Production and characterization of monoclonal antibodies to virulent *Mycobacterium bovis*

by

Gary Dean Kuchinka

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GENERAL INTRODUCTION

Bovine tuberculosis is caused by Mycobacterium bovis which is a member of the M. tuberculosis complex that includes M. tuberculosis, M. microti, and M. africanum. Disease usually includes a progressive granulomatous lesion of the thoracic cavity with possible involvement of other organs of the body often resulting in death. Following inhalation, the organism is ingested by macrophages which fail to destroy the organism. Death of the macrophage stimulates T-lymphocytes involved in cell mediated immunity (CMI) followed by localization of T-cells and granulocytes, tissue destruction, and mineralization and caseous nodule formation. Mycobacterium bovis infection in cattle has been almost eliminated due to eradication programs but it continues to be a problem in domestic animals in developing countries and in captive exotic animals. Bovine tuberculosis is a serious problem in animals in zoological parks and primate colonies where M. bovis has been detected with increased frequency from exotic ruminants and from non-human primates. Due to the high monetary and aesthetic value of these animals eradication of infected animals is not an acceptable approach. Alternative control possibilities could include early detection of disease and subsequent treatment.

Currently, effective vaccines do not exist and the diagnostic efficacy of tuberculin skin tests has been limited due to lack of highly specific diagnostic antigens. Antigens obtained by classical

biochemical methods are complex and contain non-specific determinants. Since CMI is important in controlling bovine tuberculosis, antigens involved in T-cell proliferation could be important in diagnosis where delayed type hypersensitivity (DTH) develops. Exposure of T-cells to antigens specific for stimulation could prime immune responses for future exposure to M. bovis and provide additional host resistance to disease. Methods to survey antigens from M. bovis would facilitate identification of specific serodiagnostic determinants and/or possible antigens that stimulate T-cell response.

Hybridoma technology and monoclonal antibody (McAb) production is an approach that allows identification of antigenic determinants of M. bovis. Monoclonal antibodies with binding activity to determinants specific for M. bovis, and that compete with and inhibit binding of serum antibody, could be used in serodiagnostic enzyme-linked immunosorbent assays (ELISA). In addition, corresponding antigen could be isolated through McAb linked affinity chromatography and used in combination with the McAb in ELISA or alone as a skin test antigen. Immunologic reagents with improved specificity could minimize problems associated with cross-reactivity in assays used to evaluate immune responses. Monoclonal antibodies could also be used as tools to detect gene products of an expression library that may be associated with humoral and cell mediated responses or to detect mycobacterial components in animal tissue. Alternatively, McAb that detect cross-reactive determinants could be used to identify and possibly remove

cross reactive components in antigenic preparations. Monoclonal antibodies also have taxonomic and epidemiological applications involving strain identification of field isolates.

LITERATURE REVIEW

Monoclonal antibodies (McAb) have been produced to investigate the antigenic composition of mycobacteria. Available information indicates that organisms used for immunization and hybridoma production have included *M. tuberculosis*,[1,7,11,12,17,25,32,33] *M. leprae*,[3,14,16 19,24,25,44] *M. lepraemurium*,[2] *M. bovis* BCG,[3,25,28,29,34] a field isolate of *M. bovis*,[29] and *M. avium-M. intracellulare-M. scrofulaceum* (MAIS) complex serovars 4, 8, and 9.[30] The initial objective of producing McAb to *M. tuberculosis* was to differentiate between strains of *M. tuberculosis* and of *M. bovis*.[7] Another objective was to use McAb to isolate antigens of *M. tuberculosis* by immunosorbent affinity chromatography and to study antigen specificity for use in skin-tests and/or serodiagnosis of tuberculosis.[12,32] Subsequent objectives were to provide a tool for identification of *M. tuberculosis* antigen in serodiagnosis[1,25,33] or immunochemical characterization of *M. tuberculosis* antigen,[1,33] to identify shared antigens among mycobacteria,[11] and to detect and isolate peptide antigens expressed in DNA cloning experiments.[17] Similarly, the objectives of producing McAb to *M. bovis* BCG have been to develop a tool for identification of *M. bovis* BCG antigen in serodiagnosis,[25] to isolate antigen by immunosorbent affinity chromatography for partial characterization,[28] to detect antigen in sonicated extracts of *M. bovis* that do not occur in related species,[29] to detect cross-reactive determinants,[3] or to

detect cell wall and B and C glycolipids.[34] In general, McAb have been used for detection and physical isolation of antigenic components.

Various mycobacterial species and corresponding antigen preparations have been utilized to establish hybridoma cell lines that produce murine McAb (Table 1). Mycobacterium bovis BCG antigen preparations contained dry weight sonicated whole cells (SWC),[25] sonicated heat killed whole cells (SHKWC),[28] and sonicate supernatant (SON 1S) obtained by sonicating cells that were grown on Lowenstein-Jensen medium[26] plus sodium pyruvate followed by centrifugation at 1000 x g for 20 minutes at 4°C.[29,38] For one immunization schedule, SON 1S from M. bovis (field isolate 117/79) was utilized.[29,38] Similarly, lyophilized whole cells were resuspended, ultrasonicated, and centrifuged at 20,000 x g for 15 minutes to prepare soluble sonicate antigen (SON 20S).[3] Also, cell walls have been extracted from M. bovis BCG for use in immunization.[34]

Mycobacterium tuberculosis H37Rv has been used to prepare pressate antigen (PRES 100S) obtained by passing harvested organisms through a bacterial press followed by centrifugation at 100,000 x g.[7] Other M. tuberculosis antigen preparations included intact whole cells (WC),[25] dry weight SWC,[25] whole cells autoclaved at 100°C (HKWC) and dried at 37°C,[17] supernatant obtained after sonication and centrifugation at 60,000 x g (SON 60S),[5,6] bacterial press extract (BPE) obtained after rupturing cells in a French press at 14,000 lb/in^2 with removal of insoluble material by centrifugation at 40,000 x g

TABLE 1. List of monoclonal antibodies produced for various mycobacterial species including culture media and antigen preparations used in immunization

Organism	Medium[a]	Immunization Schedule			Monoclonal antibody	Reference
		Antigen[b] (amount)	Day	Route[c,d]		
M. bovis BCG	Sauton	SWC (100 ug)	0	i.p.	F24-2	25
		"	30	i.p.		
		"	341	i.p.*		
		SWC (500 ug)	362	i.v.		
M. bovis BCG (Glaxo)		SHKWC (1 mg)	0	i.p.@	SA-12	28
		"	21	i.p.@		
		"	3 d.p.f.	i.p.*		
M. bovis BCG (Glaxo)	Lowenstein-Jensen (4-6 wks.)	SON 1S (50 ug)	0	i.p.*	MB2, MB3	29,38
		"	21	i.p.		
M. bovis 117/79[#]	Lowenstein-Jensen (4-6 wks.)	SON 1S (50 ug)	0	i.p.*	MB5, MB7, MB11 MB13, MB17	29,38
		"	21	i.p.		
M. bovis BCG		SON 20S (50 ug) + ?	0	i.p.@	B6	3
		"	7	i.p.@		
		"	14	i.p.		
M. bovis BCG		cell wall (?)	0		TB41, TB44	34
			3 d.p.f.	i.v.*		

M. tuberculosis H37Rv	Sauton	PRES 100S	(100 ug)	0	i.p.	TB68, TB71, TB72	7
				15	i.p.*	TB73, TB77, TB78	
M. tuberculosis S1#	Sauton	HKWC (10^8)		210	i.v.		

[a]Medium used for growing mycobacteria (Culture period indicated in parenthesis); *M. leprae* and *M. lepraemurium* were grown *in vivo*.

[b]Abbreviations for antigen preparations are: SWC, sonicated whole cells; SHKWC, sonicated heat killed whole cells; SON 1S, sonicate supernatant after centrifugation at 1000 x g (or 20,000, 72,000, 100,000 x g); PRES 100S, pressate supernatant obtained after centrifugation at 100,000 x g; WC, whole cells; BPE, bacterial press extract; CCF-RaBCG ppt., precipitate corresponding to rabbit anti-BCG antibody obtained from concentrated culture filtrate; CCF-HYTad, Concentrated culture filtrate HYT antigen depleted; CFS, culture filtrate solids; SLWC, sonicated lyophilized whole cells; 10KP and 30KS, pellet obtained after disrupting cells and centrifugation at 10,000 x g and supernatant after re-centrifugation at 30,000 x g; P, pellet; RSON 20S, Supernatant obtained after re-sonicating a pellet and centrifugation at 20,000 x g.

[c]Abbreviations: i.p., intraperitoneal; i.v., intravenous, d.p.f., days prior to fusion; wks., weeks; l., liquid; s.q., subcutaneous; f.p., foot pad; i.d., intradermal.

[d]Symbols: *=boost inoculation; @=adjuvant used; #=field isolate; $=absorbed with HYT 27 and HYT 28.

