CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Sept. 1996, p. 563–566 Vol. 3, No. 5
1071-412X/96/$04.00+0

Comparison of Two 2,3-Diacyl Trehalose Antigens from *Mycobacterium tuberculosis* and *Mycobacterium fortuitum* for Serology in Tuberculosis Patients

MARÍA TERESA TÓRTOLA,[1] MARIE ANTOINETTE LANÉELLE,[2] AND NURIA MARTÍN-CASABONA[1]*

Servicio de Microbiología y Parasitología, Ciudad Sanitaria y Universitaria Vall d'Hebron, Barcelona, Spain,[1] and Département des Glycoconjugués et Biomembranes LPTF du CNRS, Toulouse, France[2]

Received 1 February 1996/Returned for modification 8 April 1996/Accepted 18 June 1996

Immunoglobulin G antibodies against two 2,3-diacyl trehalose (DAT) antigens from *Mycobacterium tuberculosis* (DAT_T) and *Mycobacterium fortuitum* (DAT_F) were studied by enzyme-linked immunosorbent assay of 356 serum samples. The sera were obtained from non-tuberculosis-infected individuals (282 serum samples) and tuberculosis patients (74 serum samples). Non-tuberculosis-infected individuals were healthy people (120 serum samples; positive purified-protein-derivative skin test, 60 patients; negative purified-protein-derivative skin test, 60 patients) patients with nontuberculosis lung disease (59 serum samples), contacts of sputum-smear-positive tuberculosis patients (57 serum samples), and human immunodeficiency virus-infected patients with nontuberculosis lung disease (46 serum samples). Of the 74 patients with tuberculosis, 14 were human immunodeficiency virus infected. The sensitivity of the method using DAT_T was 44.5%, and that with DAT_F was 48.6%. The specificities with both antigens were 99.1%. There were no significant differences between the mean values for both antigens ($P = 0.2815$). We therefore concluded that both antigens were interchangeable. As *M. fortuitum*, a fast-growing mycobacterium, could be a good source of antigen DAT, these results deserve consideration in the serology of tuberculosis.

A large number of studies of mycobacterial cell wall have been conducted (7, 9, 10, 12, 16, 18) to obtain species-specific antigens to detect antibodies in sera of tuberculosis patients and use them in attempts to develop an early diagnostic method or to study the evolution of these antibodies in the patient over time (14).

Several antigens have been used for tuberculosis serology with great variability in specificity and sensitivity. One of them, 2,3-diacyl trehalose (DAT), has shown in various studies involving tuberculous patients specificities of 100% (5), 88 to 100% (8), and 96.0% (14). At the present, this is therefore the most promising glycolipid antigen for clinical use.

This glycolipid, obtained from *Mycobacterium tuberculosis*, was first called SL-IV because it was described as 2,3-diacyl trehalose 2′-sulfate (10). Recent studies have demonstrated that the lipid is not homogeneous, and after purification, its structure was revised as a DAT (3, 13). Recently, an almost identical DAT was also discovered in *Mycobacterium fortuitum* (11). As this glycolipid was more abundant and easier to obtain from *M. fortuitum* than from *M. tuberculosis*, it was deemed necessary to further verify whether the DATs from *M. fortuitum* (DAT_F) and *M. tuberculosis* (DAT_T) had the same serological reactivity.

(This work was presented in part at the 15th Annual Meeting of the European Society for Mycobacteriology, Athens, Greece, 26 to 30 June 1994 [abstract OP26].)

MATERIALS AND METHODS

Population studied. A total of 356 serum specimens were studied. These sera came from two groups: non-tuberculosis-infected individuals and tuberculosis patients.

(i) Non-tuberculosis-infected individuals. The non-tuberculosis-infected group included healthy people (120 serum specimens: positive purified-protein derivative [PPD] skin test, 60 patients; negative PPD skin test, 60 patients) patients with nontuberculosis lung disease (59 serum specimens), contacts of patients with positive sputum smears (57 serum specimens), and HIV-infected patients with nontuberculosis lung disease (46 serum specimens).

(ii) Tuberculosis patients. Seventy-four serum samples were obtained from tuberculosis patients before treatment was started; of these, 14 were from HIV-infected patients.

The skin test was performed using 5 IU of PPD-RT 23, and a reaction equal to or above 6 mm was classified as positive.

