberculosis. To clarify the exact antigenic epitope in cord factor, recognized by a rabbit anti-cord factor IgG antibody, and to ascertain the most sensitive and specific diagnostic test antigen, rabbits were immunized with two kinds of cord factors isolated from Mycobacterium tuberculosis or Mycobacterium avium and the reactivities of the sera were tested against cord factors or the component mycolic acid methyl esters by ELISA. The serum from rabbits immunized with M. tuberculosis cord factor was highly reactive against M. tuberculosis cord factor, but less reactive against M. avium cord factor. In contrast, the serum from rabbits immunized with M. avium cord factor was highly reactive against M. avium cord factor but less reactive against M. tuberculosis cord factor. Moreover, the serum from rabbits immunized with M. tuberculosis cord factor reacted against mycolic acid methyl esters, especially methoxy mycolic acid methyl ester. On the other hand, the serum from rabbits immunized with M. tuberculosis cord factor was less reactive against trehalose-6-monomycolate and not reactive against sulfolipid (2,3,6,6‘-tetraacyl trehalose 2‘-sulfate). From these results, it was concluded that the anti-cord factor IgG antibody, produced experimentally in rabbits, recognized the differences in the cord factor structures, i.e. the hydrophobic moiety rather than the carbohydrate moiety. It was also noted that the serum from rabbits immunized with M. tuberculosis cord factor was highly reactive against methoxy mycolic acid as an epitope. This paper is the first to describe how the anti-cord factor IgG antibody can recognize the mycolic acid subclasses, which differ according to the species of mycobacteria. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Mycobacterium; Serodiagnosis; Antibody; Cord factor; Mycolic acid

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Abbreviations: FAB/MS, first atom bombardment mass spectrometry; MAC-TDM, cord factor (trehalose-6,6‘-dimycolate) isolated from Mycobacterium avium; Mt-TDM, cord factor (trehalose-6,6‘-dimycolate) isolated from Mycobacterium tuberculosis; PBS-T, phosphate-buffered saline containing 0.05% (v/v) Tween-20; SL, sulfolipid (2,3,6,6‘-tetraacyl trehalose 2‘-sulfate); TB, tuberculosis; TLC, thin layer chromatography; TMM, trehalose-6-monomycolate; w/o/w, water in oil in water

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1. Introduction

Tuberculosis (TB) is one of the most important infectious diseases in humans. The incidence of TB had decreased continuously in advanced countries since the 1940’s. However, the decreasing rate of the TB incidence has dropped steadily since the 1980’s, owing partially to HIV co-infection and the appearance of drug resistant *Mycobacterium tuberculosis*. More recently, another reason has also been suggested concerning the decrease in the host immune resistance for tuberculous infection. Tuberculous immunity has long been understood as an aspect of cellular immunology but the contribution of the humoral response has not been fully investigated. Previously, Kato reported [1-3] that both anti-cord factor antibodies produced in experimental animals and IgM antibodies produced in rabbits after administration of cord factor as a methylated bovine serum albumin complex, recognized trehalose as an epitope by the precipitation test. However, anti-cord factor antibody could not be detected in sera of TB patients. It was recently reported [4,5] that the anti-cord factor IgG antibody was detectable by ELISA in active and inactive TB patients and was useful for the early clinical diagnosis of tuberculous diseases. Cord factor is a unique and ubiquitous surface molecule in mycobacteria, which may contact with the host phagocytic cells at the early step of infection. Therefore, cord factor may induce the immune response against host animals quickly. However, cord factor is a very hydrophobic molecule containing mycolic acid, a very long branched chain fatty acid for which the exact antigenic epitope or binding site recognized by the antibody is unknown. In general, such a highly hydrophobic molecule would not be expected to elicit an immune response similar to hydrophilic antigens such as large molecular mass proteins or carbohydrate complexes. However more recently, mycolic acid has been reported to be a CD1b-restricted antigen, based on the proliferative response of T-cells [6]. Therefore, it was considered that mycolic acid might play a critical role in the immune response against cord factor. We attempted to produce anti-cord factor antibody in rabbits experimentally, using two kinds of cord factors as antigen, which differed in mycolic acid subclasses. In this paper, it was demonstrated that anti-cord factor IgG antibody could be produced in rabbits immunized with cord factor in the form of a 'water in oil in water' (w/o/w) micelle without any protein carrier and that the epitope or binding site of anti-cord factor IgG antibody was mycolic acid.

