

Immunochemical Properties of Anti-Cord Factor Antibody

MASAHIKO KATO

Research Institute of Tuberculosis, Toneyama Hospital, National Sanatorium, Toyonaka,
Osaka, Japan 560

Received for publication 14 September 1972

Sera of man and animals infected with tubercle bacilli were tested for precipitating antibody to trehalose-6,6'-dimycolate (cord factor) of *Mycobacterium tuberculosis* H37Rv. None of the sera of mice and rabbits infected with *M. tuberculosis* H37Rv reacted with cord factor, whereas the sera of animals vaccinated with cord factor-methylated bovine serum albumin complex demonstrated a precipitin reaction with cord factor. Antibody against cord factor was not detected in the sera of cases of active, convalescent, and pneumonectomized human tuberculosis, indicating that the anti-cord factor antibody is not associated with tuberculous infection in man and animals. Precipitin activity against cord factor was localized in 19S macroglobulin (immunoglobulin M) fraction of the serum of rabbits vaccinated with cord factor-methylated bovine serum albumin complex. Analytical gel filtration and immunodiffusion studies indicated that the anti-cord factor precipitin dissociated from the cord factor-antibody complex is an immunoglobulin M antibody.

A previous paper from this laboratory (2) has reported that the vaccination with trehalose-6,6'-dimycolate (cord factor)-methylated bovine serum albumin (MBSA) complex induced in mice and rabbits the production of anti-cord factor antibody which precipitates cord factor and neutralizes its toxicity either in vivo or in vitro. Live BCG vaccine failed to elicit such an immune response to cord factor. Since the binding capacity of antibody to cord factor was completely lost by the treatment of anti-cord factor antiserum with 2-mercaptoethanol, it was assumed that the antibody is mainly composed of 19S immunoglobulin M (IgM).

In the present study, these previous findings are extended by titrating anti-cord factor precipitin activity in sera of animals infected with virulent *Mycobacterium tuberculosis* and of patients with pulmonary tuberculosis and secondly by analyzing the immunochemical properties of antibody produced in rabbits vaccinated with cord factor-MBSA complex. Evidence is presented which indicates that the antibody against cord factor is not produced in man and animals infected with virulent tubercle bacilli and that the antibody is produced predominantly in the IgM fraction of serum under the present vaccination schedule.

MATERIALS AND METHODS

Animals. Male albino mice of random-bred dd0 stock (1) 4 to 6 weeks of age were maintained on a

standard commercial diet (Oriental Pellet Diet MF, Oriental Yeast Industrial Co., Tokyo, Japan) and water. Rabbits of both sexes weighing 3 kg were obtained from a commercial source and maintained on an Oriental Pellet Diet RC-5.

Serum of tuberculous patients. The source of serum was male inpatients 25 to 45 years of age with either sputum-positive, convalescent, or pulmonary tuberculosis treated by pneumonectomy. The serum was prepared and heat inactivated just prior to testing.

Vaccination with cord factor-MBSA complex. An aqueous suspension of the complex of cord factor and MBSA prepared by the previously reported method (2) was emulsified with an equal volume of Freund incomplete adjuvant and injected subcutaneously into mice and rabbits twice a week for 3 weeks. The serum was prepared 10 to 14 days after the last injection.

Preparation of gamma globulin fraction. The gamma globulin fraction of the immune rabbit serum was prepared by the method of Kekwick (4). A solution (30 mg/ml) in 0.2 M glycine-sodium hydroxide buffer (pH 8) was stored frozen at -25 C.

Infection. Mice and rabbits were infected intravenously with a 7-day culture of virulent human *M. tuberculosis* H37Rv in Tween-albumin medium. The inoculum contained 10^8 to 5×10^8 viable units for the infection of mice and 10^7 to 5×10^7 viable units for the inoculation into rabbits. The serum was collected at 4 to 6 weeks after testing for cutaneous hypersensitivity to tuberculin purified protein derivative (PPD). Vaccination with live BCG vaccine was done as previously reported (2).

Sensitization to heat-killed H37Rv. Rabbits

were sensitized to heat-killed human tubercle bacilli H37Rv in the following manner. H37Rv cells were suspended in 0.85% saline and autoclaved. The bacterial suspension containing 2 mg (dry weight) of bacilli was emulsified with an equal volume of Freund incomplete adjuvant and subcutaneously injected into rabbits twice a week for 3 weeks. After a strong skin hypersensitivity to tuberculin PPD had developed, the serum was collected and subjected to antibody analysis.

