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Studies of Mycobacterial Antigens, with Special Reference to *Mycobacterium leprae*

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Eight individual antigens were detected in soluble antigen preparations from *Mycobacterium leprae* bacilli by using pools of serum samples from lepromatous leprosy patients as antibody reagents in crossed immunoelectrophoresis. Two of these antigens were analyzed further. Antigen no. 1 gave an elution pattern on Sephadex G-200 corresponding to a molecular weight of 285,000. This antigen was also present in three slow-growing and eight fast-growing mycobacterial species. There was a reaction of complete identity in immunological tests using lepromatous serum pools as well as with rabbit antisera raised against *M. leprae* and *M. smegmatis*. Antigen no. 21 of *M. leprae* showed antigenic heterogeneity when compared with other species. Three types of antigenic determinants were detected; one, called 21A, was shared by all mycobacteria; another, called 21B, was limited to antigen no. 21 of *M. leprae*; a third, called 21C, was present in all mycobacteria except the leprosy bacillus. This submolecular heterogeneity may indicate a separate taxonomic position of *M. leprae* among the mycobacteria.

Biopsies of nodules from lepromatous leprosy patients have constituted the only source of bacilli of the noncultivable bacillus *Mycobacterium leprae* since its discovery in 1873. In spite of inherent difficulties with this source of material such as limited amounts and the possibility of antigenic deficiency (5, 30), progress in antigenic analysis of *M. leprae* has been noted. Carbohydrate antigens, also found in other mycobacteria, have been identified (1, 2, 6, 7, 19, 20, 29). They have been called NEG2 (1, 2), polysaccharide I (6,7),and the β- and δ-antigens (19, 20). NEPR, an *M. leprae*-specific protein antigen with a molecular weight of more than 200,000, has been identified by Abe (1, 2). Using a plaque assay technique, a separated fraction of *M. leprae* containing one single protein antigen was found to be strain specific (21).

Larger amounts of *M. leprae* bacilli grown in vivo in armadillos have recently become available (10, 28). Antigen extracts of such bacilli have been studied in two-dimensional immunodiffusion analysis (26), revealing as many as four *M. leprae*-specific antigens (28). In previous investigations, one *M. leprae* antigen was found to carry determinants unique to the leprosy bacillus (11). This antigenic heterogeneity at the molecular level indicated the possibility of an alternative approach to taxonomic studies of the leprosy bacillus. The results of such studies reported here suggest a position of *M. leprae* among the mycobacteria different from the slow-growing or fast-growing mycobacterial clusters.

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MATERIALS AND METHODS

Mycobacterial antigen preparations. Strains of both slow-growing and fast-growing mycobacterial species (Table 1) were cultured on Sauton medium solidified with 1.5% agar. Harvested bacteria were washed three times in PBS (phosphate-buffered saline; 0.12 M NaCl, 0.03 M phosphate, pH 7.3) and suspended in 10 ml of PBS per g (wet weight). Soluble antigens were prepared by ultrasonication for 30 min, using a Branson Sonifier B-12 (Danbury, Conn.), and insoluble residues were removed by centrifugation. Protein determinations of soluble antigen preparations were performed by using Folin-Ciocalteau reagent (17).

Antigen preparations of *M. leprae* were obtained from bacilli grown in vivo in armadillos (10, 28). Cutaneous nodules were extracted three times with PBS to remove soluble material by using an Ultra-Turrax homogenizer (Janke & Kunkel KG, IKA-Werk, Staufen, Breisgau, model TP 18-10). The insoluble residue consisting of tissue material and leprosy bacilli was then dehydrated, and the bacilli were extracted by using oil-chloroform (Ricella 917-chloroform, 8:2) (18, 25). Combined extracts were centrifuged, and the pellet was washed twice with acetone and then with ether. The pellet was suspended in PBS (1 ml/20 mg) and ultrasonicated. The soluble antigens were separated from insoluble residues by centrifugation. Extracts of normal armadillo tissue were negative in crossed immunoelectro-

TABLE 1. *Immunological comparison of antigen no. 21 from different mycobacterial species using lepromatous serum pools or anti-M. smegmatis antiserum as antibody reagents*