The diagnosis of all tuberculous patients was bacteriologically confirmed by means of isolation and identification of *M. tuberculosis*, except for 14 patients, for whom the diagnosis of tuberculosis was made by clinical symptoms, radiology, and histology, including Ziehl-Neelsen stain with favorable evolution after treatment. Among the tuberculous patients, 65 had pulmonary disease, 5 had pleuritis, 2 had lymph node tuberculosis, 1 had pericarditic tuberculosis, and 1 had disseminated tuberculosis.

The nontuberculosis lung diseases were 36 cases of pneumonia, 7 cases of pulmonary fibrosis, 4 cases of bronchiectasis, 3 cases of sarcoidosis, 3 cases of pleural effusion, 2 cases of chronic bronchitis, 2 cases of pulmonary cancer, 1 case of asthma, and 1 case of bronchospasm.

Antigens. Two DATs were used: one was obtained from *M. tuberculosis* (DAT_T) (supplied by F. Papa and H. L. David, Pasteur Institute, Paris, France) (3, 13), and the second was isolated from *M. fortuitum* ATCC 6841 (DAT_F) by one of us (M. A. Lanéelle) of the Centre National de la Recherche Scientifique in Toulouse, France (11).

Methods. The enzyme-linked immunosorbent assay (ELISA) method was carried out as previously described (8), and both antigens were used in the same way for the detection of immunoglobulin G (IgG)-specific antibodies. IgG-specific immunoglobulin was detected by performing the ELISA in microplates. The antigens were dissolved in hexane (100 ng per well), dried for 2 h at 37°C, saturated with 5% bovine serum albumin (BSA) overnight at 4°C, and washed with phosphate-buffered saline (PBS) (pH 7.4). We used polystyrene microtiter plates from Nunc (Roskilde, Denmark). Sera were diluted 1/250 in 0.5% BSA, and 100 μl was added to each well. After 90 min of incubation and washing the plate, 100 μl of goat anti-human IgG–β-galactosidase conjugate (Biosis, Compiègne, France) diluted 1/1,000 in PBS was added. After 2 h of incubation and washing with PBS, 100 μl of *o*-nitrophenyl β-mercaptoethanol (6 μl/ml) dissolved in a buffer (0.1 M K_2HPO_4, 1 mM $MgSO_4$, 2 mM $MnSO_4$, 2 mM Mg Tritriplex) was added. After 1 h of incubation, A_{410} values were determined with a Dynatech MR 700 apparatus. The incubation temperature was always 37°C. All tests were carried out in duplicate by two workers on different days. In each plate, three control serum samples with previously established absorbancy results were included—a negative control with an optical density (OD) of 0.036, an intermedi-

* Corresponding author. Mailing address: Laboratorio de Microbiología y Parasitología, Ciudad Sanitaria y Universitaria Vall d'Hebron, 08035 Barcelona, Spain. Phone: 34-3-4280947. Fax: 34-3-4285952.

TABLE 1. Classification of sera presenting different responses with the two antigens

Serum sources	No. of serum samples[a]			
	Group 1	Group 2	Group 3	Total
Non-tuberculosis-infected individuals				
Healthy people	4	0	1	5
PPD skin test positive	2	0	1	3
PPD skin test negative	2	0	0	2
Nontuberculosis lung disease patients	0	0	3	3
Contacts of sputum-smear-positive tuberculosis patients	0	0	3	3
HIV-positive non-tuberculosis-infected patients	0	2	1	3
Tuberculosis patients	3	12	14	29
HIV negative	3	8	11	22
HIV positive	0	4	3	7
Total	7	14	22	43

[a] Groups: 1, negative for both antigens; 2, positive for both antigens; 3, discrepant results (positive for one antigen and negative for the other in relation to the cutoff point).

ately positive control with an OD of 0.540, and a highly positive control with an OD of 1.300—and when the correlation between the controls was less than 95%, the plate was discarded and the test was repeated.

The cutoff point of the ODs between samples from healthy people and those from tuberculosis patients was established to be 0.191 in a previous work (14) for IgG-specific antibodies against DAT_T. Using the same methodology (14), we established the cutoff point for the new antigen. An OD value of 0.183 was obtained for IgG-specific antibodies against DAT_F. Differences in their ODs equal to or greater than 0.100 were considered to be a different result. Differences in their ODs below 0.100 were considered to be due to the variability of the technique. Both antigens were tested on the same day in the same plate for each serum sample.