2. Materials and methods

2.1. Isolation of antigenic glycolipids and mycolic acid subclasses

*M. tuberculosis* Aoyama B was cultivated at 37°C for 5-6 weeks in Sauton medium (pH 7.2), containing 0.5% (w/v) L-asparagine, 0.2% (w/v) citric acid, 0.05% (w/v) K$_2$HPO$_4$$\cdot$3H$_2$O, 0.05% (w/v) MgSO$_4$$\cdot$7H$_2$O, 0.005% (w/v) ammonium ferric citrate and 6% (v/v) glycerol. *M. avium* serotype 4 was cultivated at 37°C for 5-6 weeks in Middlebrook 7H9 broth (Difco Laboratories). The cultures were autoclaved for 10 min at 121°C and bacterial cells were collected by centrifugation. To extract the lipids, cells were sonicated and extracted with chloroform:methanol (4:1, 3:1 and 2:1, v/v) successively [7]. Cord factor, trehalose-6-monomycolate (TMM) and Sulfolipid (SL: 2,3,6,6'-tetraacyl trehalose 2'-sulfate) were isolated from the crude extractable lipids of *M. tuberculosis* or *M. avium*. Each glycolipid was purified repeatedly by thin layer chromatography (TLC) of silica gel (Uniplate, Analtech, Newark, DE, USA) until a single spot was obtained. The developing solvents were chloroform:methanol:acetic acid (80:20:6, 90:10:6, v/v) and chloroform:methanol:water (90:10:1, v/v) [8]. Mycolic acids of *M. tuberculosis* were liberated by alkali hydrolysis (10% KOH, w/v) from the chloroform:methanol-extracted residues. After methylation with benzene:methanol:H$_2$SO$_4$ (10:20:1, v/v) at 90°C for 3 h, each subclass of α-, methoxy and keto mycolic acid methyl esters was separated by TLC of silica gel, developed three times with the solvent system benzene or n-hexane:diethyl ether (90:15, v/v).

The molecular species of mycolic acid were determined by fast atom bombardment mass spectrometry (FAB/MS) of the intact molecule with a JMS-SX102A double focusing mass spectrometer (JEOL, Tokyo, Japan).
2.2. Immunization of rabbits

Rabbits (male albino, Japan SLC, Shizuoka, Japan) were used for antibody production. Two kinds of cord factors were used as antigens. One from *M. tuberculosis* (Mt-TDM) and the other from *M. avium* (MAC-TDM). Each cord factor suspended with phosphate-buffered saline was emulsified with an equal volume of a Freund-type incomplete adjuvant (Difco Laboratories) and ground to prepare a water in oil emulsion. After that, saline containing 0.2% (v/v) Tween-80 was added and ground again to form a w/o/w micelle, according to the method described previously [9,10]. A 2 ml of w/o/w micelle containing 300 μg of each cord factor was injected into the dorsal skin subcutaneously at four sites at 7-day intervals. 10 days after the last injection, terminal bleeding was performed by cardiac puncture. Pooled blood was kept at 2°C for 12 h after incubation at 37°C for 1 h. Sera were separated by centrifugation at 14 000×g for 1 h, inactivated by heating at 56°C for 30 min and stored at −25°C.

2.3. ELISA

ELISA was carried out on a 96-well microplate (Falcon 3915, Becton Dickinson, Lincoln Park, NJ, USA). As antigen, the purified cord factor or mycolic acid methyl ester was dissolved in n-hexane. Each antigenic solution was placed in a well and the plates were allowed to dry at room temperature overnight. Blocking was done with phosphate-buffered saline containing 0.05% (v/v) Tween-20 (PBS-T) for 1 h. Then 50 μl of serum diluted with PBS-T was added to each well and the plate was incubated for 1 h at room temperature. Serum was aspirated and washed three times with PBS-T. After that, 50 μl of the second antibody, peroxidase-conjugated goat affinity purified antibody to rabbit IgG Fc (Cappel, ICN Pharmaceuticals, Aurora, OH, USA) diluted 500 times with PBS-T, was added and incubated for 1 h at room temperature. The second antibody was aspirated and washed three times with PBS-T, 50 μl of substrate (10 mg of o-phenylenediamine and 4 μl of 30% hydrogen peroxide in 10 ml of citrate phosphate buffer, pH 4.8) was added and incubated at room temperature. After 10 min, the reaction was stopped with 6 N-HCl. The absorbance was read at 492–630 nm on a microplate reader (MTP-100P, Corona Electric, Tokyo, Japan) [4].

2.4. Absorbance

Absorbance tests were carried out with Mt-TDM or methoxy mycolic acid methyl ester of *M. tuberculosis*. Excess amounts of Mt-TDM (10 μg well$^{-1}$)
or methoxy mycolic acid methyl ester (60 µg well⁻¹) were added to each well and dried. After 50 µl of the serum from rabbits immunized with Mt-TDM had been added to each well, the plate was incubated at 4°C overnight. After that, the supernatant was transferred to another plate and the main ELISA test was performed against Mt-TDM (10 µg well⁻¹) or methoxy mycolic acid methyl ester (60 µg well⁻¹) as antigen.

