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Results of the Third Immunology of Leprosy/Immunology of Tuberculosis Antimycobacterial Monoclonal Antibody Workshop

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An international workshop was sponsored by the World Health Organization to screen new antimycobacterial monoclonal antibodies and to identify antibodies which could be recommended as standard reagents giving consistent results under differing assay conditions. Fifty-eight antibodies were submitted to the workshop by eight independent laboratories. Nineteen of the antibodies recognized antigens distinct from those identified in earlier workshops, defining at least 10 new protein antigens. Monoclonal antibodies characterized in the workshop provide a set of convenient reagents for further characterization of mycobacterial antigens.

In 1984 and 1985, two international workshops sponsored by the World Health Organization Scientific Working Groups on the Immunology of Leprosy (IMMLEP) and Immunology of Tuberculosis (IMMTUB) evaluated the available murine monoclonal antibodies directed to mycobacterial antigens (3, 4). After 55 antibodies were screened, five distinct protein antigens (subunit molecular masses of 65, 36, 35, 18, and 12 kDa), were identified in extracts from *Mycobacterium leprae* and seven antigens (71, 65, 38, 23, 19, 14, and 12 kDa) were identified in extracts from *Mycobacterium tuberculosis*. The monoclonal antibodies were then used to screen recombinant DNA expression libraries, leading to the cloning of genes encoding the appropriate antigens and the opening of a new era of molecular research in mycobacterial disease (15, 16). Genes for almost all of these antigens have been characterized by nucleotide sequence analysis, and a substantial amount of information regarding the antigenicity and biochemistry of this set of mycobacterial proteins has been obtained (1, 13–16).

A striking feature of the workshops was the finding that although highly complex protein mixtures were used for immunization, there was extensive overlap in specificity of antibodies generated independently in different laboratories, thus prompting the idea that these antibodies defined the immunodominant antigens of the leprosy and tubercle bacilli. However, subsequent studies have demonstrated that while these initial proteins are certainly prominent targets of the immune response, many other mycobacterial antigens can be detected by using T lymphocytes, human sera, and additional antibodies (10, 13). In particular, it was reported that generation of monoclonal antibodies in different inbred mouse strains (BALB.B10 or CBA/J instead of BALB/c) resulted in recognition of a novel repertoire of specificities (8). In this report, we describe the results of a third workshop that was held in order to evaluate new monoclonal antibodies generated since the 1985 workshop.

Fifty-eight antimycobacterial monoclonal antibodies were submitted to the workshop by eight independent laboratories. These were coded and sent to two reference laboratories for screening by Western blot (immunoblot) assays and enzyme-linked immunosorbent assays (ELISAs). The results were collated in a third laboratory, and inconclusive or contradictory findings were repeated. Mycobacterial strains (see Table 1, footnote *b*) were grown on Sauton's medium, and protein samples for immunoassay were prepared by two procedures: (i) bacteria were killed by heating at 75°C for 15 min, and extracts were prepared by ultrasonication in distilled water, and (ii) proteins were precipitated from culture media by addition of ammonium sulfate (80% saturation), resuspended in phosphate-buffered saline (pH 7.4) (PBS), and filtered through 0.2-μm-pore-size membranes. Protein extract from *M. leprae* (batch CD146) was supplied by R. J. W. Rees through the IMMLEP *M. leprae* Bank (National Institute for Medical Research, London, United Kingdom). For ELISAs, polystyrene microtiter plates with high binding capacity (Greiner, Nürtingen, Germany) were coated with 1 μg of sonicated antigen per well in PBS and incubated for 16 h at 4°C. The plates were washed with PBS containing 0.05% Tween 20 (PBST) and incubated for 1 h at 37°C with monoclonal antibodies diluted in 1% bovine serum albumin in PBST (culture supernatants were used at a dilution of 1:5, and ascitic fluids were used at 1:1,000). Antibody binding was detected by using peroxidase-conjugated sheep anti-mouse immunoglobulin G (IgG) (heavy plus light chains) (Institut Pasteur, Paris, France) diluted 1:1,000

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TABLE 1. Characterization of monoclonal antibodies to mycobacteria