Antibody determination. The precipitin test (2) was used to measure the anti-cord factor antibody in human and animal sera. All sera were inactivated by heating at 56 C for 30 min before testing. An aqueous emulsion of cord factor (100 μ g/0.05 ml) was added to 0.5 ml of the increasing dilutions of serum in 0.2 M glycine-sodium hydroxide buffer (pH 8) containing 0.1% gelatin and allowed to stand at 2 C for 7 days. The precipitin titers were expressed as the reciprocal of the highest serum dilution giving definite macroscopic precipitation.

Gel filtration. Gel filtration of immune rabbit serum and gamma globulin solution was done with Sephadex G-200 (Sephadex Fine Chemicals, Inc., Uppsala, Sweden) column equilibrated with 0.2 M glycine-sodium hydroxide buffer (pH 8). A 0.2-ml amount of either serum or gamma globulin solution was applied to a Sephadex column (1.5 by 30 cm). The column was eluted with the same buffer at 4 C at a flow rate of 3 ml/hr. After a reading of the absorbancy of 1-ml samples at 280 nm, the precipitin content in each fraction was determined by adding an appropriate amount of aqueous emulsion of cord factor and measuring the protein content in the formed precipitate by the previously reported procedure (2).

Immunochemical analyses of cord factor-antibody complex. To dissociate the specific antibody from the cord factor-antibody complex, the immune precipitate was washed twice with ice-cold 0.85% saline and suspended in 0.2 M glycine-sodium hydroxide buffer (pH 8) containing 15% NaCl. After standing overnight at 2 C, the suspension was centrifuged at 4,000 rev/min for 15 min in a refrigerated centrifuge. This separated the immune complex into a surface lipid pellicle (cord factor) and a clear, bottom layer which contained the antibody. The antibody solution collected by suction with a syringe was subjected to protein determination by the modified Folin method (5), analytical gel filtration on Sephadex G-200 column, and immunodiffusion studies in agarose gel. Goat antisera specific for rabbit whole serum, IgM, IgG, and IgA (Miles Laboratories, Inc., Kankakee, Ill.) were used.

RESULTS

Antibody titration in various sera. Table 1 shows that 100% of sera of mice and rabbits vaccinated with cord factor-MBSA complex demonstrated a precipitin reaction against the aqueous emulsion of cord factor, whereas neither the sera of animals infected with virulent human *M. tuberculosis* H37Rv, the sera of rabbits sensitized to heat-killed H37Rv nor normal and BCG-vaccinated animal sera

TABLE 1. Serum precipitin test to cord factor

Serum source	No. of positive tests/ no. tested	Precipitin titer ^a
Mice		
Cord factor-MBSA-vaccinated	12/12	1:40-1:160
BCG-vaccinated	0/10	
H37Rv-infected	0/6	
Normal	0/10	
Rabbits		
Cord factor-MBSA-vaccinated	20/20	1:40-1:320
BCG-vaccinated	0/5	
Killed H37Rv-injected	0/3	
H37Rv-infected	0/5	
Normal	0/10	
Tuberculosis patients		
Sputum-positive	0/10	
Convalescent	0/8	
Pneumectomized	0/5	

^a Range of highest serum dilution giving a definite macroscopic precipitation by the addition of the aqueous emulsion of 100 μ g of cord factor.

gave a precipitin reaction against cord factor emulsion. The sera of sputum-positive patients with far-advanced or moderate pulmonary tuberculosis contained no precipitin antibody to cord factor. The fact that no anti-cord factor antibody was detected in the serum of either convalescent or pneumectomized tuberculosis patients excludes the possibility that a trace amount of anti-cord factor precipitin is circulating in the serum of tuberculosis patients but is absorbed by tubercle bacilli in the pathological foci. The above results indicate that vaccination with cord factor-MBSA complex elicits in animals a definite precipitating antibody to cord factor, whereas infection with virulent tubercle bacilli, as well as vaccination with live BCG vaccine, fails to induce the precipitin antibody to cord factor in either man or animals.