Mycobacterial strain		Lepromatous serum pools[a]			Anti-M. smegmatis antiserum		
		M. leprae[b]	*M. avium intracellulare*	*M. smegmatis*	*M. leprae*[c]	*M. avium intracellulare*	*M. lactis*
Slow growing							
M. avium	NCTC 8551	X	I	I	X	I	I
M. av.intrac.	ATCC 25153	X	I	I	X	I	I
M. gordonae	NCTC 102 67	X	I	I	X	I	I
Fast growing							
M. smegmatis	NCTC 333	X	I	I			
M. phlei	NCTC 8156	X	I	I	X	I	I
M. vaccae	ATCC 15483	X	I	I	X	I	I
M. diernhoferi	ATCC 19340	X	I	I	X	I	I
M. ranae	NCTC 2891	X	I	I	X	I	I
M. chelonei	NCTC 946	X	I	I	I	X[d]	X[d]
M. lactis	J.S. no. 831	X	I	I	X	I	I
M. thermoresist.	NCTC 10409	X	I	I	X	I	I
M. leprae		I	X[b]	X[b]	I	X[c]	X[c]

[a] Abbreviations: X, Cross-reactivity with partial identity; I, complete identity.

[b] All cross-reactivities noted with lepromatous serum pools as antibody reagents were seen as a spurring by *M. leprae* antigen no. 21 over the others (Fig. 2C).

[c] All cross-reactivities noted using anti-*M. smegmatis* antiserum were seen as a spurring by antigen no. 21 of slow and fast growers over the corresponding *M. leprae* antigen (Fig. 3B).

[d] Cross-reactivities with *M. chelonei* were observed as a spurring over antigen no. 21 of this strain by the others.

phoresis against high-titered anti-*M. leprae* sera from leprosy patients.

Leprosy sera. Serum samples from lepromatous patients diagnosed clinically or by histopathological examination (22–24) were pooled. LS II (lepromatous serum pool no. II) consisted of 43 individual sera and LS III of 37 serum samples from clinically diagnosed lepromatous cases. LS IV was a pool of three individual sera, two lepromatous leprosy and one borderline lepromatous case, selected for their high titers against *M. smegmatis* antigen no. 40 (11). LS V was made up of seven lepromatous borderline and two lepromatous sera with very high titers of antibodies against *M. smegmatis* antigens. Serum samples and serum pools were stored at −20 C until use.

Rabbit antiserum. Antisera against *M. smegmatis* and *M. duvalii* were raised in rabbits by injection subcutaneously of 2 mg of antigen with Freund incomplete adjuvant every other week. When sufficient titers were reached, the rabbits were bled twice a week for 2 months while the immunizations were continued. Serum samples were pooled after crossed immunoelectrophoretic analysis and stored at −20 C.

Crossed immunoelectrophoresis. The method of crossed immunoelectrophoresis as described by Laurell (15) was used with modifications (3, 4, 16). For comparison of antigenic cross-reactivity, the tandem technique was used. The intermediate gel technique was used to define antibody activity of serum samples in *M. smegmatis*, *M. duvalii*, or *M. leprae* reference systems. Antigens in eluates of chromatographic columns were detected by the fused rocket technique.

Gel filtration. Sephadex G-200 gel filtration of *M. leprae* antigens was performed by using a column having an inner diameter of 25 mm and length of 950 mm. The gel was equilibrated with 0.2 M ammonium bicarbonate buffer, pH 8.0, and 3.5-ml fractions were eluted at a rate of 13.5 ml/h. The column was calibrated for determination of molecular weights. Fractions from filtration experiments with mycobacterial antigens were freeze dried, and the contents were dissolved in small volumes of immunoelectrophoresis buffer for analysis.

RESULTS

Antigens detected by lepromatous serum pools. Crossed immunoelectrophoretic precipitation patterns using serum pools from lepromatous leprosy patients were obtained with antigen preparations from *M. leprae*, *M. avium intracellulare*, and *M. smegmatis* (Fig. 1). The latter two were taken as representatives of slow-growing and fast-growing mycobacteria, respectively. By including increasing concentrations of lepromatous serum pools in the intermediate gel of an *M. smegmatis* reference system described earlier (11), four precipitin lines could be assigned corresponding numbers: antigens no. 1, 21, 40, and 41 (Fig. 1C). Using the tandem crossed immunoelectrophoretic technique, the same antigens were also identified in *M. avium intracellulare* and *M. leprae* antigen preparations (Fig. 1A, B). The other precipitin lines of *M. leprae* were designated

letters d, e, f, and g (Fig. 1A). Further comparisons between *M. leprae*, *M. avium intracellulare*, and *M. smegmatis* indicated that antigens d, e, and f were present in *M. avium intracellulare* and that antigens d and e were present in *M. smegmatis* (Fig. 1).