TABLE 1 (Continued)

Organism	Medium[a]	Immunization Schedule			Monoclonal antibody	Reference
		Antigen[b] (amount)	Day	Route[c,d]		
M. tuberculosis H37Rv	Sauton	WC ($2x10^7$)	0	i.p.	F29-17, F29-19,	25
		"	30	i.p.	F29-23, F29-29,	
		WC ($1x10^8$)	208	i.p.	F29-44, F29-47,	
					F29-50	
M. tuberculosis H37Rv	Sauton	SWC ($2x10^7$)	0	i.p.	F23-4, F23-24,	25
		"	30	i.p.	F23-30, F23-41,	
		"	122	i.p.*	F23-49	
		"	140	i.v.		
M. tuberculosis	Long synthetic (2-6 wks.)	HKWC (1 mg)	0	i.p.@	HGT 1, HGT 2	17
		SON 60S (100 ug)	14	i.p.	HGT 3a, HGT 4,	
		"	28	i.p.	HGT 6	
		"	4	i.p.*		
		SON 60S (50 ug)	3 d.p.f.	i.p.		
M. tuberculosis H37Rv	Sauton (l.)	BPE (100 ug)	0	i.p.	HAT 1, HYT 4,	1,33
		"	14	i.p.*	HYT 6, HYT27	
		"	84	i.p.	HYT 29	
M. tuberculosis H37Rv	Sauton (l.)	CCF-RaBCG ppt.	0	i.p.	HYT 28	33
		"	7	s.q.		
		"	14	s.q.*		
		"	28	i.p.		

M. tuberculosis H37Rv	Sauton (l.)	CCF-HYTad[$] (100 ug)	0	i.p.	HAT 3	1
		"	14	i.p.		
		"	28	i.p.*		
		"	3 d.p.f.	i.p.		
M. tuberculosis H37Ra	Difco 7H9	WC ($2x10^7$ c.f.u.)	0	i.p.	TB23	7
		"	28	i.p.*		
		"	93	i.p.		
M. tuberculosis H37Ra		CFS (50 ug) + HKWC (5 mg)	0	i.p.[@]	TB5-1, TB5-2 TB5-20	12
		CFS (200 ug)	28	i.p.*		
M. tuberculosis H37Ra	Proskauer-Beck (l.)	CFS (100 ug) + HKWC (200 ug)	0	i.p.[@]	TB-C-1,TB-C-2 TB-C-3, TB-C-4	11,32
		CFS (? ug)	28	s.q.	TB-C-5, TB-C-6	
		"	42	s.q.*	TB-C-7, TB-C-8	
		CFS (400 ug)	70	i.p.	TB-C-9, TB-C-10	
M. tuberculosis H37Ra	Proskauer-Beck (l.)	CFS (100 ug)	0	i.p.[@]	TB-C-11, TB-C-12	32
		"	28	s.q.	TB-C-13, TB-C-14	
		"	42	s.q.*	TB-C-15, TB-C-16	
		CFS (400 ug)	70	i.p.	TB-C-17	
M. tuberculosis H37Ra		Antigen 5 (? mg)	0	i.p.[@]	TB-C-18, TB-C-19	32
		Antigen 5 (1 mg)	14	?		
			? (rest 4-6 wks.)	?		
		Antigen 5 (? mg)	4 d.p.f.	?*		
M. leprae	Armadillo	WC ($5x10^4$)	0	f.p.*	F31-20, F31-28	25
		WC ($1x10^8$)	190	i.v.	F31-29, F31-30	
M. leprae	Armadillo	WC ($2x10^7$)	0	i.d.	F41-1,	25
		"	14	i.d.*		
		WC ($1x10^8$)	202	i.v.		

TABLE 1 (Continued)

Organism	Medium[a]	Immunization Schedule: Antigen[b] (amount)	Day	Route[c,d]	Monoclonal antibody	Reference
M. leprae	Armadillo	SWC ($2x10^8$)	0	i.d.&i.p.@	F26-3, F26-4, F26-6, F26-7	25
		"	10	i.d.*		
		SWC ($1x10^9$)	42	i.v.		
M. leprae	Armadillo	SWC ($2.5x10^8$)	0	i.d.&i.v.@	F30-5, F30-11, F30-14	25
		"	17	i.d.*		
		SWC ($1x10^9$)	47	i.v.		
M. leprae	Armadillo	SLWC ($1x10^9$)	0	i.p.	ML30, ML34	16
		"	30	i.p.		
		"	60	i.p.*		
		"	90	i.p.		
M. leprae	Armadillo	SON 100S (100 ug)	0	i.p.@	ML02, ML09	16
		" (? ug)	?	i.p.@		
		" (? ug)	168	i.p.*		
		?	3 d.p.f.	i.p.@		
M. leprae	Armadillo	10KP (50 ug) + 30KS (100 ug)	0	i.p.	IVD2, IVE12	14
		"	6	i.p.		
		"	12	i.p.*		
		"	22	i.p.		

M. leprae	Armadillo	SON 20S (50 ug) + P (50 ug)	0	i.p.[@]	L1, L2, L3, L4	33
		"	7	i.p.[@]		
		"	14	i.p.*		
		"	30	i.v.		
M. leprae	Armadillo	SON 20S (50 ug)	0	i.p.[@]	L7, L8, L9, L10	33
		"	7	i.p.[@]		
		"	14	i.p.*		
		"	180	i.v.		
M. leprae	Armadillo	RSON 20S	immunize and rest for 180 days		L14, L21, L22 L25	4
		RSON 20S	3 d.p.f.*			
M. leprae	Armadillo	SON 27S (50 ug)	0	i.p.[@]	SA1. D2D	43
		"	7	i.p.		
		"	14	i.p.		
		"	21	i.p.*		
		"	3 d.p.f.	i.v.		
lepraemurium	Mice or Rats	WC ($1x10^8$)	0	i.v.*	A-494	2
		11	11	i.v.		

for 1 hour,[1,33] and an immunoprecipitate from concentrated culture filtrate (CCF).[1,33] The immunoprecipitate was obtained by crossed immunoelectrophoresis (CIE) with CCF electrophoresed in second dimension into an agarose gel containing rabbit anti M. bovis BCG (RaBCG) serum followed by excision from the gel. The CCF was prepared from M. tuberculosis H37Rv grown in Sauton fluid medium[42]. The cells were removed by filtration and antigen was precipitated using ammonium sulphate, resuspended in PBS, and centrifuged at 44,000 x g to remove insoluble material.[33] Three McAb HYT 6, HYT 27 and HYT 28, resulting from immunization with BPE and hybridoma production, were then used to construct an immunosorbent matrix by coupling to CNBr-activated Sepharose 4B.[1,33] The CCF was depleted of antigens corresponding to HYT 6, HYT 27, and HYT 28 after passage through the affinity column. The CCF effluent (CCF-HYTad) was then used for immunization and hybridoma production.[1] Mycobacterium tuberculosis strain S1 HKWC was used as a boost inoculum in one immunization schedule.[7] Antigens from M. tuberculosis H37Ra were used in the form of WC,[7] dried HKWC,[11,12,32] and culture filtrate solids[11,12,32] (CFS) prepared after pellicle growth in Proskauer-Beck synthetic medium[26] for eight to ten weeks followed by filtration, dialysis of the filtrate against water, and lyophilization.[10] Antigen 5 from M. tuberculosis H37Ra unheated culture filtrate, obtained after absorption onto an immunosorbent column prepared using goat antiserum,[8,9] was also used for immunization in hybridoma production.[32] The goat antiserum was prepared using

Antigen 5 that was obtained after acrylamide gel separation of material recovered by concanavalin A-Sepharose chromatography.