Gel filtration studies. To determine the immunoglobulin type of the anti-cord factor antibody in rabbit immune serum, the serum was filtered through a Sephadex G-200 column. Figure 1 illustrates the pattern of elution showing three protein peaks corresponding to 19S macroglobulins (IgM) and 7S immunoglobulins (IgG) and albumin, respectively. Precipitin activity to cord factor was localized only in the IgM zone. Gel filtration was performed with the supernatant fluid of immune serum after the sedimentation of cord factor-antibody complex formed by the addition of different

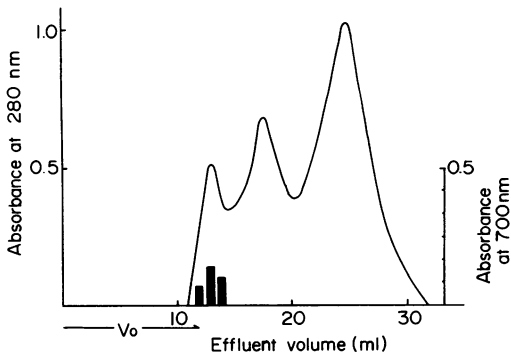


FIG. 1. Sephadex G-200 gel filtration of rabbit anti-cord factor serum. A 0.2-ml amount of serum was filtered through a Sephadex column (1.5 by 30 cm) in 0.2 M glycine-sodium hydroxide buffer (pH 8) at 4 C (flow rate 3 ml/hr). The distribution of the precipitin activity to cord factor (vertical bars) was defined by the Folin absorbancy at 700 nm of protein contained in the immune precipitate which was formed by the addition of 250 μ g of cord factor in 0.125 ml of water to 0.5 ml of each fraction. V_0 , Void volume.

amounts of cord factor. IgM peak decreased in direct proportion to the amount of cord factor added to the serum to bind to antibody, indicating that the precipitin antibody to cord factor is predominantly associated with 19S macroglobulins (Fig. 2). A Sephadex G-200 gel filtration pattern of the gamma globulin fraction of rabbit anti-cord factor serum also showed that the precipitin activity to cord factor was found only in the IgM zone.

Immunochemical studies of cord factor-antibody complex. A number of attempts to dissociate the antibody from the immune complex was made next. The use of Sephadex G-200 column equilibrated with 0.2 M glycine-hydrochloride buffer (pH 2.3) and the elution with the same buffer failed to dissociate the antibody from cord factor-antibody complex. It was finally noticed that the use of 0.2 M glycine-sodium hydroxide buffer (pH 8) containing 1 to 3 M NaCl resulted in an incomplete dissociation of the immune complex into cord factor and antibody during the gel filtration. Cord factor dissociated from the immune