Statistical analysis. For statistical analysis we used the Pearson correlation coefficient, the matched Student *t* test, variance analysis, and the interclass correlation coefficient. A *P* value of ≤ 0.05 was considered significant.

RESULTS

Three hundred thirteen serum samples (87.9%) exhibited the same response to the DATs and did not show a difference greater than 0.100 in their ODs. The results of the remaining 43 serum samples could be classified in three groups (Table 1): group 1, sera with an OD below the cutoff point for both antigens (7 samples), group 2, sera with an OD above the cutoff point for both antigens (14 samples), or group 3, sera that could be classified as either positive or negative depending on the antigen used (22 samples [6.1%]). We will refer to the third group as discrepant. Of this group, 13 samples were positive for DAT_T and 9 samples were positive for DAT_F.

Groups 1 and 2 in the above paragraph had the same result in relation to the cutoff point. We focus our explanation on the results of group 3 (the discrepant group), for which a positive or negative result was obtained depending on the antigen used. These results are specified and presented in Tables 2 and 3.

Non-tuberculosis-infected individuals. Table 2 shows the characteristics of sera of non-tuberculosis-infected individuals displaying discrepant responses with the two antigens.

(i) Healthy people. The discrepant result among healthy people was from a woman with bronchospasm and a negative PPD skin test. No clinical symptoms of tuberculosis were detected after a period of 4 years.

(ii) Patients with nontuberculosis lung disease. We had three nontuberculosis lung disease patients with discrepant results. In all three cases ELISA showed an OD above the cutoff point with DAT_T but not with DAT_F. So far the patients have no clinical symptoms of tuberculosis disease.

(iii) Contacts of sputum-positive tuberculosis patients. Only three contacts of sputum-positive tuberculosis patients had discrepant results; of these, two had a positive reaction with DAT_F and one had a positive reaction with DAT_T. The individual providing serum sample 839 had a negative PPD skin test but 2 months later had a positive result of 20 mm. The serum sample was obtained when the positive PPD skin test reaction was detected. Chemoprophylaxis with isoniazid was

TABLE 2. Characteristics of sera of non-tuberculosis-infected individuals showing discrepant responses with the two antigens

Serum source(s)	Serum sample	Sex[a]	Age (yr)	Underlying disease	PPD result[b]	OD result	
						DAT_T	DAT_F
Healthy person	235	F	51	None	Neg	0.490	0.120
Nontuberculosis lung disease patients	893	M	47	Sarcoidosis	Neg	0.276	0.140
	902	F	30	Pneumonia	ND	0.350	0.160
	1037	F	31	Pneumonia	ND	0.225	0.100
Contacts of sputum-smear-positive tuberculosis patients	825	M	21	None	17 mm	0.100	0.350
	836	M	25	None	14 mm	0.065	0.280
	839	F	16	None	Neg to 20 mm	0.355	0.100
HIV-positive non-tuberculosis-infected patient	783	M	29	IVDU[c]	Neg	1.864	0.110

[a] F, female; M, male.
[b] Neg, negative; ND, not done.
[c] IVDU, intravenous drug user.

TABLE 3. Characteristics of sera of tuberculosis patients showing discrepant responses with the two antigens

Patient HIV status	Serum sample	Sex[a]	Age (yr)	Underlying disease	PPD result (mm)[b]	Tuberculosis location	OD result	
							DAT_T	DAT_F
Negative	22	M	18		12	Lung	1.340	0.080
	53	M	51	Diabetes	15	Lung	0.140	0.265
	108	M	72	Asthma	ND	Lung	0.220	0.095
	146	M	26		ND	Lung	0.100	0.215
	156	M	26		16	Lung	0.275	0.175
	227	F	25		11	Lung	0.060	0.215
	423	F	20		12	Lung	0.189	0.606
	460	M	62	Bronchitis	20	Lung	0.230	0.120
	488	M	24		18	Lung	0.750	0.080
	495	M	21		14	Lung	0.260	0.095
	869	M	33		25	Lung	0.350	0.070
Positive	717	F	26	IVDU[c]	Neg	Disseminated	0.097	0.313
	755	M	34	IVDU	Neg	Lung	0.121	0.406
	948	M	32		Neg	Lung	0.170	0.343

[a] M, male; F, female.
[b] Neg, negative; ND, not done.
[c] IVDU, intravenous drug user.

started, and no tuberculosis symptoms developed over a control period of 6 months.