Although McAb were produced to investigate antigens of M. leprae or M. lepraemurium, some of these McAb detect antigens of M. bovis BCG. Mycobacterium leprae used for antigen preparation was isolated from livers of infected armadillos. Antigens obtained from M. leprae included WC,[25] dry weight SWC,[25] sonicated lyophilized whole cells (SLWC),[16] sonicate supernatant (SON 100S) obtained after ^{60}Co irradiation of cells followed by ultrasonication and centrifugation at 100,000 x g for 1 hour,[16] or centrifugation of fractions 10KP and 30KS.[14] The 10KP fraction was prepared by extracting acetone treated cells in a lithium acetate-ethylenediaminetetraacetic acid (EDTA) solution for 2 hours at 45°C and cell wall material and damaged cells were removed by centrifugation at 10,000 x g for 10 minutes. The remaining supernatant was centrifuged for 20 minutes at 30,000 x g and the final supernatant was dialyzed against H_2O, lyophilized, and resuspended in phosphate buffered saline (PBS) to obtain 30KS. Other centrifuge fractions were prepared by ultrasonicating lyophilized whole cells and centrifuging at 20,000 x g for 15 minutes to obtain soluble sonicate (SON 20S).[3] The remaining pellet was resonicated and after centrifugation at 20,000 x g, the final pellet (P)[3] and supernatant (RSON 20S)[3] were also used for immunization. Mycobacterium leprae was also sonicated and centrifuged at 27,000 x g for 30 minutes to obtain supernatant antigen (SON 27S).[43] Mycobacterium lepraemurium WC, grown

in vivo in a susceptible mouse or rat strains, were also used as antigen for immunization and McAb production.[2]

Mycobacterial antigen preparations included heat killed or non-heat killed organisms, and ranged from intact whole cells to disrupted cells and resulting fractions. Immunization schedules using mycobacterial antigens consisted of repeated inoculations and a final boost injection three to four days prior to fusion and hybridoma production (Table 1). Time for immunization, including boost, varied from 11 days to 362 days however a 30 to 90 day interval was most common. Freunds incomplete adjuvant was frequently used with the initial inoculation. Routes of inoculation for various immunizations were intraperitoneal, intravenous, subcutaneous, intradermal, or in one schedule via the foot pad.[25]

Techniques used to produce McAb to mycobacteria involve fusion of spleen cells obtained from immunized mice with immortal myeloma cells.[20,27] Myeloma cell lines used in hybridoma production have included P3-NSI/1-AG4-1,[3,4,7,11,12,14,16,29,32,34,38,43] Sp2/0,[17,25] and P3X63-Ag8.653.[2,28] Myeloma cell line P3-NSI/1-Ag4-1 is a variant clone from myeloma P3K that produces MOPC 21 protein and is resistant to 20 ug/ml 8-azaguanine.[23] P3-NSI/1-Ag4-1 does not produce immunoglobulin (Ig) heavy chain and produces kappa light chain but it is not secreted. Myeloma P3X63-Ag8, derived from P3K, is resistant to 20 ug/ml 8-azaguanine and secretes MOPC 21 IgG1 (kappa),[21] and was used to generate Sp2/0 after fusion with spleen cells from mice immunized

with sheep red blood cells.[35] Sp2/0 does not express Ig heavy or light chains.[22] Myeloma P3X63-Ag8 was repeatedly cloned and subclone P3X63-Ag8.653 was isolated which does not express Ig heavy or light chain.[18] An additional myeloma cell line, FOX-NY was a spontaneous mutant from P3-NSI/1-Ag4-1.[37] Stimulated spleen B-cells that produce antibody were fused with myeloma cell using inactivated Sendai virus[21] or by polyethylene glycol.[13] Fusion of plasma membranes results in formation of a cell containing two or more nuclei. At cell division, the nuclei fuse and a hybrid cell results. Hybridomas are differentiated from myeloma cells and unfused cells after growth in selective medium. In order to multiply in culture, a cell must replicate its DNA. There are two pathways in which purines and pyrimidines are synthesized for incorporation into DNA.[36] In the de novo pathway, ribose 5-phosphate, glycine, aspartate, glutamine, CO_2, and carbon atoms from tetrahydrofolate are utilized to produce inosinate (IMP) which is a precursor in synthesis of deoxyadenylate (dATP) and deoxyguanylate (dGTP) that are incorporated into DNA. Tetrahydrofolate also is a methyl donor in the synthesis reaction converting deoxyuridylate (dUMP) to deoxythymidylate (dTMP) that is needed in DNA synthesis. In the salvage pathway, adenine phosphoribosyl transferase (APRT) catalyzes transfer of the ribose phosphate moiety of 5-phosphoribosyl-1-pyrophosphate (PRPP) to adenine forming adenylate (AMP) and thymidine kinase (TK) catalyzes formation of dTMP from thymidine and ribose phosphate.[31] Cells can synthesize DNA via the salvage pathway if

adenine and thymidine are supplied in the medium. Aminopterin blocks conversion of dihydrofolate to tetrahydrofolate in the de novo pathway and cells growing in the presence aminopterin synthesize DNA by salvage pathways. FOX-NY myeloma cells are deficient in APRT and can not survive in aminopterin supplemented medium. In B-cells from Robertsonian (8.12) 5 Bnr [Rb(8.12)] mice the heavy chain Ig locus on chromosome 12 and the APRT locus on chromosome 8 are genetically linked.[37] After fusing B-cells with FOX-NY myelomas, cells are grown in medium supplemented with aminopterin, adenine, and thymidine (AAT); only hybridomas that produce antibody have APRT and can survive. Supernatants from cell cultures are screened to detect antibody with activity to the target antigen. Cell cultures with supernatants positive for antibody activity are expanded and corresponding McAb are characterized.

Monoclonal antibodies obtained from actively secreting hybridomas were partially characterized by determining immunoglobulin (Ig) isotype, evaluating binding activity to other mycobacterial and non-mycobacterial antigens, and by estimating the molecular mass of corresponding antigenic components. Immunoglobulin subclass determination of McAb was performed using immunodiffusion[2,3,12,14,16,25,29,32] or isotyping antisera techniques[17,28] (Table 2). The majority of McAb produced have been Ig class M (IgM). Other Ig subclasses included IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3. Two McAb produced with binding activity to M. bovis antigens

were Ig class A (IgA).[29,32] Generally, immunization periods of 30 days or less resulted in production of hybridomas secreting IgM McAb.

Binding activities of McAb to mycobacterial and non-mycobacterial antigens were evaluated by radioimmunoassay (RIA), ELISA, and immunofluorescence. Bacterial antigen SON 1S was used to evaluate MB2, MB3, MB5, MB7, MB11, MB13, and MB17 binding in RIA.[29,38]

TABLE 2. Immunoglobulin subclass of monoclonal antibodies

Subclass						
IgM	IgM	IgG1	IgG2a	IgG2b	IgG3	IgA
F24-2	TB-C-1	TB23	SA-12	TB71	B6	MB17
MB2	TB-C-2	TB68	MB5	TB5-20	F23-4	TB-C-18
MB3	TB-C-3	TB72	F23-41		ML02	
MB7	TB-C-4	TB78	F23-49		ML09	
MB11	TB-C-5	F23-24	F29-29		L9	
MB13	TB-C-6	F26-6	TB-C-13		L10	
TB73	TB-C-7	F29-23	TB-C-14			
TB77	TB-C-8	F29-50	TB-C-15			
F23-30	TB-C-9	TB5-2	L4			
F26-3	TB-C-11	HGT 1	L25			
F26-4	TB-C-11	HGT 2				
F26-7	TB-C-12	HGT 4				
F29-17	TB-C-16	HGT 6				
F29-19	TB-C-17	HYT 6				
F29-44	TB-C-19	HYT 27				
F29-47	HGT 3a	HYT 28				
F30-5	HAT 1	HAT 3				
F30-11	IVD2	ML30				
F30-14	IVE12	L7				
F31-20	ML34	L14				
F31-28	L1	L21				
F31-29	L2	L22				
F31-30	L3	SA1.D2D				
F41-1	L8					
TB5-1	A-494					

Antigens detected using MB5 were restricted to M. bovis vallee, AN-5, and 44 field isolates of M. bovis. The remaining MB McAb were used to demonstrate binding activity to a wide range of mycobacteria, and in the case of MB3 and MB17, to detect E. coli intracellular antigens. Inhibition evaluations using SON 1S confirmed binding activity patterns of MB McAb to mycobacterial antigens. MB5 binding activity was inhibited by M. bovis field strain 2309 and strain vallee antigens but not by M. bovis BCG. In contrast, MB3, and MB17 binding was inhibited by each mycobacterial sonicate tested. Binding of SA-12 to corresponding radiolabeled BCG-a antigen was inhibited by M. bovis BCG (Glaxo), M. tuberculosis SON 200S, and weekly by M. fortuitum, M. intracellulare, and M. kansasii SON 200S.[28] Mycobacterium bovis BCG HKWC also inhibited SA-12 binding to corresponding antigen BCG-a. Sonicates from non-mycobacterial species did not inhibit SA-12 binding activity. As determined by RIA using PRES 100S from bacteria tested[7], TB68 was used to detect antigens of M. bovis and M. tuberculosis. Monoclonal antibodies TB23, TB73, TB77, and TB78 were used to detect M. tuberculosis complex antigens and antigens from other mycobacterial species, where use of TB73 and TB77 also detected E. coli antigen. Of the McAb recognizing antigens of M. bovis, use of TB23, TB77, and TB78 detected antigen of M. bovis vallee and did not detect antigens of BCG, TB68 and TB73 detected antigen in both strains. Only antigens of M. tuberculosis were detected using TB71 and TB72. The ability of PRES 100S from mycobacterial species to inhibit ^{125}I labeled McAb binding to

homologous antigen was also determined to evaluate binding activity. TB68 and TB23 were inhibited by M. bovis vallee and M. tuberculosis H37Rv antigens, but not by M. bovis BCG antigens. High concentrations of M. bovis vallee antigen inhibited TB71 and TB72 binding to M. tuberculosis H37Rv antigen. As for McAb produced with the intent to detect M. leprae antigens, use of ML02, ML09, ML30, and ML34 detected M. bovis SON 100S and other mycobacterial antigens in RIA.[16] Binding patterns and inhibition of radiolabeled ML McAb by other ML McAb indicated that the same determinant was detected using ML01 and ML02. Binding of ML34 was inhibited by ML01/ML02 suggesting that it recognized a similar determinant. ML34 was not inhibited by any other ML McAb.