Comparative studies of antigen no. 1 in mycobacterial species. Lepromatous S III was used as an antibody source at a concentration of 2% in the antibody gel for comparisons between antigen no. 1 of different mycobacterial species. There was a reaction of complete identity between antigen no. 1 of *M. leprae* and that of *M. avium*, *M. avium intracellulare*, *M. gordonae*, *M. smegmatis*, *M. vaccae*, *M. diernhoferi*, *M. duvalii*, *M. rhodesiae*, and *M. chitae* (Fig. 2A). Similar experiments with *M. avium intracellulare* in tandem crossed immunoelectrophoresis with *M. gordonae*, *M. phlei*, and *M. ranae* as well as *M. smegmatis* in tandem with *M. av-*

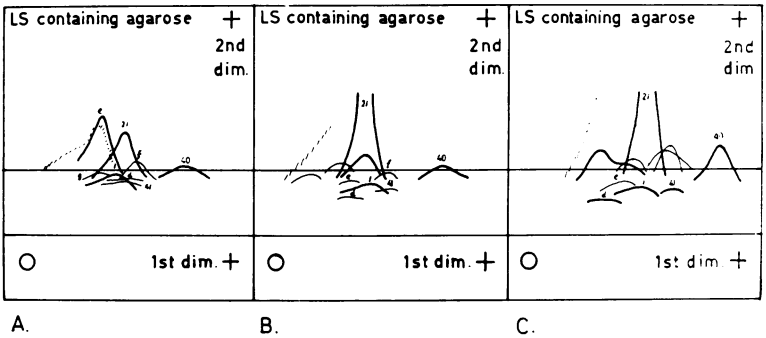

FIG. 1. *Crossed immunoelectrophoresis of mycobacterial antigen preparations against pools of serum samples from lepromatous leprosy patients. (A) M. leprae; (B) M. avium intracellulare; (C) M. smegmatis antigens. Identification of antigens as indicated by numbers and letters was performed in separate experiments using the tandem crossed immunoelectrophoretic technique.*