(iv) HIV-infected nontuberculous patients. The discrepant result among HIV-infected nontuberculous patients was from a male whose serum sample, 783, was obtained for control of his HIV infection. When the serum was obtained, the PPD skin test was negative; 1 year later, a PPD skin test result of 12 mm was detected and chemoprophylaxis with isoniazid was started. No tuberculosis disease developed in the following 3 years.

Tuberculosis patients. Table 3 shows the characteristics of the sera of tuberculosis patients with discrepant responses with the two antigens.

(i) HIV-negative group. Serological results were discrepant in 11 HIV-negative tuberculosis patients. All patients included in this tuberculosis group had pulmonary tuberculosis and a positive PPD skin test, except for two patients whose PPD skin tests were not recorded in their clinical files.

(ii) HIV-positive group. Three HIV-infected patients with tuberculosis had discrepant results. These three patients had a positive reaction with DAT_F but not with DAT_T. Tuberculosis diagnosis was confirmed by culture and identification of *M. tuberculosis.*

Statistical results. Including all the groups, the antigens are correlated positively, with a Pearson correlation coefficient of 0.6259. We could not reject the hypothesis of no difference between the mean values ($P = 0.2815$, Student's t test). In the variance analysis the component was significant for the subjects but not for the antigens. An interclass correlation coefficient of 0.5900 was obtained.

Data on the sensitivity, specificity, and predictive values of both antigens were derived from healthy people and tuberculosis patients. Both antigens have the same specificity (99.1%), with similar positive and negative predictive values (DAT_T, 97.0 and 74.3%, respectively; DAT_F, 97.2 and 75.7%, respectively). The sensitivity of DAT_F (48.6%) was slightly higher than the sensitivity of DAT_T (44.5%).

DISCUSSION

It has been proposed that the antigen DAT_T should be species specific for *M. tuberculosis*, as specific immune sera against DAT_T obtained from rabbits exhibited no reaction with 37 species of mycobacteria, with only *M. tuberculosis* and *Mycobacterium africanum* reacting (15). Other workers (17) detected that the DAT_T antigen had a weak reaction with rabbit antisera against *M. fortuitum*. Recently DAT has been obtained from *M. fortuitum* (11); this fact could explain this weak reaction.

In previous works (5, 8, 14), the sensitivity of DAT_T ranged between 46.8 and 51.9% and the specificity ranged between 88% and 100%. Our results were within the same range, since the sensitivity was 44.5% for DAT_T and 48.6% for DAT_F and the specificity was 99.1% for both antigens.

From the statistical calculations we concluded that the two antigens were interchangeable and that observed differences were due not to the antigens but to the subjects. At present we cannot explain these differences in the subjects.

Several studies have focused on the chemical characterization of DAT_T and DAT_F (1–3, 6, 11, 13). They arrived at the same conclusion: that the only carbohydrate component of the trehalose is esterified, in positions 2 and 3 of the same glucosyl residue with long-chain fatty esters. The fatty esters are a mixture of linear and branched-chain homologs. Interestingly, in both cases, specific acids of mycobacteria (2-methyl branched acids) are the main components. Thus, the only differences that can be noted result from the lipid moieties: the existence of two or three methyl branches in DAT_T acyl residues as opposed to one methyl branch for DAT_F. Moreover, 3-OH fatty acid has not been detected in DAT_F, whereas it is a major component in DAT_T. As the acyl moiety is not of importance for the serological response and the acid composition presents major analogies, it is not surprising that no difference was observed with DATs from the two origins. The presence of specific mycobacterial fatty acyl residues such as phthienoic and polymethyl branched acids could give a good stability of these trehalose esters toward host esterases.

In order to easily obtain an efficient antigen for serology, two possibilities can be considered: (i) using the DAT_F antigen in the serology of tuberculosis and (ii) using synthetic antigens. It seems that *M. fortuitum* contains larger amounts of acyl trehaloses (11) than does *M. tuberculosis* (6). Since these two antigens are structurally related and interchangeable, the antigenic compound isolated from *M. fortuitum* would be an alternative to the same antigen from *M. tuberculosis*. A synthetic antigen

with a structure similar to that of DAT has been obtained (4). Its use could be of interest in the serology of tuberculosis.

ACKNOWLEDGMENTS

This work was supported by a grant from CIRIT (AR 1991-317), Departament d'Ensenyament, Generalitat de Catalunya, Spain.

We are thankful to H. L. David for the revision of the manuscript. We also thank Tobias Willet for correcting the English in the manuscript.