Binding activities of McAb were evaluated by ElISA and IF. As determined by ELISA, use of TB44 detected glycolipid C from M. bovis BCG and not glycolipid A and B antigens,[34] and B6 was broadly reactive to SON 20S antigen form a range of mycobacteria.[3] The F23 McAb and F24-2 were used to detect M. bovis BCG and M. tuberculosis antigens as determined by ELISA using SON 20S and by IF with HKWC antigen.[25] Use of all F23 McAb, except F23-44, detected antigens from more mycobacteria than F24-2. In addition, F24-2 was used to detected antigen form chloroform-water extract of M. bovis BCG in ELISA. Detection of M. bovis BCG antigen by use of F29-23 and F29-50 was evident only in IF using HKWC antigen, and antigens from other mycobacteria were also detected. The McAb F29-17, F29-19, F29-29, F29-

44, and F29-47 indicated stronger binding activity to M. bovis, M. tuberculosis, and other mycobacteria in ELISA using HKWC instead of sonicates, and this observation was supported in IF using HKWC antigen. Overall, F26, F30, and F31 McAb had low specificity. Fusion 41 hybridomas were screened using HKWC in IF and use of F41-1 detected M. bovis and other mycobacterial antigen in IF and not in ELISA. Antigen 5 and Antigen 6 was detected in ELISA using TB-5-1, TB-5-2, and all TB-C.[11,12,32] McAb TB-C-1, TB-C-2, TB-C-5, TB-C-6, TB-C-7, TB-C-8, TB-C-11, TB-C-12, TB-C-16, and TB-C-17 were reactive to CFS from a broad range of mycobacteria, and TB-C-3, TB-C-4, TB-C-9, TB-C-10, and use of TB-C-19 also detected CFS antigen from some mycobacteria, but reacted stronger to antigen 5 and 6. Only use of TB-C-13, TB-C-14, TB-C-15 and TB-C-18 detected Antigen 5 and 6 specifically. Formaldehyde-killed whole cells were used to evaluate HYT McAb by ELISA.[1,33] Monoclonal antibody HYT 28 was used and only detected antigens of M. tuberculosis of all the mycobacteria tested, whereas use of HYT 4, HYT 27, and HYT 29 identified antigen of M. bovis BCG and antigen of other mycobacteria. Similarly. HYT 6, HAT 1, and HAT 3 were used to detect CCF and BPE antigens from a range of mycobacteria. Binding activity of HGT McAb were evaluated by ELISA using mycobacterial SWC antigen and HGT 4 exhibited strong binding to M. bovis BCG and M. tuberculosis and moderate binding activity to M. kansasii and M. scrofulaceum. Use of HGT 1, HGT 2, and HGT 6 did not detect antigens of M. bovis, although SWC antigen of M. tuberculosis and other mycobacteria were detected.

HGT 3a binding activity was limited to M. tuberculosis of the mycobacteria tested.

McAb obtained after immunization with M. leprae antigens that detected M. bovis antigens, with exception of L7, were of low specificity and could be used to detect antigen from numerous mycobacteria.[3] Binding activity of L7 was greater with M. bovis SON 20S antigens than to M. tuberculosis, M. leprae, and M. scrofulaceum SON 20S antigens.[3] Sonicates from eleven species of mycobacteria were used to determine binding of L21 and L22 in ELISA.[4] Antigens from each of M. bovis BCG, M. tuberculosis H37Rv, M. gastri, M. intracellulare, M. leprae, M. nonchromogenicum, M. phlei, M. simiae, M. smegmatis, M. ulcerans, and M. vaccae were detected by use of L21 and L22. L21 and L22 were also used to detect whole bacilli in IF. In ELISA using 30KS antigens, IVD2 had greater binding activity as compared to IVE12.[14] As detected by IF using WC antigens, A-494 was also broadly reactive to mycobacterial species, as well as to antigens of Nocardia.[2]

Molecular weight (MW) of antigenic components corresponding to McAb were estimated using immunoblot procedures[39] (Table 3). The MB McAb that was used to detect antigens of M. bovis were analyzed by immunoblot techniques under reducing conditions using SON 1S antigen from M. bovis BCG, M. tuberculosis, M. africanum, M. avium, and M. paratuberculosis.[28] Antigenic components detected by use of MB5 were from a field isolate of M. bovis and included one major component of

Table 3. Characterization of monoclonal antibodies to mycobacteria[a,b]

Monoclonal Antibody	Molecular Weight (kilo)[c]	Nature of Antigen[d]	Immunoblot Specificity	Reference
F23-24	>200			25
F26-3	>200			25
F26-6	>200			25
F29-17	>200	non-protein		25
F29-19	>200	non-protein		25
F29-23	>200	non-protein		25
F29-44	>200	non-protein		25
F29-50	>200	non-protein		25
F31-20	>200	non-protein		25
MB3+	200-20	carbohydrate	CR	29
HAT 1	71	protein	CR	1
HAT 3	71 (m.b.)	protein	CR-B	1
L7	70	protein	CR-L	3
TB78	65 (m.b.)	protein	CR-L	41
L14	65	protein	CR	4
L25	65	protein	CR	4
ML30	65,55,50-28(s)	protein	CR	40
L21%	65,12	protein	CR	4
L22%	65,12	protein	CR	4
HGT 1	60-45 (m.b.)		CR-B	17
HGT 2	60-45 (m.b.)		CR-B	17
HGT 4	60-28 (m.b.)		CR-L	17
HYT 29	57,33,32	protein		33
ML34	50-40 (s)	carbohydrate/lipid	CR	16,40
ML02	50-40 (s)	carbohydrate/lipid	CR	16,40

[a]Abbreviations: s, smear; m.b., multiple bands; CR, cross reactive (-L, limited; -B, broad); Mtb-C, M. tuberculosis complex.

[b]Symbols: +=cross reactive with tissue antigens; ^=cross reactive with bovine serum albumin; ~=additional component detected in M. africanum and M. avium; %,#,*,@,$,&=Monoclonal antibodies (McAb) to the same antigen.

[c]Molecular weight was determined by immunoblot procedures.

[d]As indicated by susceptibility to proteolytic digestion.

Table 3 (Continued)

Monoclonal Antibody	Molecular Weight (kilo)[c]	Nature of Antigen[d]	Immunoblot Specificity	Reference
ML09	50-40 (s)	carbohydrate		16
HGT 6	45,43		CR-B	17
F29-29	40	protein	CR-L	41
L1[#]	40-30 (s)	carbohydrate	CR	3
L9[#]	40-30 (s)	carbohydrate	CR	3
L10[#]	40-30 (s)	carbohydrate	CR	3
TB72[*]	38	protein	Mtb-C	41
HYT 28[*]	38	protein	Mtb-C	41
HGT 3a	38		Mtb-C	17
TB71	38	protein	Mtb-C	41
HYT 27	33,32	protein	CR	1
MB5	29.8,22.9,19.9		M. bovis	29
TB-C-1	26.8 (m.b.)	carbohydrate	CR	11
SA1.D2D	23	protein	CR-B	41
TB23[@]	19	protein	CR-L	41
F29-47[@]	19	protein	CR-L	41
HYT 6[@]	19	protein	CR-L	1,41
TB-C-13	19	protein	? (weak activity)	41
HYT 4	19-17	protein		33
MB17	17.4 (16~)		CR	29
F23-41	<15			25
TB68[$]	14	protein	Mtb-C	41
F24-2[$]	14	protein	Mtb-C	41
F23-49[$]	14	protein	Mtb-C	41
SA-12	12	protein	CR-L	41
B6[&]	6-4.5 (s)	carbohydrate	CR	3
L3[&]	6-4.5 (s)	carbohydrate/lipid	CR	3,40
L4[&]	6-4.5 (s)	carbohydrate	CR	3

29.8 kilo (k) and two minor components of 22.9 k and 19.9 k. Multiple bands in the range of 100 to 20 K were detected by use of MB3 from the field isolate of M. bovis and each of the mycobacterial cell sonicates examined. Broadly reactive MB17 was used to detect a major 17.4 k component in each mycobacterial cell sonicate, and a minor 16 k component in M. africanum and M. avium.

The F24, F23, F26, F29 and F31 McAb were analyzed in immunoblot using the same sonicates from the homologous organism as used in immunization and hybridoma production.[25] Antigens were electrophoresed in the presence of SDS under reducing conditions. Use of F23-24, F26-3, F26-6, F29-17, F29-19, F29-23, F29-44, F29-50 and F31-20 detected a component with MW greater than 200 k. Use of F24-2, F23-41 and F23-49 detected antigens from M. bovis BCG sonicate with MW in the range 15 k to 14 k. Components detected by use of F29-29 and F29-47 were 4 k and 19 k respectively. In general, F29 McAb reacting with antigens of high MW were heat and SDS stable and insensitive to pronase treatment. Antigens detected by use of F29-29, F29-47, F23-49, and F24-2 were sensitive to proteolytic digestion indicating protein composition.

