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Characterization of Antibody-Reactive Epitopes on the 65-Kilodalton Protein of *Mycobacterium leprae*

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Twenty-three monoclonal antibodies (MAbs) prepared in seven different laboratories were studied, all of which recognized the 65-kilodalton (kDa) protein of *Mycobacterium leprae* as determined by Western blotting or gel immunoradioassay or both. Fourteen of the MAbs recognized different epitopes, as evaluated by cross-competition studies using radiolabeled MAb and unlabeled inhibitors; the species specificity of these epitopes was defined by nitrocellulose dot blot immunoassays with bacterial sonic extract antigen preparations from 23 species of mycobacteria. Each of the 14 distinct MAbs recognized a 65-kDa protein produced by a lysogenized *Escherichia coli* Y1089 host containing cloned rDNA which included the gene for the *M. leprae* 65-kDa protein. Of the 14 distinct MAbs, 1 recognized an epitope found only on *M. leprae*, and the others recognized epitopes present on as few as 8 or as many as all 23 of the mycobacterial species studied. Identification of these distinct 65-kDa protein epitopes and use of the MAbs which recognize them should assist future structural studies of this protein and characterization of the T-cell reactive and serodiagnostically useful portions of the molecule.

The structure of epitopes that stimulate T cells as opposed to the structure of those which interact with antibodies has been studied for very few bacterial diseases. Mycobacterial diseases represent the largest single category of bacterial diseases in which cellular immunity is fundamental to resistance and recovery from the infection. The 65-kilodalton (kDa) protein molecule is a prominent component of *Mycobacterium leprae* and *Mycobacterium tuberculosis*. Humans make antibodies to the molecule during leprosy and tuberculosis (8, 11), and T-cell clones responsive to the 65-kDa protein of *M. leprae*, *M. tuberculosis*, and *Mycobacterium bovis* have been isolated from leprosy patients and their contacts (R. R. P. deVries et al., Lepr. Rev., in press; A. H. J. Kolk et al., Lepr. Rev., in press) and from tuberculosis patients and purified-protein-derivative-responsive individuals (2; S. Mustafa and T. Godal, Lepr. Rev., in press). These antibody-reactive and T-cell-stimulating epitopes of the 65-kDa protein may prove relevant to efforts to develop rapid diagnostic procedures, improved skin test reagents, and vaccines. Several laboratories have produced monoclonal antibodies (MAbs) to the 65-kDa molecule of *M. leprae* (3, 4, 7; World Health Organization Workshop, Letter, Infect. Immun. **48**:603–605, 1985). The gene encoding the protein has been cloned in λgt11 bacteriophage (13), and recently the total sequence of the protein has been determined (9). Lysogens of *Escherichia coli* containing this gene express the protein in antigenic form, allowing isolation of the protein in quantities much greater than those previously available from the nonculturable leprosy bacillus. These developments suggested the possibility of defining at a molecular level the structural basis of some of the epitopes of the 65-kDa protein molecule that react with available MAbs, as well as of epitopes capable of stimulating T cells. Such studies may facilitate an understanding of differences in the primary, secondary, or tertiary structure of the two types of epitopes and may identify regions of the molecule recognized by hosts infected with the leprosy bacillus.

This study examined the number of different epitopes recognized by 23 MAbs known to recognize the 65-kDa protein of the leprosy bacillus and the mycobacterial species specificity of the MAbs shown to recognize different epitopes.

MATERIALS AND METHODS

MAbs. All of the MAbs studied recognized the 65-kDa protein of *M. leprae*, as identified by Western blotting (6) or gel immunoradioassay (10). The MAbs were obtained from the following sources: IIC8, IIIC8, IIIE9, IVD8, IIH9, and IVD2, T. M. Buchanan (3, 4; World Health Organization Workshop, Letter); Y1.2, E3.14, T2.3, and C1.3, V. Mehra and B. R. Bloom (World Health Organization Workshop, Letter); SL12, SL14, SL22, SL26, and SL28, W. J. Britton; ML-30, J. Ivanyi (7); E423, T. Gillis; F47-10, F67-2, F67-13, and F67-18, A. J. Kolk; and MOC2 and MOC6, O. Closs.