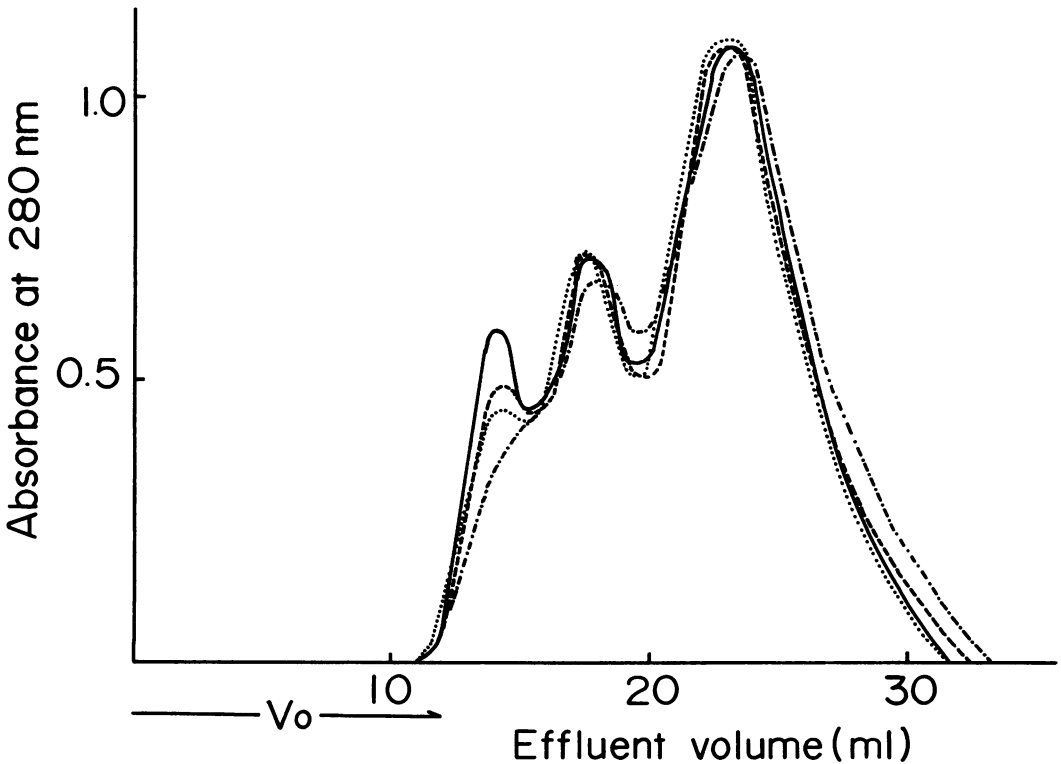


FIG. 2. Sephadex G-200 gel filtration of the supernatant fluid of rabbit anti-cord factor serum after sedimenting the cord factor-antibody complex. To 0.2 ml of rabbit anti-cord factor serum were added various levels of cord factor ranging from 200 to 1,000 μ g and were allowed to stand at 2 C for 7 days. The cord factor-antibody complex was precipitated by a centrifugation at 2,000 rev/min for 1 hr at 2 C, and the supernatant fluid was filtered through a Sephadex column (1.5 by 30 cm) in 0.2 M glycine-sodium hydroxide buffer (pH 8) at 4 C (flow rate 3 ml/hr). V_0 , Void volume.

complex was absorbed on the top portion of column and irregularly eluted from the column with several bed volumes of buffer. Attempts to purify the anti-cord factor antibody were made by incubating the immune complex in a buffer containing high concentration of salt. Incubation of the cord factor-antibody complex in 0.2 M glycine-sodium hydroxide buffer (pH 8) containing 15% NaCl permitted the recovery of the purified anti-cord factor antibody in yields of 30 to 40%.

The analytical gel filtration of this purified antibody demonstrated one single peak at the IgM zone (Fig. 3). When the eluates were tested for anti-cord factor precipitin activity, the peak was found to be composed of 100% pure precipitin to cord factor.

The purified anti-cord factor antibody produced a single precipitin line against goat anti-rabbit serum in agarose gel (Fig. 4). This precipitin line fused with that formed between anti-cord factor and goat anti-rabbit IgM serum. Anti-cord factor did not react with the anti-rabbit IgG serum. Although not shown in the figure, anti-rabbit IgA serum also failed to form precipitin line against anti-cord factor antibody. Thus, it may be concluded that the anti-cord factor antibody produced in rabbit serum under the present vaccination schedule contained only IgM.

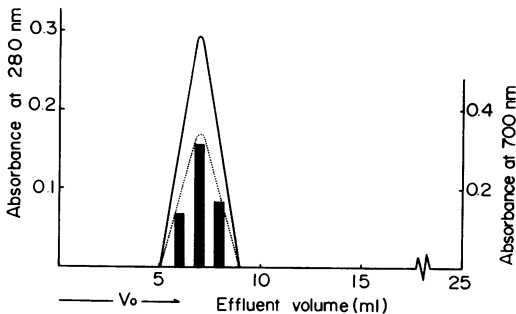


FIG. 3. Sephadex G-200 gel filtration of the purified anti-cord factor antibody. Antibody (0.27 mg of protein) in 0.2 ml was applied to a Sephadex column (0.9 by 30 cm) and eluted with 0.2 M glycine-sodium hydroxide buffer (pH 8) at 4 C (flow rate 3 ml/hr). One milliliter fraction was collected and precipitin activity against cord factor was determined by adding 250 μ g of cord factor in 0.125 ml of water to 0.5 ml of eluate and by measuring the protein content in the formed precipitate by a modified Folin method. Solid line: absorbance at 280 nm; dotted line, Folin absorbance at 700 nm of protein contained in the immune precipitate with added cord factor; V_0 , void volume.

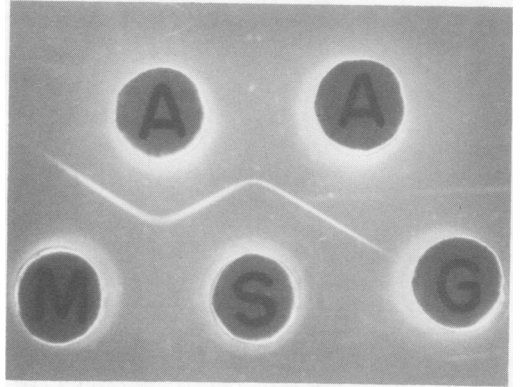


FIG. 4. Immunodiffusion pattern of the purified anti-cord factor antibody in agarose gel. A: Anti-cord factor antibody (33.4 μ g of protein); S, goat anti-rabbit serum; M, goat anti-rabbit IgM; G, goat anti-rabbit IgG.