Group	Molecular mass (kDa)[a]	Antibody			Reactivity[b]			Comment[c]
		Code[d]	Designation[e]	Isotype	*M. tuberculosis*	*M. leprae*	Other mycobacteria	
I	85	11	CBA4	IgG1	+	−	+ (B)	
		33	HBT8	IgG1	+	−	−	
II	70	3	HAT1	IgM	+	−	+ (B)	Antigen previously identified as DnaK heat shock protein[f]
		5	HAT3	IgG1	+	−	+ (B)	
III	65	9	HAT5	ND[g]	+	−	+ (B)	Antigen previously identified as GroEL heat shock protein[h]
		10	CBA1	IgG1	+	+	+ (B)	
		43	CW1F2E8	IgG1	+	+	+ (B)	
		55	H2.16	IgG1	+	−	+ (B)	
IV	58	22	HBT3	IgG2a	+	−	−	
V	45	12	21-6D7	ND	+	−	+ (B)	
		32	3805F9	IgG3	+	−	+ (B)	
		34	HBT10	IgG1	+	−	+ (L)	
VI	38	4	HAT2	IgG1	+	−	+ (L)	Antigen previously identified as PstS phosphate transport protein[i]
		15	F67-19	IgG1	+	−	−	
		41	HBT12	IgG1	+	−	−	
		42	C38.D1	IgG1	+	−	−	
VII	35	8	MMP-I3C3	IgG1	−	+	−	Antigen previously identified (MMP-I)[j]
		35	F86-2	IgG1	+	−	+ (B)	
		54	H61.3	IgG2b	+	−	−	
VIII	33	20	F67-1	IgG1	+	+	+ (B)	
		25	F126-5	IgG1	+	+	+ (B)	
		28	HBT7	IgG2a	+	+	−	
IX	28	26	MCP-III1B2	IgG1	−	+	−	
		53	H60.15	IgG1	+	−	+ (L)	
X	24	44	L24.b4	IgG1	+	−	+ (L)	
		45	C24.b1	IgG1	+	−	−	
XI	23	30	F116-5	IgG1	+	+	+ (B)	Antigen previously identified (SOD)[k]
XII	22	14	MMP-II1G4	IgG1	−	+	−	
XIII	20	39	HBT11	IgG1	+	−	−	
XIV	19	6	21-2H3	ND	+	−	+ (L)	Antigen previously identified[l]
		18	HBT2	IgG1	+	−	+ (L)	
XV	10	51	CS-01	IgG3	+	+	+ (B)	

[a] Molecular masses were estimated by SDS-polyacrylamide gel electrophoresis.

[b] Reactivity was tested on extracts from 17 species of mycobacteria by ELISA and on 9 species of mycobacteria (*M. tuberculosis* H37Rv, *M. leprae*, *M. bovis* BCG, *M. avium*, *M. intracellulare*, *M. fortuitum*, *M. scrofulaceum*, *M. marinum*, and *M. kansasii*) by immunoblotting techniques. The designation B or L indicates broad or limited cross-reactivity, respectively.

[c] Monoclonal antibodies were screened for reactivity with previously identified antigens by using recombinant proteins provided by Raju Lathigra and Ying Zhang (MRC Tuberculosis and Related Infections Unit, London, United Kingdom). Monoclonal antibodies recognizing the same antigen in previous workshops are identified in corresponding footnotes.

[d] Antibodies submitted to the workshop were assigned a random code number to conceal their origins during the testing procedures.

[e] MMP-I3C3, MCP-III1B2, MMP-II1G4, CW1F2E8, and CS-01 (6) were contributed by Patrick J. Brennan and Becky Rivoire, Colorado State University, Fort Collins; 21-6D7, 21-2H3 and 3805F9, were contributed by Sotiros Chaparas, National Institutes of Health, Bethesda, Md.; H2.16, H61.3 and H60.15 (2) were contributed by Guido Damiani, University of Genoa, Genoa, Italy; F67-19 (11, 12) and F86-2, F67-1, F126-5, and F116-5 (7, 11) were contributed by Arend Kolk, Royal Tropical Institute, Amsterdam, The Netherlands; CBA4, HBT8, HAT1, HAT3, HAT5, CBA1, HBT3, HBT10, HAT2, HBT12, C38.D1, HBT7, L24.b4, C24.b1, HBT11, and HBT2 (1, 8) were contributed by Åse Andersen and Jørgen Bennedsen, Statens Seruminstitut, Copenhagen, Denmark.