Each MAb was purified by staphylococcal protein A or sizing chromatography, and the purified antibody was radiolabeled with ^{125}I by a chloramine T (5) or Bolton-Hunter (1) procedure. An antigenic preparation of sonicated *M. leprae* (12) was coated to Immulon I Removawells (Dynatech Laboratories, Inc.). The binding of each ^{125}I-labeled antibody to the antigen-coated wells was evaluated for inhibition by a 1:100 dilution of ascitic fluid of each MAb. MAbs with different inhibition patterns from those of all of the other MAbs were presumed to recognize different epitopes, and

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when more than one antibody recognized a given epitope, a single representative antibody was chosen for further study. In some instances in which the radiolabeled antibody reacted poorly with antigen bound to polystyrene plates, the antibody was reacted with the same antigen bound to nitrocellulose. Inhibition of this binding by unlabeled MAb at a 1:100 dilution was evaluated by autoradiography.

Evaluation of mycobacterial species specificity. Bacterial sonic extract antigens of each of 23 mycobacterial species (see Table 2) were prepared as previously described (12) and adjusted to a protein concentration of 1 mg/ml. One microgram of protein in a 5-μl volume was spotted onto nitrocellulose in a template of the 23 species plus a buffer negative control per paper strip and allowed to air dry. The nitrocellulose template strips were blocked at 37°C for 1 h with 5% nonfat milk in phosphate-buffered saline (PBS) and then incubated with a 1:500 to 1:2,000 dilution of ascitic fluid containing the appropriate MAb in PBS containing 1% nonfat milk at room temperature for 30 min. The template strips were drained of antibody and washed for 5 min in PBS with gentle shaking at room temperature, followed by washing for 15 min in 0.5 M NaCl in phosphate buffer containing 0.05% Tween 20 and a second 5-min wash with PBS. The goat anti-mouse immunoglobulin-peroxidase conjugate (Cooper Biomedical, Inc.) was diluted 1:2,000 in 1% nonfat milk–PBS and incubated with the template strips at room temperature for 30 min. The strips were then drained and washed once at room temperature with PBS for 5 min, followed by four washes of 10 min each in 0.5 M NaCl in PBS with 0.05% Tween 20 and a final wash in PBS for 5 min. The washed template strips were reacted for 30 to 45 min with a 0.015% H_2O_2 color development solution (4-chloro-1-naphthol; Bio-Rad Laboratories) until the dots developed a purple color. The templates were then washed with distilled water several times, air dried, and stored in the dark, or in some instances they were stored in distilled water before air drying and storage. The dot reactions were graded as negative or positive in an intensity range of 1 (low) to 4 (high).

Preparation of bacterial lysates containing the *M. leprae* 65-kDa protein or some of its epitopes. λgt11 recombinant lysogens were generated in *E. coli* Y1089. Strain Y1089 was grown in LB broth (1% tryptone, 0.5% yeast extract [Difco Laboratories], 1% NaCl [pH 7.5]) supplemented with 0.2% maltose at 32°C with aeration overnight to saturation. The bacteria were infected with the λgt11 phage at a multiplicity of five phage per bacterium by incubating for 20 min at 32°C in LB broth (pH 7.4) supplemented with 10 mM $MgCl_2$. The phage contained cloned recombinant DNA coding for expression of one or more epitopes on the 65-kDa protein of *M. leprae* (13). The infected cells were plated in a soft agar overlay (LB broth with 0.6% agar) onto an LB plate (LB broth with 1.5% agar) at a density of approximately 200 per plate and incubated at 32°C. Individual colonies were picked with sterile toothpicks, and one-half of each colony was placed onto plates to be incubated at 45°C and then onto plates to be incubated at 32°C. Colony progeny which grew at 32°C but not at 45°C were assumed to be lysogens. Single colonies of lysogens were grown overnight in LB broth at 32°C, and this growth was used to seed additional LB broth at a ratio of 0.5 ml per 50 ml of broth. A subculture of the overnight growth was placed onto LB plates incubated at 45 and 32°C to confirm the temperature sensitivity of the lysogen. During log-phase growth at approximately 100 Klett units, the temperature was quickly raised to 42 to 45°C, and the culture was incubated for 20 min with continued shaking to permit phage multiplication. The culture was then supplemented with isopropyl-β-D-thiogalactopyranoside (2 to 10 mM) if it was required for synthesis of the protein of interest and incubated at 38°C for 1 h. The cells were harvested by centrifugation at 24 to 37°C for 5 min at 5,000 × *g*. The cell pellet was quickly suspended in 10 mM Tris buffer (pH 8)–2 mM phenylmethylsulfonyl fluoride–4 mM EDTA at 1/12.5 of the original culture volume, followed by freezing in liquid nitrogen, thawing, and centrifugation to obtain the supernatant containing the 65-kDa protein or its epitopes of interest.