3. Results

3.1. Isolation of glycolipids and mycolic acids

Glycolipids and mycolic acid methyl esters were isolated and purified from *M. tuberculosis* Aoyama B or *M. avium* serotype 4. The TLC patterns of these glycolipids and mycolic acid methyl esters are shown as a single spot in Fig. 1. Negative FAB/MS spectra of each mycolic acid subclass from Mt-TDM showed cluster ions corresponding to quasimolecular ions [RCOO]⁻. These cluster ions were clearly detected at m/z 1108–1220 in α-mycolic acid ranging from C₇₆:2 to C₈₄:2, 1196–1308 in methoxy mycolic acid ranging from C₈₁:1 to C₈₉:1 and 1236–1306 in keto mycolic acid ranging from C₈₄:1 to C₈₉:1 as shown in Fig. 2. Based on [RCOO]⁻ ions, the most abundant molecule in α-mycolic acid was C₇₈:2 and C₈₀:2, in methoxy mycolic acid C₈₅:1 and in keto mycolic acid C₈₇:1. The chemical structures of glycolipids and mycolic acids are shown in Fig. 3 [10–12].

3.2. Production of anti-cord factor IgG antibody and cross reactivity against Mt-TDM and MAC-TDM

After subcutaneous injections of 300 µg of cord factor in w/o/w emulsion at four sites at 7-day intervals, rabbits produced anti-cord factor IgG antibody distinctly. Antibody titers against cord factor were estimated by ELISA. The serum from rabbits immunized with Mt-TDM showed high titers and wide linearity against Mt-TDM in proportion to the concentration of antigen but did not react significantly against MAC-TDM (Fig. 4). On the other hand, the serum from rabbits immunized with MAC-TDM was highly reactive against MAC-TDM but less reactive against Mt-TDM (Fig. 5). The control serum from rabbits reacted against neither Mt-TDM nor MAC-TDM. Therefore, the two kinds of sera from rabbits immunized with a different type of TDM were reactive against each homologous antigen specifically and had almost no cross reactivity against the heterologous antigen, Mt-TDM or MAC-TDM.

3.3. Cross reactivity against trehalose-containing glycolipids of *M. tuberculosis*

Cord factor, TMM and SL are characteristic trehalose-containing glycolipids of *M. tuberculosis*. As shown in Fig. 6, the serum from rabbits immunized with Mt-TDM had a weak reactivity against TMM and practically no reactivity against SL, compared with its reactivity against cord factor.

3.4. Reactivity against each subclass of mycolic acid from *M. tuberculosis*

Since the sera from rabbits immunized with cord factor were reactive against each homologous mycoloyl glycolipids, it was predicted that the antigenic epitope recognized by the antiserum from rabbits might be the mycolic acid moiety. Therefore, the reactivity of each mycolic acid methyl ester against the serum from rabbits immunized with Mt-TDM was tested. As shown in Fig. 7, all three subclasses of mycolic acid methyl ester reacted in proportion to the antigen concentration against the serum from rabbits immunized with Mt-TDM, although generally the titers were lower than with Mt-TDM antigen itself. Among the three subclasses of mycolic acid from *M. tuberculosis*, the methoxy mycolic acid methyl ester showed the highest reactivity compared to α- and keto mycolic acid methyl esters. The order of reactivity was methoxy > α- > keto mycolic acid methyl ester.

Fig. 1. Negative FAB/MS spectra of α-, methoxy and keto mycolic acids from *M. tuberculosis*. Mass spectra were recorded with a JMS-SX102A double focusing mass spectrometer. (A) α-mycolic acid, (B) methoxy (MeO-) mycolic acid and (C) keto mycolic acid.
(A) Cord factor (trehalose-6,6'-dimycolate)

(B) TMM (trehalose-6-monomycolate)

(C) SL (2,3,6,6'-tetraacyl trehalose 2'-sulfate)

(D) 

\[
\begin{align*}
\text{\(\alpha\)-mycolic acid} & : & 
\text{CH}_3(CH_2)_nCH=CH(CH_2)_3CHCOOH & C_{2n+49} \\
\text{methoxy mycolic acid} & : & 
\text{CH}_3(CH_2)_nCH=CH(CH_2)_3CH(OCH_3) & C_{2n+46} \\
\text{keto mycolic acid} & : & 
\text{CH}_3(CH_2)_nCH=CH(CH_2)_3CH(OH) & C_{2n+46} \\
\text{diester mycolic acid} & : & 
\text{CH}_3(CH_2)_nCH=CH(CH_2)_3CH(OH) \cdot \text{C}((CH_2)_n)_2CH(COOH) & C_{2n+45}
\end{align*}
\]
3.5. Absorbance

Absorbance tests were carried out to clarify the antigenic epitope of cord factor. The serum from rabbits immunized with Mt-TDM was absorbed with methoxy mycolic acid methyl ester or Mt-TDM before main ELISA tests. Absorbance values of the pre-treated sera were distinctly less than those of the non-absorbed control, as shown in Fig. 8. The degree of inhibition of the serum pre-treated with Mt-TDM was much stronger than that of the serum pre-treated with methoxy mycolic acid methyl ester. These results indicated that anti-cord factor antibody and anti-methoxy mycolic acid antibody share the antigenic determinant(s).