The McAb TB-C-1 was used in immunoblot analysis with CFS form M. tuberculosis, M. gordonae, M. intracellulare, M. kansasii, and M. scrofulaceum.[11] Four components were detected in M. tuberculosis and M. intracellulare with MW of 58 k, 28.5 k, 26.8 k, and 25.5 k. The 58 k component was also detected in M. scrofulaceum and the 28.5 k

component was detected in M. kansasii. The 26.8 k component was detected in CFS from each of the five mycobacterial species tested. Antigens recovered by TB-C-1 affinity chromatography consisted of arabinomannan and arabinogalactan. The 19 k component was weakly detected by use of TB-C-13 and was considered protein in composition.[41]

To estimate the MW of antigenic components corresponding to HYT McAb, CCF, BPE, and a saline extract from M. tuberculosis were electrophoresed under reducing conditions.[1,33,41] Immunoblot analysis using CCF and BPE indicated similar results where use of HYT 29 detected 57 k, 33 k and 32 k components, HYT 28 identified a 38 k component, HYT 27 was used to detected 33 k and 32 k components, and use of HYT 4 detected 19 k and 17 k components. When a saline extract of M. tuberculosis was used in immunoblot analysis, use of HYT 4 and HYT 27 detected similar components as were detected in CCF and BPE preparations, a 32 k component was detected using HYT 29, and use of HYT 28 failed to detect antigens. The HYT McAb were also evaluated using CIE techniques and could be grouped on the basis of binding activity as they were after immunoblot analysis.[33] The CIE precipitate reactive with HYT 28 was indistinguishable from the CIE precipitate used to immunize mice for hybridoma production. The antigens CCF, BPE, or crude cell wall extract (CCWE) from M. bovis BCG, M. kansasii, M. avium, M. fortuitum, M. intracellulare and M. marinum were also used to evaluate binding activity of HYT and HAT McAb. HYT 28 was used to detect a 38 k component in each antigen preparation from M. bovis BCG,

whereas use of HYT 27 detected antigens of M. bovis in only CCF and CCWE. In addition, M. avium antigens in CCF and CCWE, M. fortuitum antigens in CCF and BPE, M. intracellulare antigens in BPE and CCWE, and components in CCF, BPE, and CCWE from M. kansasii and M. marinum were detected using HYT 27. A 71 k component was detected by use of HAT 1 in CCF and BPE from each mycobacteria tested except M. fortuitum, in CCWE from each mycobacteria tested except M. kansasii and M. marinum, and from sonicated Nocardia asteroides. HYT 6 was used to detect a 19 k component in each antigen preparation from each mycobacterial species tested except M. fortuitum. Multiple components were detected by use of HAT 3 in each antigen preparation from each mycobacterial species tested as well as from sonicated N. asteroides and Rhodococcus erythropolis. Using CCF, HYT27, HYT28, and HAT1 detected determinants of M. tuberculosis H37Rv that were heat sensitive as determined by ELISA and immunoblot results.[1] All determinants detected using HYT and HAT McAb were pronase sensitive.[1] Lectin chromatography using a sepharose-concanavalin A matrix was performed to isolate antigens from M. tuberculosis H37Rv CCF which were detected by use of HYT6 and HAT1 implying associated carbohydrate.[1]

Sonicated M. tuberculosis was used as antigen under reducing conditions in immunoblot to determine MW of components detected by use of HGT McAb.[17] Multiple bands detected by use of HGT 1/ HGT 2 were in range 60 to 45 k, HGT 3a was used to detect a single 38 k component, 60-28 k components were detected using HGT 4, and HGT 6 was used to

identify 45 k and 43 k components. Immunoblots were performed using antigens from M. bovis BCG, M. kansasii, M. avium, M. intracellulare, M. phlei, M. scrofulaceum, M. smegmatis, M. vaccae, E. coli, and N. asteroides. A 38 k component was detected in M. bovis and M. tuberculosis by use of HGT 3a. Use of HGT 4 detected multiple components in M. bovis, M. tuberculosis, M. kansasii, and N. asteroides. Use of low specificity HGT 6 detected antigens in N. asteroides and each mycobacterial species tested except M. vaccae and M. phlei. Similarly, use of HGT 1/HGT 2 detected components in E. coli and N. asteroides and each mycobacterial species tested except M. vaccae.

Antigens used in immunoblot analysis of B6 included sonicate supernatant (SON 20S), the corresponding pellet (P), and supernatant from resonicated pellet (RSON 20S).[3] The McAb B6 was used to detect a 6 kD component in SON 20S and P and was considered to be carbohydrate.

Of the McAb directed against M. leprae, those with binding activity to M. bovis were also analyzed by immunoblot techniques. Sonicate supernatant antigen was used under reducing conditions to estimate MW of components corresponding to ML McAb.[16,40] Antigenic components detected by use of ML30 were 65 k, 55 k, and 50 to 28 k and the antigen was sensitive to subtilisin treatment. Antigens detected using ML02/ML09 or ML34 were 50-40 k and were resistant to digestion by subtilisin, implying polysaccharide or glycolipid. Antigens from mycobacteria other than M. leprae were detected by use of ML02, ML09,

ML30, and ML34.

Antigens used in immunoblot analysis of L1-10, L14, and L25 included sonicate supernatant (SON 20S), the corresponding pellet (P), and supernatant from resonicated pellet (RSON 20S) of M. leprae.[3,4] Use of L3 and L4 detected a 6 k component in SON 20S and P, L1, L9, and L10 were used to detect components 40 to 30 k, and L7 identified a 70 k component. Antigens of M. bovis and M. tuberculosis were also detected by use of L4, L7, and L9. Antigenic components approximately 40-30 k and 6 k were resistant to subtilisin proteolysis and 70 k and 16 k components were sensitive to proteolysis. Use of cross-reactive L14 and L21 detected a 65 k antigen, and use of L22 and L25 detect 65 k and 12 k components. The 65 k antigen was also sensitive to proteolysis and was considered protein in nature.

The World Health Organization (WHO) established workshops designed to characterize McAb produced against mycobacteria.[41] The MW of antigens were determined using immunoblot procedures and McAb were classified as protein in nature on the basis of sensitivity to proteolytic digestion by subtilisin. The McAb SA-12, produced against M. bovis, was used to detect a protein antigen with MW of 12 k and exhibited cross-reactive activity. Use of TB78, TB72, TB71, TB23, and TB68 detected 65 k (multiple bands), 38 k, 38 k, 19 k, and 14 k components and each antigen was considered to be protein. The specificity of TB72, TB71, and TB68 were directed toward the M. tuberculosis complex and limited cross-reactivity was demonstrated by

use of TB78 and TB23. Monoclonal antibody SA1.D2D was used to detect a 23 k component composed of protein and exhibited broad cross-reactivity. Additional information from inhibition studies was provided by WHO. The same antigen (38 K) was detected by use of TB72, HYT 28, and TB71, although TB71 and TB72 did not identify the same determinant. The same antigen (19 K) was detected by use of TB23, F29-47, and HYT 6 and TB68, F24-2, and F23-49 were used to detect the same antigen (14 K). In addition, L1, L9, and L10 were considered to identify simular determinants on the same antigen[3] and use of L21 and L22 detected the same antigen.[4]

Considering the common use of purified protein derivative (PPD) as a skin test antigen, it is of interest to evaluate McAb binding activity to PPD. The MB McAb were used to analyze PPD antigens in RIA and MB3 detected antigens of M. bovis, M. avium, and M. paratuberculosis.[29] In contrast, use of MB5 and MB17 did not detect antigens in the PPD tested.[15] The McAb TB72, TB71, TB68, TB78, TB23, ML30, and ML34 were also evaluated in RIA using PPD. Only use of TB68 and ML30 detected antigen of M. bovis PPD. Culture filtrate from M. tuberculosis was used to prepare PPD and, when evaluated in ELISA, TB-C-1 detected antigens where as TB-C-13 did not detect antigen.[11] As determined by ELISA and immunoblot, use of HYT6 and HAT3 detected components in tuberculin PPD.[1] Antigens corresponding to HYT 27, HYT 28, and HAT 1 were sensitive to heat treatment (similar to that used in preparation of PPD) and were not detected by immuoblot procedures.