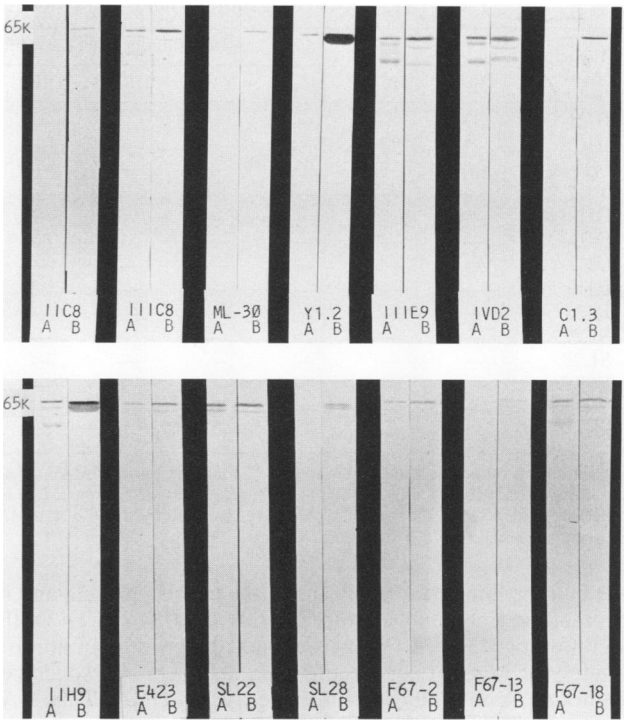

FIG. 1. Western blot results with MAbs. Lanes B, Crude bacterial lysates of *E. coli* Y1089 lysogenized with λgt11 rDNA clone Y3178; lanes A, latex spheres containing the 65-kDa protein partially purified from the bacterial lysates.

Lysogens of the λgt11 phage rDNA clone Y3178 which expressed a protein of approximately 65,500 daltons (and no detectable β-galactosidase) were used to produce lysates for interaction with MAbs (Fig. 1). Each of the MAbs presumed to recognize different epitopes was reacted in a Western blot (6) against crude lysates and against latex spheres containing covalently coupled MAb IIC8 and the 65-kDa protein and its antigenic fragments recognized by MAb IIC8 (Fig. 1). The spheres contained the 65-kDa protein approximately 1,000-fold purified compared with that in the crude lysates. In addition, each of the 14 different MAbs was reacted with a lysate of *E. coli* Y1089 lysogenized with λgt11 containing rDNA from an irrelevant yeast protein gene, as a control for recognition of *E. coli* products by the MAbs.

RESULTS

Twenty-three MAbs were tested by cross-competition experiments, and 14 different MAbs were identified. The inhibition results obtained for these 14 antibodies are shown in Table 1. All 14 MAbs recognized a band of approximately 65 kDa in the crude lysates from lysogens of clone Y3178, and 11 of the 14 also recognized one or more bands of ≤65

TABLE 1. Cross-competition studies of MAbs that recognize 14 epitopes on the 65-kDa protein of *M. leprae*

^{125}I-labeled MAb	% Inhibition produced by diluted (1:100) ascitic fluid containing the indicated MAb[a]:													
	IIC8	IIIC8	ML-30	Y1.2	IIIE9	IVD2	C1.3	IIH9	E423	SL22	SL28	F67-2	F67-13	F67-18
IIC8	89	—	—	—	—	—	—	—	—	—	—	—	—	—
IIIC8	—	80	—	—	—	—	—	—	—	—	—	—	—	—
ML-30	—	—	93	—	—	—	—	—	—	—	—	—	—	—
Y1.2	—	—	—	83	—	—	—	—	—	—	—	—	—	—
IIIE9	—	—	—	—	90	67	—	—	—	—	—	—	—	—
IVD2	—	—	—	—	51	80	—	—	—	—	—	—	—	—
C1.3	—	—	—	—	—	—	74	—	24	—	—	—	—	—
IIH9	—	—	—	—	—	22	—	83	—	—	—	—	—	—
E423	—	—	—	—	—	—	—	—	100	—	—	—	—	—
SL22	—	—	20	—	—	—	—	—	—	90	—	—	—	—
SL28	—	—	—	—	—	—	—	—	—	—	100	—	—	—
F67-2	—	—	—	—	—	—	—	—	—	—	—	100	—	—
F67-13	—	—	—	—	—	—	—	—	—	—	—	—	100	—
F67-18	—	—	—	—	—	—	—	—	—	—	—	—	—	100