4. Discussion

Cord factor is a highly hydrophobic molecule containing mycolic acid on the mycobacterial surface and is not likely to behave as an antigenic molecule like hydrophilic proteins or carbohydrates. Although cord factor is one of the mycobacterial virulence factors, it is also a potent adjuvant active substance that modulates cellular or humoral immunological responses [13–18]. Cord factor was found first as a sole component of virulent TB bacilli. Later, it was reported to be distributed widely among Mycobacterium, Nocardia and Rhodococcus species belonging to the Actinomycetales [19,20]. The production of an antibody against cord factor in mice and rabbits was first described by Kato [1–3], using a methylated bovine serum albumin complex antigen and the immunological properties of this anti-cord factor antibody have been investigated. Kato [1–3] demonstrated that the anti-cord factor IgM antibody from rabbits was detected by a precipitin reaction with an aqueous emulsion, when the methylated bovine serum albumin complex of cord factor was injected subcutaneously. It was suggested that the antigenic epitope may be the trehalose moiety, since the pre-treatment of the antisera with trehalose inhibited the precipitin reaction against cord factor. It was also demonstrated that the major biological activities of cord factor were inhibited by the administration of anti-cord factor antibody in vivo and in vitro. Nevertheless, by the precipitation test, any anti-cord factor antibody could not be detected in the sera of TB patients. In our study, rabbits were im-

![Fig. 3. Chemical structures of glycolipids and mycolic acid subclasses. (A) cord factor, (B) TMM, (C) SL, (D) mycolic acid subclasses of M. tuberculosis and M. avium.](https://academic.oup.com/femspd/article-abstract/24/2/141/756812)
munized with hydrophobic antigen in the form of w/o/w micelle without protein carriers. This method differs from that reported by Kato. Rabbits produced high titers of anti-cord factor IgG antibody by multiple immunization with two kinds of cord factors. As shown in Figs. 4 and 5, both of the antibodies from rabbits immunized with Mt-TDM or MAC-TDM reacted against each homologous antigen specifically and the cross reactivity was minimal to each other. These results suggested that each antibody recognized the difference between the two cord factors, which differ in mycolic acid structure. Actually, Mt-TDM is composed of $\alpha$-, methoxy and keto mycolic acids as its hydrophobic part, while the mycolic acid composition of MAC-TDM contains $\alpha$-, keto and diester subclasses [12]. Furthermore, the reactivity of the anti-cord factor IgG antibody from rabbits immunized with Mt-TDM was tested in detail. Cord factor, TMM and SL are characteristic glycolipids containing trehalose that occur commonly in \emph{M. tuberculosis} [21]. The anti-cord factor IgG antibody from rabbits immunized with Mt-TDM was shown to be highly reactive against Mt-TDM but less reactive against TMM and not reactive against SL (Fig. 6). Moreover, the mycolic acid moiety itself was reactive against the anti-cord factor IgG antibody from rabbits, although the reactivity of mycolic acid methyl esters was weaker than that of cord factor in general, owing possibly to their extreme hydrophobic properties. The methoxy mycolic acid methyl ester among three subclasses of mycolic acids in \emph{M. tuberculosis} reacted strongly and specifically with this antibody in a dose-dependent fashion (Fig. 7). These results suggested that mycolic acid residues play an important role as the epitope of our anti-cord factor IgG antibody. Moreover, this was supported by the absorbance readings with Mt-TDM or methoxy mycolic acid (Fig. 8). In this study, anti-cord factor IgG antibody in rabbits was
References


produced by multiple injections of cord factor in the form of w/o/w micelle. The antibody was characterized by its ability to recognize mycolic acid as epitope. In particular, the antibody from rabbits immunized with Mt-TDM reacted specifically against methoxy mycolic acid, one of the major characteristic subclass of mycolic acids in M. tuberculosis. These results prompt us to develop a more sensitive and more specific ELISA test for serodiagnosis of TB using semi-synthetic cord factor antigen composed of a single subclass of mycolic acid, such as methoxy mycolic acid. Furthermore, the biological activities of such anti-cord factor IgG antibodies produced in rabbits that recognize mycolic acids should be examined in the context of regulation of cellular or humoral immunity and virulence.