[f] Antigen also recognized by antibody 51A (4, 14, 15).

[g] ND, not determined.

[h] Antigen also recognized by antibodies IIH9, IIIE9, IVD8, IIC8, Y1.2, ML30, TB78, and D5H (3, 4, 14–16).

[i] Antigen also recognized by antibodies TB71, TB72, and HYT28 (1, 4).

[j] Antigen also recognized by antibody ML04 (3, 6).

[k] SOD, superoxide dismutase; antigen also recognized by antibody D2D (4, 17).

[l] Antigen also recognized by antibodies F29-47, HYT6, 66, and TB23 (4, 15).

in PBST with bovine serum albumin and a colorimetric assay with tetramethylbenzidine substrate. For Western blot analysis, proteins were separated by electrophoresis in 12.5% (wt/vol) polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) and transferred onto nitrocellulose sheets (Schleicher & Schuell BA85) in a semidry blotting apparatus according to the instructions provided by the manufacturer (Ancos, Olstykke, Denmark). The blots were incubated with monoclonal antibodies diluted in PBS containing 0.5 M NaCl and 0.05% Tween 20 (culture supernatants were diluted 1:25, and ascitic fluids were diluted 1:500). Antibody binding was detected by using peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark) at a dilution of 1:1,000 and a colorimetric assay. In addition to this standard protocol adopted in the primary reference laboratory, antibodies were screened by similar Western blot assays in two other laboratories. Previous experience has indicated that antibodies which give

consistent results independent of minor assay variations provide the most useful reagents for coordination of research in different laboratories (3, 4).

Five of the antibodies were directed towards nonprotein antigens (lipoarabinomannan in the case of several mycobacterial species [5], phenolic glycolipid in the case of *M. leprae*, and glycopeptidolipids in the case of *Mycobacterium avium* [9]) and were not further analyzed in the present study. Of the remaining 53 antibodies, 32 were found to give consistent results when different techniques were used in different laboratories, and these were separated into 15 groups on the basis of the subunit molecular weight of the antigen determined by Western blotting. The results are summarized in Table 1.

Several of the antibodies recognized proteins already identified in previous workshops. Experiments using recombinant antigens demonstrated that the group II and group III antibodies recognized the mycobacterial 70-kDa (DnaK) and 65-kDa (GroEL) heat shock proteins (14), while group VI antibodies were directed to the PstS protein of *M. tuberculosis* (1). Interestingly, antibody HAT2 differed from the other monoclonal antibodies directed to this protein in showing reactivity with *Mycobacterium intracellulare* in addition to members of the *M. tuberculosis* complex. The single member of group XI recognized the 23-kDa antigen identified as the superoxide dismutase enzyme of *M. tuberculosis* (17), and the group XIV antibodies were directed to a previously described *M. tuberculosis* 19-kDa antigen (4, 15). One member of group VII (MMP-I3C3) was specific for *M. leprae* and is directed to the 35-kDa antigen reported previously (3).

The remaining 19 antibodies recognized antigens distinct from those identified in earlier workshops. From the Western blot results, it is clear that these define at least 10 new protein antigens. Further experiments involving competitive binding assays or tests with individual recombinant antigens will be required in order to determine whether antibodies binding to proteins of similar molecular weight are, in fact, directed towards the same or distinct antigens. Several of the epitopes recognized were found to be species specific and may be of use in development of improved diagnostic assays. It will also be important to investigate the potential role of these additional defined antigens in protective and pathological aspects of T-cell-mediated immune responses to mycobacteria.

Subject to available reserves, samples of antimycobacterial monoclonal antibodies will be made available by IMM LEP and IMMTUB to qualified investigators upon receipt of a short (one-page) description summarizing the intended experiments and addressed to IMMLEP/IMMTUB Secretary, World Health Organization, 1211 Geneva 27, Switzerland.

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