SECTION I. OPTIMIZATION OF HYBRIDOMA PRODUCTION AND PARAMETERS FOR DETECTION OF ANTIBODY TO *Mycobacterium bovis*

SUMMARY

Somatic cell hybridization parameters including inoculum concentration for immunizing mice, cell suspensions in fusions, and media composition were optimized for production of hybridomas secreting monoclonal antibody. Heat- killed whole cell inoculum at 5 mg/ml wet weight was adequate for immunizing Robertsonian mice to generate primed B-lymphocytes. Hybridoma colonies were obtained in approximately 90% of the wells when a myeloma-B-lymphocyte fusion mixture was resuspended in culture medium containing FOX-NY myelomas and plated. Addition of amphotericin B (3 mg/ml to cell culture medium) did not influence the viability of hybridomas. Enzyme linked immunosorbent assay parameters were adjusted to detect anti-Mycobacterium bovis binding activity to increase accuracy for selecting stable hybridomas. Heat killed M. bovis cells were bound to microtiter plates by wet binding overnight at 4^{o}C. Supernatant containing monoclonal antibody was used as primary antibody. Goat anti-mouse antibody (H+L) conjugated to horseradish peroxidase was used at serial dilution 1 to 500. Optical density values recorded after 30 minutes of enzyme-substrate reaction were used to determine a positive test reaction. Two hybridoma cell lines selected as positive controls had optical density values 5 fold greater than negative control values. Optical density values of test supernatants from other viable hybridomas with stable antibody production were similar. Supernatants were used at dilution 1 to 9 in

enzyme linked immunosorbent assay for initial screening of hybridomas. Optimization of enzyme-linked immunosorbent assay parameters allowed efficient use of monoclonal antibodies for detection of Mycobacterium bovis antigens.

INTRODUCTION

Development of somatic cell hybridization techniques and production of monoclonal antibodies[3,4,7] (McAb) provides the tools necessary for analyzing bacterial antigens on a molecular level. The complex antigen structure of mycobacteria can be explored by using McAb directed against various antigenic determinants of this organism. The majority of McAb detecting mycobacterial antigens have been produced to M. tuberculosis and M. leprae,[15] and few McAb exist that detect antigens from virulent M. bovis. Myeloma cell lines used in hybridoma production have been based on a hypoxanthine phosphoribosyl transferase selection system where the locus for this selectable marker is located on the X chromosome.[1] An alternative selection strategy utilizes the FOX-NY myeloma cell line and Robertsonian (8.12) 5 Bnr [Rb(8.12)] mice.[12] In Rb(8.12) mice, the heavy chain immunoglobulin locus on chromosome 12 is genetically linked to the adenosine phosphoribosyl transferase (APRT) selectable marker locus of chromosome 8. Hybridoma selection based on APRT activity requires presence of both heavy chain and selectable marker loci. Thus, an added advantage of this system is that hybridomas not producing immunoglobulin, encoded by the fused B-cell, are eliminated as a result of simultaneous loss of APRT activity.

In order to maximize the number of hybridomas obtained after a fusion experiment, comparisons of inoculum concentrations used to immunize mice, fusion mixtures, and of media compositions were made.

Success of a fusion experiment was indicated by the number of hybridoma colonies per fusion plate and percentage of colony supernatants containing antibody with activity to M. bovis ATCC 19210 heat-killed whole cell (HKWC) antigen. Antibody activity in supernatants from hybridomas was evaluated by using an enzyme linked immunosorbent assay (ELISA). Parameters were adjusted in the ELISA to allow selection of stable hybridomas producing antibody, which included, reading time after initiation of enzyme-substrate reaction, establishing a positive control, and dilution of hybridoma supernatant used for screening fusions.

MATERIAL AND METHODS

Bacteria

Mycobacterium bovis ATCC 19210 (neotype) was obtained from the American Type Culture Collection. Organisms were grown on Middlebrook and Cohn 7H10 agar base medium (Difco Laboratories Inc., Detroit, MI) to which Middlebrook OADC enrichment (Difco Laboratories Inc., Detroit, MI) and pyruvate (4.1 g/L) was added,[6] or Middlebrook and Cohn 7H10 agar base medium (BBL Microbiology Systems, Cockeysville, MD) to which pyruvate (8.2 g/L), L-asparagine (5.0 g/L), L-glutamic acid (6.0 g/L), and dextrose (30 g/L) was added. Cultures were incubated at 37° C. Cells were harvested at 6-8 weeks and used for antigen preparation.

Antigen Preparation

Harvested cells were washed twice by resuspension in sterile deionized and distilled H_2O followed by centrifugation at 10,000 rpm. The resulting pellet was weighed, resuspended in sterile phosphate buffered saline (PBS) to cell suspensions of 10 mg/ml and 50 mg/ml (wet weight), and autoclaved for 10 minutes at 121° C. The HKWC antigen was used for immunizing mice, for ELISA evaluations, and for immunoblotting.

Animal Inoculation

Female Robertsonian [Rb(8.12)] mice (Jackson Labs., Bar Harbor, MA), 8-12 weeks old, were used for immunization.[12] The amount of M. bovis ATCC 19210 HKWC antigen and immunization schedule used for four different fusions are listed in Table 1. The concentration of inoculum used to immunize mice for fusion 01 was 10 mg M. bovis HKWC per ml PBS and 100 ul (1 mg) was given by intraperitoneal injection at 7-day intervals for six injections. Three days prior to fusion, an injection of 200 ul (2 mg) was given. A modified immunization schedule was adopted in fusion 02. Mice were immunized as in fusion 01; then the mice were injected with 5 mg HKWC at 55 days and with 15 mg HKWC at 62 days (three days prior to fusion). In fusion 03 and 06, mice were given intraperitoneal injections of 100 ul (5 mg) at 1 week intervals for six weeks using an inoculum containing 50 mg/ml PBS. Three days prior to fusion, mice were injected with 200 ul M. bovis HKWC antigen adjusted to 75 mg/ml PBS (15 mg). Inoculum used for injecting mice for fusion 01, 02, and 03 was from M. bovis grown on Middlebrook and Cohn medium containing OADC enrichment; inoculum used for fusion 06 was from cells grown on modified Middlebrook and Cohn base medium to which pyruvate, asparagine, glutamate, and dextrose were added.

TABLE 1. Inoculation schedules for mice given heat killed *Mycobacterium bovis*[a]

Fusion Number	Immunization Schedule	
	Amount	Day
01	1 mg	0
	"	7
	"	14
	"	21
	"	28
	"	35
	2 mg	42
02	1 mg	0
	"	7
	"	14
	"	21
	"	28
	"	35
	2 mg	42
	5 mg	55
	15 mg	62
03, 06	5 mg	0
	"	7
	"	14
	"	21
	"	28
	"	35
	15 mg	42

[a]Inoculations given through intraperitoneal route.

Hybridoma Production

The mice were euthanatized and spleens were removed from mice aseptically, rinsed in sterile PBS, transferred to a petri dish containing 10 ml of sterile PBS, and minced to obtain separate spleen

cells. A single mouse was used for each fusion experiment. The spleen cell suspension was transferred to a centrifuge tube and FOX-NY myeloma cells (Hyclone Labs., Logan, UT) were mixed at a ratio of 1:2, myeloma to spleen cells.[12] The myeloma-spleen cell suspension was centrifuged at 300 rpm for 10 minutes; supernate was discarded, and 0.5 ml of 45% polyethylene glycol 1540 (EM science, Gibbstown, NJ) in sterile PBS was mixed with the pellet. After 60 seconds at 37°C, the cell suspension was diluted with 20 ml of Dulbecco's Minimum Essential Medium[10] (Gibco Ltd., Grand Island, NY) (DMEM). Then fused cell suspension was added to DMEM containing 7.5×10^{-5}M adenine, 8×10^{-7}M aminopterin, 1.6×10^{-5}M thymidine, streptomycin sulfate (100 mg/L), penicillin (1×10^{5} units/L), and 15% horse serum (DMEM-AAT).

One-third the number of cells from a spleen of a nonimmunized mouse and peritoneal macrophages (to approximately 250 cells per ml) were added to the fused cell suspension in fusions 01, 02, and 03. For comparison (fusion 6), approximately 1×10^{8} FOX-NY myeloma cells were added to a fused cell suspension. Fusion suspensions were distributed into 96-well tissue culture plates (Costar, Cambridge, MA), and hybridoma cell cultures were incubated in a humid, 5% CO_2 atmosphere at 37°C. After 8 to 10 days, hybridoma supernatants were screened for presence of antibody with activity to M. bovis HKWC using an ELISA. Hybridoma colonies producing antibody that detected M. bovis antigens were further maintained in 24-well culture plates using DMEM-AAT with amphotericin B (3 mg/L) (DMEM-AATF) and screened again for activity by

ELISA; the cells were recloned twice by limiting dilution with DMEM-AAT in 96-well plates. Single colonies corresponding to supernatants with strong positive ELISA reactions to M. bovis were transferred to 24-well plates. Cells were then transferred to 25 cm^2 tissue culture flasks containing DMEM-AATF. Aliquots of cells were suspended in DMEM containing 15% horse serum and 10% dimethylsulfoxide (DMSO) and cryopreserved at -70°C or in liquid nitrogen.