DISCUSSION

Previous findings on the antibody formation to trehalose-6,6'-dimycolate (cord factor) of *M. tuberculosis* by the vaccination with cord factor-MBSA complex (2) and the failure of live BCG vaccine to elicit the anti-cord factor response in animals are extended in the present study by using the sera of either mice or rabbits infected with virulent *M. tuberculosis* or the sera of tuberculous patients presenting various disease states. It is clearly shown in this study that neither the infection with viable, virulent tubercle bacilli nor the sensitization to heat-killed tubercle bacilli is capable of inducing a precipitin antibody response against cord factor in laboratory animals. Moreover no antibody to cord factor was detected in the serum of tuberculous patients presenting active or convalescent states or after the resection of lung foci. These data completely exclude the possibility of the occurrence of precipitating antibody to cord factor in tuberculosis. Therefore, cord factor-MBSA complex should be considered to be an artificial immunogen which stimulates the production of a non-natural humoral antibody to cord factor.

Fractionation of anti-cord factor antiserum of rabbits and the immunochemical studies of the cord factor-antibody complex support the view that the antibody is predominantly formed in the IgM fraction of serum. Although it remains to be determined whether this IgM-specific immune response is characteristic of the cord factor-type haptenic glycolipid or merely results from the present sched-

ule of immunization and serum preparation, these findings are consistent with the previous finding (2) that the treatment of antiserum with 2-mercaptoethanol resulted in a complete loss of precipitin activity to cord factor. Experiments on the immunoglobulin response to cord factor at different stages of immunization with cord factor-MBSA antigen and the response elicited by different immunization procedures are in progress.

A most surprising finding in the present study is that the antibody to cord factor is associated with the IgM fraction of rabbit serum and that the ratio of antibody to cord factor on weight basis is 1.0 in the equivalence zone of the precipitin reaction (2). This means that approximately 300 molecules of cord factor of molecular weight of 2,900 ($C_{138}H_{366}O_{17}$) are bound to one molecule of IgM (molecular weight = 900,000) to form an immune precipitate. Although it seems difficult to make any assumption at present on the mechanism of binding of cord factor to IgM, the hydroxyl groups of trehalose in the cord factor molecule seem to participate in the binding to IgM because it was found in a previous study (2) that the antigenic determinant group in the precipitin reaction between cord factor and antiserum is α -D-trehalose. Neither totally acetylated cord factor nor mycolic acid reacted with anti-cord factor serum. From this previous result and the present finding that the immune complex dissociates into antigen and antibody in a high salt concentration, it appears likely that cord factor binds to IgM by hydrogen bond or some ionic interactions. An alternative possibility is that cord factor tends to

aggregate in the presence of antibody and that the latter serves to bind the aggregates. The precise nature of the bond between cord factor and IgM is under investigation by means of the purified anti-cord factor antibody.

Another interesting finding, obtained in parallel with those reported in this paper, is that either the active immunization with cord factor-MBSA complex or the passive transfer of rabbit anti-cord factor serum protected mice against not only the toxic effect of cord factor (2) but against the infection with virulent *M. tuberculosis*. This suggests that the anti-cord factor antibody elicits an infection-protecting effect by neutralizing the toxic action of cord factor during the course of tuberculous infection. These studies are the subject of the following paper (3).

ACKNOWLEDGMENTS

I am grateful to Y. Yamamura, Osaka University Medical School, Osaka, for the encouragement and valuable discussions.

LITERATURE CITED

1. Kato, M. 1968. Studies of a biochemical lesion in experimental tuberculosis in mice. VII. Structural and functional damage in mouse liver mitochondria under the toxic action of cord factor. *Amer. Rev. Resp. Dis.* **98**: 260-269.
2. Kato, M. 1972. Antibody formation to trehalose-6,6'-dimycolate (cord factor) of *Mycobacterium tuberculosis*. *Infect. Immunity* **5**:203-212.
3. Kato, M. 1973. Effect of anti-cord factor antibody on experimental tuberculosis in mice. *Infect. Immunity*. **7**: 14-21.
4. Kekwick, R. A. 1940. The serum proteins in multiple myelomatosis. *Biochem. J.* **34**:1248-1257.
5. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.