[a] Inhibition by ascitic fluid of binding of ^{125}I-labeled purified MAb. MAbs IVD8, SL12, SL26, and MOC2 gave cross-competition results identical to those obtained with IIIE9, and all appeared to recognize the same epitope. MAb E3.14 produced a reaction pattern similar to that of IIH9, and MOC6 was also inhibited by IIH9. MAbs SL14, T2.3, and F47-10 were inhibited by many of the MAbs. —, Inhibited less than 20%.

kDa on the latex beads containing the 65-kDa protein and its proteolytic fragments captured by MAb IIC8 (Fig. 1). Of the 14 different MAbs, 1 (Y1.2) recognized a product of approximately 65 kDa found in the *E. coli* Y1089 host lysogenized with λgt11 containing the unrelated yeast DNA. This MAb reacted with a 17-amino-acid synthetic peptide prepared by Anderson et al. (D. C. Anderson, R. A. Young, and T. M. Buchanan, Lepr. Rev., in press) from the *M. leprae* 65-kDa protein sequence (data not shown). This suggests that the epitope recognized by this MAb is indeed located on the 65-kDa protein of *M. leprae* but that this epitope may also be found on one or more *E. coli* proteins with a size of approximately 65 kDa. The dot blot immunoassay studies of species specificity for the 14 different MAbs are shown in Table 2. The group of MAbs represented by IIIE9 (IIIE9, IVD8, SL12, SL26, and MOC2) recognized an epitope found only on the *M. leprae* 65-kDa protein molecule. MAb IVD2 recognized a portion of the same epitope recognized by IIIE9, as shown by cross-competition studies (Table 1), but reacted with 13 other mycobacterial species in addition to *M. leprae*. The other MAbs recognized epitopes found on as few as 8 (F67-13) to as many as all (IIC8) of the 23 species of mycobacteria tested. The sequences of the epitopes recognized by MAbs IIC8 and IIIC8 have been confirmed by

TABLE 2. Species specificity in dot blot immunoassays of MAbs which recognize 14 separate epitopes on the 65-kDa protein of *M. leprae*

Mycobacterial species	Reactivity of MAb[a]:													
	IIC8	IIIC8	ML-30	Y1.2	IIIE9	IVD2	C1.3	IIH9	E423	SL22	SL28	F67-2	F67-13	F67-18
M. bovis BCG	4	–	1	3	–	–	–	4	4	4	3	4	1	4
M. bovis	3	–	–	1	–	2	3	1	3	3	1	2	–	3
M. chelonei	4	3	1	2	–	3	–	2	1	3	2	4	1	4
M. diernhoferi	2	1	–	1	–	–	–	1	1	1	2	–	–	1
M. duvalii	3	2	–	2	–	2	1	1	2	2	2	1	–	2
M. flavescens	4	4	–	3	–	3	3	3	3	3	2	3	–	4
M. fortuitum	2	1	–	1	–	–	–	1	1	1	2	–	–	1
M. gastri	4	4	1	4	–	3	–	1	4	4	2	2	–	4
M. gordonae	3	3	–	2	–	3	3	3	3	3	2	3	–	3
M. intracellulare	3	1	–	2	–	–	–	1	2	2	2	1	–	2
M. kansasii	3	–	–	2	–	–	2	2	3	3	3	2	–	2
M. leprae	4	4	4	4	4	3	4	4	4	4	4	4	2	4
M. marinum	2	1	–	–	–	–	–	1	1	1	2	1	1	1
M. nonchromogenicum	2	1	–	–	–	–	–	–	1	1	2	1	–	1
M. peregrinum	3	2	–	1	–	1	–	–	3	1	2	1	–	2
M. phlei	2	1	–	1	–	1	–	1	–	2	1	2	–	3
M. scrofulaceum	4	4	1	3	–	3	3	3	3	4	1	4	1	4
M. smegmatis	3	2	–	1	–	1	1	1	2	2	–	3	–	3
M. terrae	3	3	–	2	–	2	1	2	2	3	4	2	–	2
M. thamnopheos	4	4	4	4	–	2	3	4	–	4	4	4	1	4
M. triviale	2	1	1	–	–	–	–	–	–	1	2	1	–	2
M. tuberculosis H37Rv	4	1	2	2	–	–	1	3	4	3	4	4	1	4
M. ulcerans	4	4	1	3	–	2	2	3	3	3	4	4	1	3
M. vaccae	2	2	–	1	–	–	–	1	1	2	1	1	–	2

[a] Reactions were graded as negative (–) or from 1 to 4 in order of increasing intensity of the dot reaction obtained after reacting the MAb, immunoenzyme conjugate, and substrate with the nitrocellulose spots, each containing 1 μg of the sonic extract antigen of a given mycobacterial species.

solid-phase peptide synthesis (Anderson et al., in press) beginning with sequence information provided by Mehra et al. (9). The peptides bearing these epitopes produced ≥40% inhibition of their immunoassays in 1-ng (IIC8) and 10-ng (IIIC8) amounts.