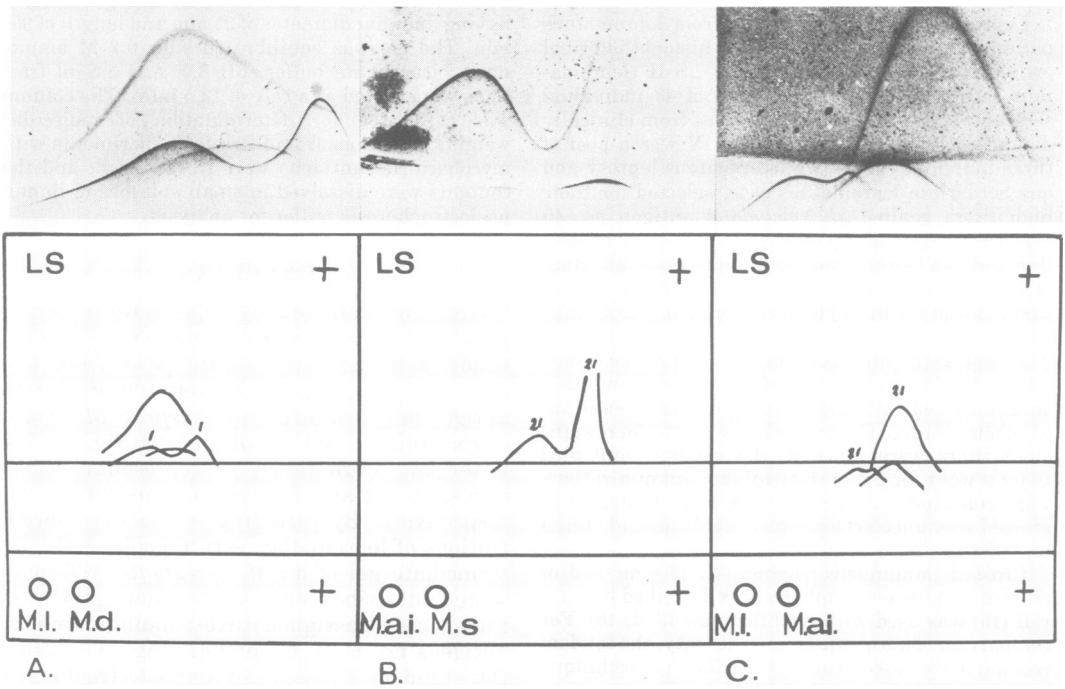

FIG. 2. *Examples of tandem crossed immunoelectrophoretic analysis of antigen no. 1 and no. 21 in different mycobacterial strains, using lepromatous serum pools (LS) as antibody reagents. Relevant antigens are shown in drawings with actual photographs above. (A) Identity reaction between antigen no. 1 of M. leprae and M. duvalii; (B) identity reaction between antigen no. 21 of M. avium intracellulare and M. smegmatis; (C) reaction of partial identity between antigen no. 21 of M. leprae and M. avium intracellulare.*

ium intracellulare, *M. diernhoferi*, and *M. duvalii* showed complete identity between all antigens no. 1. An antigen preparation from *M. kansasii* indicated slight antigenic deficiency of antigen no. 1 when compared with both *M. avium intracellulare* and *M. smegmatis* as judged by a faint spur formation by antigen no. 1 of these strains over *M. kansasii*. Identity reactions obtained with lepromatous serum pools were confirmed in comparisons between *M. leprae*, *M. avium intracellulare*, and *M. smegmatis* using rabbit antisera raised against *M. leprae* antigens and against *M. duvalii* antigens. Sephadex G-200 gel filtration experiments indicated a molecular weight of antigen no. 1 from *M. leprae* of about 285,000.

Antigen no. 21 in cultivable mycobacteria. Antigen no. 21 of *M. avium intracellulare* was compared with the corresponding antigen of both slow-growing and fast-growing mycobacterial species using 5 to 20% LS II, III, and IV as antibody reagents. The antigen was detected in three slow-growing and eight fast-growing mycobacterial species (Table 1). In all instances there was a reaction of complete immunological identity (Fig. 2B, Table 1). No antigen no. 21 was detected in direct tandem precipitation tests with *M. kansasii* or *M. duvalii* antigen preparations. Using an inhibition technique with increasing amounts included in the intermediate gel, the presence of small amounts of antigen no. 21 could be detected in *M. duvalii* antigen extracts. Antigen no. 21 of *M. smegmatis* was also compared with that of other strains. In all cases there was a reaction of complete identity between the antigens (Table 1).

***M. leprae*-specific determinants on antigen no. 21.** When antigens no. 21 of *M. avium intracellulare* and *M. smegmatis* were compared with that of *M. leprae* in tandem crossed electrophoresis using lepromatous serum pools, a reaction of partial immunological identity was observed. Antigen no. 21 of *M. leprae* spurred over those of the other two strains (Fig. 2C). Prominent spurring was seen using all four lepromatous serum pools. The partial identity was confirmed in inhibition experiments. *M. smegmatis* antigen was included in excess in the intermediate gel in tandem crossed immunoelectrophoresis with *M. leprae* and *M. avium intracellulare*. The precipitation line of antigen no. 21 of the latter strain was completely inhibited, whereas the *M. leprae* no. 21 line was essentially intact.

Comparisons of *M. leprae* antigen no. 21 were also made with those of other mycobacterial strains (Table 1). In all these experiments there was a marked spurring by antigen no. 21 from the leprosy bacillus over the other ones. The results of these immunological studies of antigen no. 21 indicate the existence of at least two types of antigenic determinants. One type is shared by all mycobacterial species, and the other one is detected only in *M. leprae*.