REFERENCES

1. **Ariza, M. A., F. Martín-Luengo, and P. L. Valero-Guillén.** 1994. A family of diacyltrehaloses isolated from *Mycobacterium fortuitum*. Microbiology **140:** 1989–1994.
2. **Ariza, M. A., and P. L. Valero-Guillén.** 1994. Delineation of molecular species of a family of diacyltrehaloses from *Mycobacterium fortuitum* by mass spectrometry. FEMS Microbiol. Lett. **119:**279–282.
3. **Baer, H. H.** 1993. The structure of an antigenic glycolipid (SL-IV) from *Mycobacterium tuberculosis*. Carbohydr. Res. **240:**1–22.
4. **Baer, H. H., and X. Wu.** 1993. Synthesis of α-α-trehalose 2,3- and 2,3′-diesters with palmitic and stearic acid: potential immunoreactants for the serodiagnosis of tuberculosis. Carbohydr. Res. **238:**215–230.
5. **Berlie, H. C., J. C. Petit, and H. L. David.** 1991. Use of the SL-IV and the PGL-Tb1 glycolipid antigens in ELISA for the diagnosis of tuberculosis in AIDS patients. Zentralbl. Bakteriol. **275:**351–357.
6. **Besra, G. S., R. C. Bolton, M. R. McNeil, M. Ridell, K. E. Simpson, J. Glushka, H. van Halbeek, P. J. Brennan, and D. E. Minnikin.** 1992. Structural elucidation of a novel family of acyltrehaloses from *Mycobacterium tuberculosis*. Biochemistry **31:**9832–9837.
7. **Brennan, P. J.** 1989. Structure of mycobacteria: recent developments in defining cell wall carbohydrates and proteins. Rev. Infect. Dis. **11**(Suppl. 2)**:**420–430.
8. **Cruaud, P., J. T. Yamashita, N. Martin-Casabona, F. Papa, and H. L. David.** 1990. Evaluation of a novel 2,3-diacyl-trehalose-2′-sulphate (SL-IV) antigen for case finding and diagnosis of leprosy and tuberculosis. Res. Microbiol. **141:**679–694.
9. **Daniel, T. M., and B. W. Janicki.** 1978. Mycobacterial antigens: a review of their isolation, chemistry, and immunological properties. Microbiol. Rev. **42:**84–113.
10. **Daffé, M., F. Papa, A. Laszlo, and H. L. David.** 1989. Glycolipids of recent isolates of *Mycobacterium tuberculosis*: chemical characterization and immunoreactivity. J. Gen. Microbiol. **135:**2759–2766.
11. **Gautier, N., L. M. López Marín, M. A. Lanéelle, and M. Daffé.** 1992. Structure of mycoside F, a family of trehalose-containing glycolipids of *Mycobacterium fortuitum*. FEMS Microbiol. Lett. **98:**81–88.
12. **Goren, M. B.** 1982. Immunoreactive substances of mycobacteria. Am. Rev. Respir. Dis. **125:**50–69.
13. **Lemassu, A., M. A. Lanéelle, and M. Daffé.** 1991. Revised structure of a trehalose-containing immunoreactive glycolipid of *Mycobacterium tuberculosis*. FEMS Microbiol. Lett. **78:**171–176.
14. **Martin-Casabona, N., T. Gonzalez Fuente, F. Papa, J. Rosselló Urgell, R. Vidal Plá, G. Codina Grau, and I. Ruiz Camps.** 1992. Time course of anti-SL-IV immunoglobulin G antibodies in patients with tuberculosis and tuberculosis-associated AIDS. J. Clin. Microbiol. **30:**1089–1093.
15. **Papa, F., P. Cruaud, and H. L. David.** 1989. Antigenicity and specificity of selected glycolipid fractions from *Mycobacterium tuberculosis*. Res. Microbiol. **140:**569–578.
16. **Puzo, G.** 1990. The carbohydrate and lipid-containing cell wall of mycobacteria, phenolic glycolipids: structure and immunological properties. Crit. Rev. Microbiol. **17:**305–327.
17. **Ridell, M., G. Wallerström, D. E. Minnikin, R. C. Bolton, and M. A. Magnusson.** 1992. Comparative serological study of antigenic glycolipids from *Mycobacterium tuberculosis*. Tubercle Lung Dis. **73:**101–105.
18. **Young, D. B.** 1988. Structure of mycobacterial antigens. Br. Med. Bull. **44:**562–583.