Enzyme-Linked Immunosorbent Assay

A modified ELISA procedure was used to screen hybridoma cell cultures[13,14]. Mycobacterium bovis ATCC 19210 HKWC antigen preparation (50 mg/ml) was used at a dilution of 100 ul (5 mg) in 5.0 ml 0.1 M Na_2CO_3, pH 9.6. The antigen solution was added to Immulon I Dynatech microtiter plates (Dynatech, Alexandria, VA) at 50 ul per well followed by an equal volume of cyanamide 1 mg/ml 0.1 M Na_2CO_3, pH 9.6. Plates were incubated at 4°C for 16 hours, washed three times with PBS, pH 7.2 and then 0.1 M NH_4Cl (100 ul/well) was added and incubated at room temperature (RT) for 30 minutes. The antigen coated plates were washed three times with phosphate buffer containing 0.5 M NaCl and 0.5% Tween 80, pH 7.5 (ELISA wash solution). For evaluating fusions 01, 02, and 03, hybridoma supernatants and negative controls (50 ul/well) were incubated with shaking at RT for 1 hour. Negative controls included: 1) DMEM-AATF, 2) conditioned medium from FOX-NY myeloma cell cultures (DMEM-CM), and 3) 15% horse serum in phosphate buffer containing 0.5 M

NaCl and 1.0% Tween 80, pH 7.5 (ELISA diluent). Hybridoma supernatants from fusion 06 and negative controls were used at serial dilution 1:10 in diluent (50 ul/well). Plates were then washed eight times with ELISA wash solution and goat anti-mouse IgG (H+L) horseradish peroxidase conjugate antibody (Kirkegaard & Perry labs., Gaithersburg, MD) at a dilution of 1:500 in diluent, was added 50 ul/well and the plates were incubated with shaking at RT for 1 hour. Plates were washed eight times with ELISA wash solution and briefly dried to remove excess fluid. Substrate-chromagen solution consisting of 0.4% H_2O_2, 2,2'-azino-di-3-ethyl-benzthiazoline sulfonate (ABTS) in 0.05 M citric acid, pH 4.0 (100 ul/well) was added and absorbance at 405 nm was recorded. The ELISA was used to screen for antibody produced by recloned hybridoma cell cultures.

Cell Culture Medium Comparison

To determine effects of amphotericin B on hybridoma viability, selected colonies were maintained in cell culture using DMEM-AAT with or without amphotericin B. Hybridoma cell lines 33 through 47 had corresponding supernatants with anti-M. bovis activity as determined by ELISA after primary screening. The 15 colonies were transferred in duplicate from fusion plates to tissue culture plates and cultured in DMEM-AAT or in DMEM-AAT containing amphotericin B (3 mg/L). Both sets of cell cultures were maintained for 12 days and cell viability was evaluated based on visual inspection. In addition, ELISA were conducted to obtain information on antibody activity.

RESULTS

Hybridoma Production

The protocol for cell fusion and for maintaining cell cultures used in fusion 01 and 02 were similar; however, the immunization schedules varied. The number of wells containing hybridoma colonies and supernatants with antibody activity to M. bovis HKWC as determined by ELISA are listed in Table 2. In fusion 01, 14 of 110 colonies

TABLE 2. Number of hybridoma colonies and supernatants containing antibody to Mycobacterium bovis obtained after fusion experiments

Fusion	Wells Seeded	# Colonies	at Day	Number positive[a]	% positive
01	768	110	14	14	13
		197	23	28	14
02	1152	93	17	32	34
		113	22	47	42
		142	37	64	45

[a]Supernatants with ELISA value greater than twice the optical density value of the negative control.

at day 14 had supernatant with antibody activity to M. bovis and at 23 days incubation, 28 of 197 hybridoma colonies had supernatant with antibody activity to M. bovis. No additional colonies were observed after 23 days.

In fusion 02, 32 of 93 colonies at day 17 and 47 of 113 colonies

at day 22 had supernatants with antibody activity to M. bovis. A total of 64 of 142 colonies had supernatants with antibody activity to M. bovis at 37 days of cell culture. The number of positive supernatants per total number of colonies in fusion 02 was greater as compared to fusion 01. In fusion 02, the animal was inoculated at 55 and 62 days with 5 mg and 15 mg of cells, respectively.

In fusion 06, approximately 90% of the wells contained colonies; however supernatants from only 182 colonies exhibited positive ELISA reactions at day 10 of culture. Mice used in fusions 02 and 06 had serum antibody titers greater than 1:320. The number of colonies obtained when FOX-NY myeloma cells were used as feeder cells (fusion 06) was greater than in fusion 01 or fusion 02 where spleen cells and macrophages were used as feeder cells.

Cell Culture Media Comparison

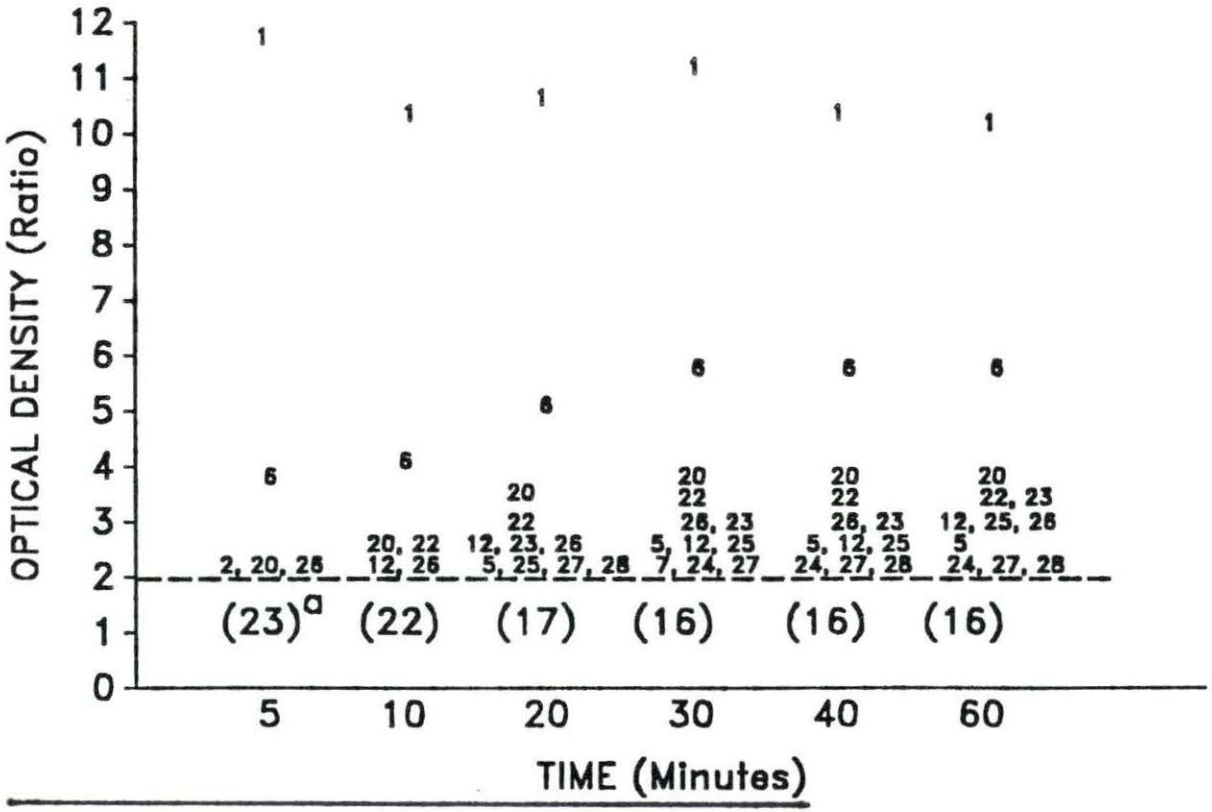
Twelve of 15 hybridoma colonies increased in cell number and remained viable when DMEM-AATF was used and one cell line produced antibody with activity against M. bovis. Similar results were obtained using DMEM-AAT indicating that amphotericin B did not contribute to loss of cell viability.

Optimization of Enzyme-Linked Immunosorbent Assay

Parameters of the ELISA were adjusted to increase accuracy in detection of stable hybridomas producing antibody against M. bovis ATCC 19210. When the ratio of the test optical density (OD) value to the negative control OD value was equal to or greater than 2, an ELISA reaction was considered positive. Negative controls consisted of PBS, DMEM-AATF, DMEM-CM, and horse serum diluted to 15% in ElISA diluent. Supernatants of 197 colonies (fusion 01) were positive on ELISA. Corresponding colonies were transferred to 24 well culture plates, maintained, and viable cell lines were retested. Ratio of OD values were calculated at 5, 10, 20, 30, 40, and 60 minute time intervals (Figure 1). Supernatants from 12 colonies were positive after 30 minute of reaction time; this number did not change at 40 and 60 minutes reaction time. Supernatants of two cell lines (numbers 1 and 6) had OD ratios of 11 and 5.75 at 30 minutes, respectively. Antibody from cell line 6 was used as a positive control for evaluating supernatants from subsequent fusions.