Determinants on antigen no. 21 common to mycobacteria except *M. leprae*. Rabbit antiserum raised against *M. smegmatis* antigens has previously been used in a reference system showing more than 50 precipitation lines against *M. smegmatis*, 39 of which were numbered, including antigen no. 21 (11). The same antiserum gave precipitin reactions also against antigen no. 21 of other mycobacterial species. Using anti-*M. smegmatis* antiserum as an antibody reagent in tandem crossed immunoelectrophoretic comparisons, this antigen of *M. avium intracellulare* as well as of *M. lactis* was shown to give reactions of complete identity with three slow-growing and six fast-growing mycobacterial species (Fig. 3A, Table 1). Identity reactions were obtained in tests between *M. gordonae* and three fast growers (*M. phlei*, *M. diernhoferi*, and *M. lactis*) as well as between *M. avium* and three fast growers (*M. smegmatis*, *M. phlei*, and *M. vaccae*). *M. chelonei* gave discordant results. In view of the regular pattern against lepromatous serum pools as antibody reagents (Table 1), the results obtained against anti-*M. smegmatis* antiserum would not imply any closer link with *M. leprae*. The phenomenon is being studied further.

Antigen no. 21 of *M. leprae* was compared in similar experiments using anti-*M. smegmatis* antiserum with the corresponding antigens of cultivable slow-growing and fast-growing mycobacteria (Fig. 3B, Table 1). A reaction of partial immunological identity was noted in all cases, with a spurring by other strains over the *M. leprae* antigen. These results indicate the existence of antigenic determinants that are present in all mycobacterial strains tested with the exception of the leprosy bacillus. The findings suggest a closer relationship between the cultivable mycobacteria than between any of these and *M. leprae*.

Antigen no. 40 in mycobacterial strains. One of the lepromatous serum pools, LS IV, consisted of three selected individual serum samples with precipitating antibodies against antigen no. 40 (11). When this lepromatous pool was used as a reagent in comparative immunological studies, a reaction of complete identity was noted between antigen no. 40 from *M. leprae*, *M. avium intracellulare*, and *M. smegmatis*. The occurrence of this antigen in all three