DISCUSSION

These studies provide the first evidence for 14 distinct epitopes recognized by a group of 23 MAbs directed at the 65-kDa molecule of *M. leprae*. In addition, this is the first report of data indicating the species distribution of each of the 14 different epitopes among 23 species of mycobacteria. These antibodies should be useful probes for elucidation of the structure of each epitope and for identification of the location of the epitopes within the full linear structure of the amino acid sequence of the total molecule. It is likely that the structural identification of species-specific antibody-reactive epitopes will aid efforts to develop rapid diagnostic tests. These MAbs may also delimit regions of the 65-kDa molecule that stimulate T cells, epitopes that will likely prove of interest for the development of vaccines and improved skin test reagents.

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LITERATURE CITED

1. **Bolton, A. E., and W. M. Hunter.** 1973. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Application to the radioimmunoassay. Biochem. J. **133**:529–539.
2. **Emmrich, F., J. Thole, J. von Embden, and S. H. E. Kaufman.** 1986. A recombinant 64 kilodalton protein of *Mycobacterium bovis* bacillus Calmette-Guerin specifically stimulates human t4 clones reactive to mycobacterial antigens. J. Exp. Med. **163**: 1024–1029.
3. **Gillis, T. P., and T. M. Buchanan.** 1982. Production and partial characterization of monoclonal antibodies to *Mycobacterium leprae*. Infect. Immun. **37**:172–178.
4. **Gillis, T. P., R. A. Miller, D. B. Young, S. R. Khanolkar, and T. M. Buchanan.** 1985. Immunochemical characterization of a protein associated with *Mycobacterium leprae* cell wall. Infect. Immun. **49**:371–377.
5. **Greenwood, F. C., W. M. Hunter, and J. S. Glover.** 1963. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. **89**:114–123.
6. **Hook, E. W., D. A. Olsen, and T. M. Buchanan.** 1984. Analysis of the antigen specificity of the human serum immunoglobulin G immune response to complicated gonococcal infection. Infect. Immun. **43**:706–709.
7. **Ivanyi, J., S. Sinha, R. Aston, D. Cussel, M. Deen, and U. Sengupta.** 1983. Definition of species specific and cross-reactive antigen determinants of *Mycobacterium leprae* using monoclonal antibodies. Clin. Exp. Immunol. **52**:528–536.
8. **Levis, W. R., H. C. Meeker, G. B. Schuller-Levis, T. P. Gillis, L. J. Marino, Jr., and J. Zabriskie.** 1986. Serodiagnosis of leprosy: relationships between antibodies to *Mycobacterium leprae* phenolic glycolipid and protein antigens. J. Clin. Microbiol. **24**:917–921.
9. **Mehra, V., D. Sweetser, and R. A. Young.** 1986. Efficient mapping of protein antigenic determinants. Proc. Natl. Acad. Sci. USA **83**:7013–7017.
10. **Poolman, J. T., and T. M. Buchanan.** 1984. Monoclonal antibody activity against native and denatured forms of gonococcal outer membrane proteins as detected within ultrathin, longitudinal slices of polyacrylamide gels. J. Immunol. Methods **75**:265–274.
11. **Thole, J. E. R., H. G. Dauwerse, P. K. Das, D. G. Groothuis, L. M. Schouls, and J. D. A. van Embden.** 1985. Cloning of *Mycobacterium bovis* BCG DNA and expression of antigens in *Escherichia coli*. Infect. Immun. **50**:800–806.
12. **Young, D. B., M. J. Fohn, S. R. Khanolkar, and T. M. Buchanan.** 1985. Monoclonal antibodies to a 28,000-molecular-wt protein antigen of *Mycobacterium leprae*. Clin. Exp. Immunol. **60**:546–552.
13. **Young, R. A., V. Mehra, D. Sweetser, T. M. Buchanan, J. Clark-Curtiss, R. W. Davis, and B. R. Bloom.** 1985. Genes for the major protein antigens of the leprosy parasite *Mycobacterium leprae*. Nature (London) **316**:450–452.