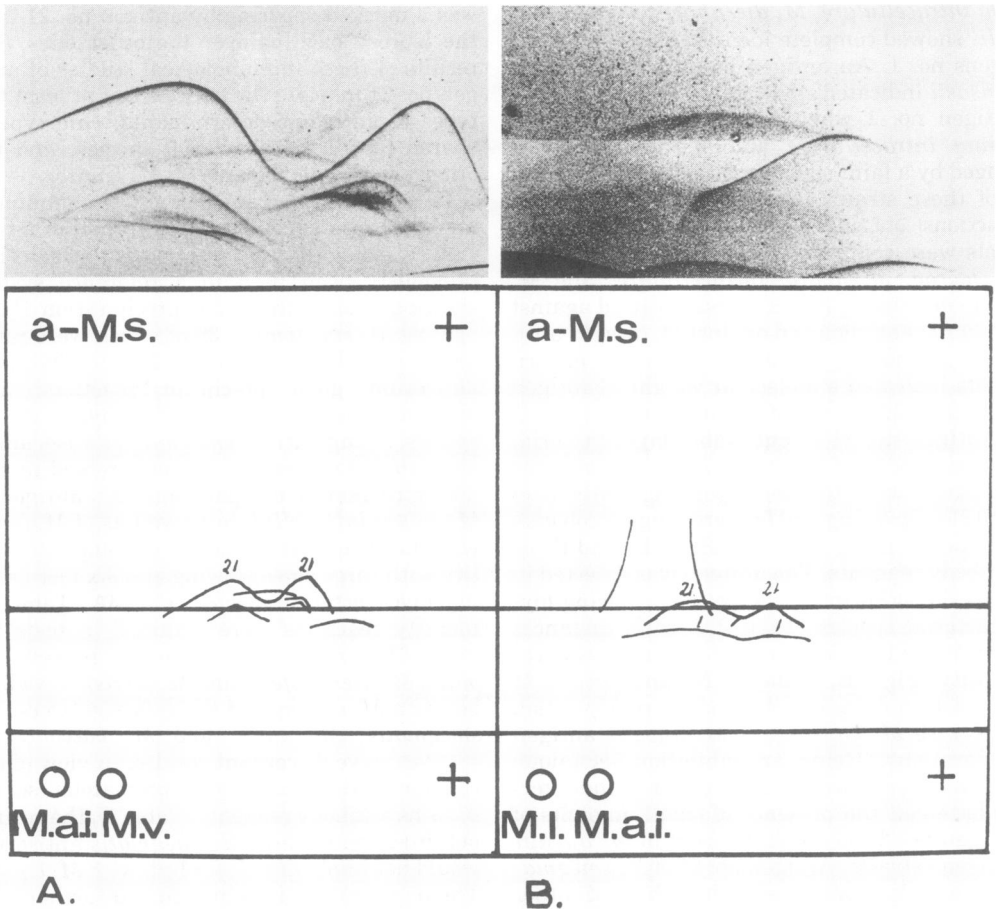

FIG. 3. *Examples of tandem crossed immunoelectrophoretic analysis of antigen no. 21 in mycobacteria, using a rabbit anti-M. smegmatis antiserum (a-M.s.). (A) Complete identity between antigen no. 21 of M. avium intracellulare and M. vaccae; (B) reaction of partial identity between antigen no. 21 of M. leprae and M. avium intracellulare.*

strains with complete identity suggests that antigen no. 40, like antigen no. 1, is shared by all mycobacteria (26).

DISCUSSION

Antigenic heterogeneity of a mycobacterial antigen was detected in previous studies (11). This antigen was labeled no. 21 in a reference system for *M. smegmatis*, and the number was also used for the corresponding antigens of other mycobacterial species. Antigen no. 21 of *M. leprae* was frequently involved in antibody responses in lepromatous leprosy patients (11). In the present investigations the existence of unique antigenic determinants on no. 21 molecules from *M. leprae* was confirmed. Such determinants, called 21B, were absent in both slow-growing and fast-growing mycobacterial species (Table 2). Using antiserum raised against a fast-growing mycobacterium, *M. smegmatis*, a third type of antigenic determinant was detected on no. 21 molecules. These determinants, called 21C, were detected in slow-growing as well as fast-growing species but were absent in *M. leprae* (Table 2). Occasional crossed immunoelectrophoretic runs using this antiserum showed a very faint spur formation by no. 21 molecules from fast growers over slow growers, but the method was not sensitive enough to document this suggested difference.

The antigenic heterogeneity of no. 21 molecules from mycobacteria indicates a unique position of the *M. leprae* antigen as compared with the other ones studied. Determinants labeled 21C are group specific, suggesting that 21B determinants more or less also express

TABLE 2. *Occurrence of three different types of determinants on antigen no. 21 in various mycobacterial strains*

Organism	Determinant		
	21A	21B	21C
Slow growing mycobacteria (3 species)	+	−	+
Fast-growing mycobacteria (8 species)	+	−	+
M. leprae	+	+	−

group specificity (Table 2). Immunological studies of the heterogeneity of individual mammalian antigens have been found to reflect the taxonomic positions of different species. Our results may have an analogous interpretation regarding the taxonomic position of *M. leprae*. The leprosy bacillus may not have any closer relationship with the representatives of the slow-growing and fast-growing mycobacteria studied. Its position in the genus *Mycobacterium* may therefore be separate from these other mycobacterial species. Similar results have been obtained recently by using a more conventional approach (26, 27) and are in line with earlier cytochemical investigations (9). A closer link with mycobacteria than with related genera is still suggested by studies of mycolic acids (8) as well as by earlier investigations (12–14).

M. lepraemurium is the other known noncultivable mycobacterium causing disease. Studies of this bacillus have shown a close relationship with slow-growing mycobacteria, particularly with *M. avium* (25). Our results would suggest that the human leprosy bacillus is no more related to the mouse leprosy bacillus than to any of the slow-growing cultivable mycobacteria, including *M. tuberculosis*. The taxonomic validity and homogeneity of the so-called slow-growing as well as fast-growing mycobacterial groups would permit such a conclusion (26).

Identification of *M. leprae*-specific antigens have been previously reported. Abe described a high-molecular-weight protein (NEPR) unique to the leprosy bacillus (1, 2). Stanford et al. have detected four different antigens that are found only in *M. leprae* (27). Since the defect in cellular immune resistance in lepromatous leprosy involves only the leprosy bacillus, it follows that the antigen(s) responsible for immunity must be *M. leprae* specific. Such antigens therefore deserve special attention regarding their role in humoral as well as cellular immunity in the various forms of leprosy.

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