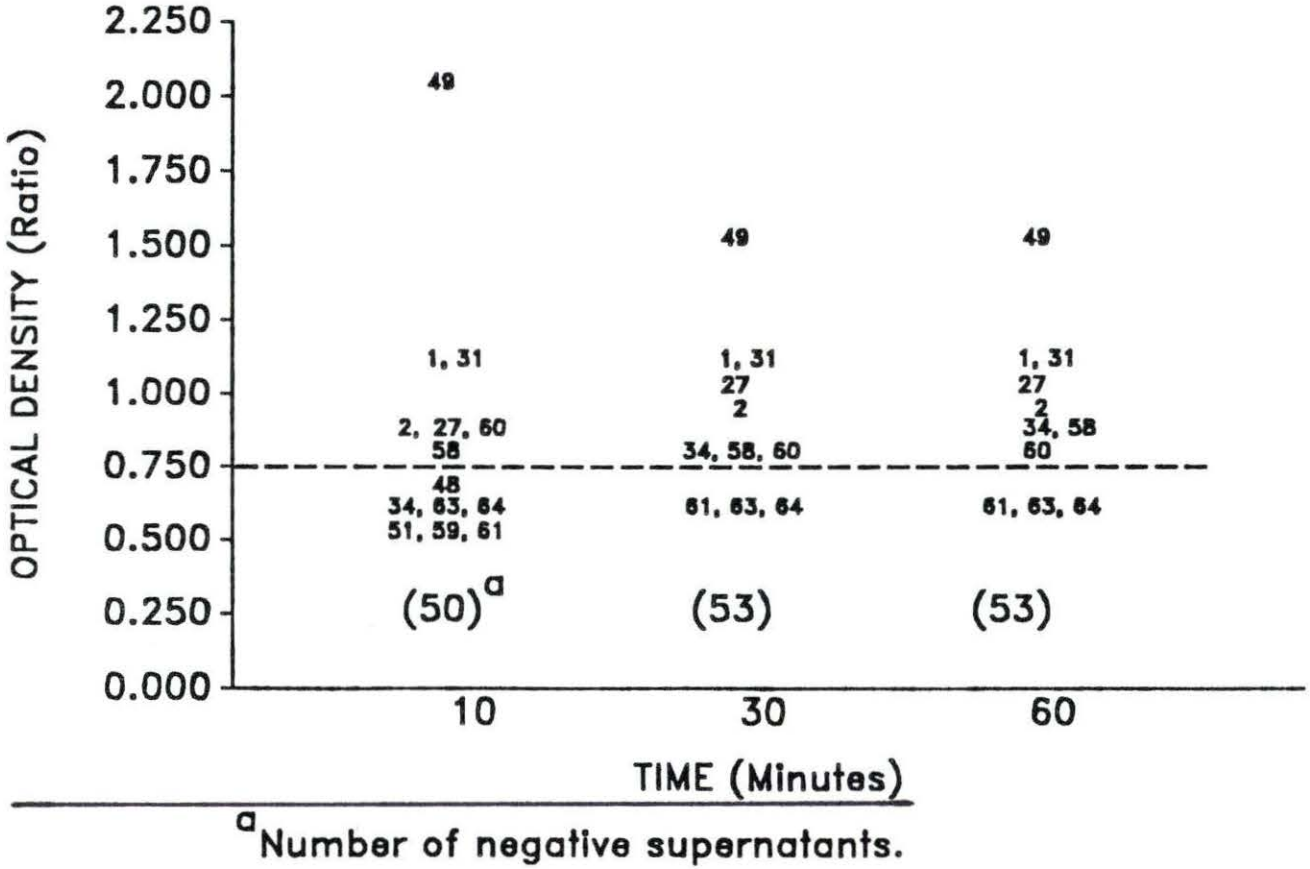
Fusion 02 was screened at 17, 21, and 37 days. Reactions were considered positive when OD ratios approached positive control 6 OD ratio (7.0 times the negative control OD value). Sixty four of 142 fusion supernatants screened had positive reactions; corresponding colonies were maintained in culture. Supernatants of viable cell lines were retested in an ELISA and ratios were calculated using the test OD value divided by positive control OD value (Figure 2). Supernatants

FIGURE 1. Results of enzyme-linked immunosorbent assay on supernatants of 28 hybridoma colonies (fusion 01) at various time intervals. Heat-killed Mycobacterium bovis (50 ug per well) was used as antigen. Monoclonal antibody binding was detected using goat anti-mouse IgG (H+L) peroxidase labeled antibody. Results are expressed as a ratio of optical density of test sample divided by optical density of negative control. Individual numbers represent hybridoma cell lines



[a]Number of negative supernatants.

FIGURE 2. Enzyme-linked immunosorbent assays on supernatants of 64 hybridoma colonies (fusion 02). Heat-killed Mycobacterium bovis (50 ug per well) was used as antigen. Monoclonal antibody binding was detected using goat anti-mouse IgG (H+L) peroxidase labeled antibody. Results are expressed as a ratio of optical density of test sample divided by optical density of positive control at various time intervals. Individual numbers represent hybridoma cell lines. Cell lines above dashed line indicate stable hybridomas producing monoclonal antibodies

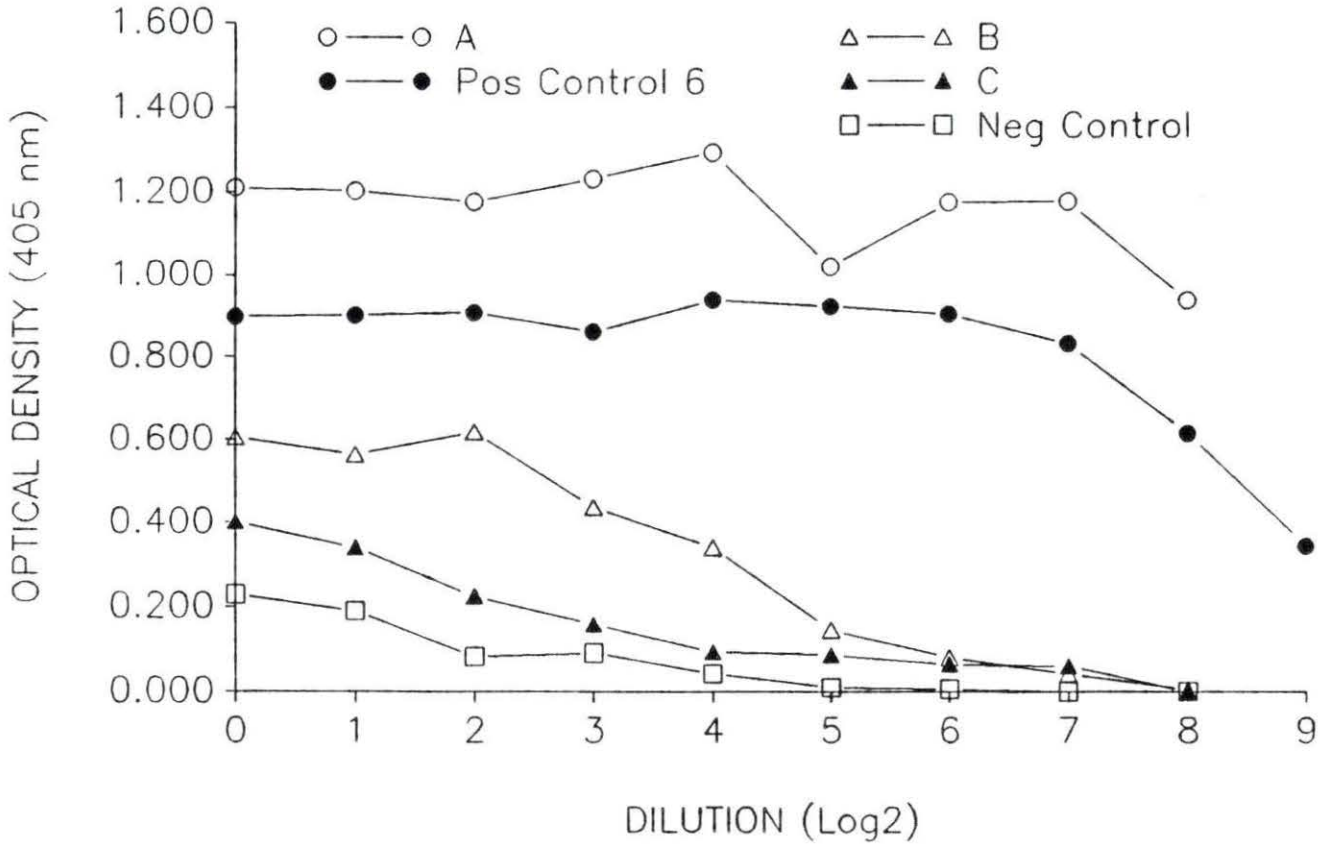


[a]Number of negative supernatants.

from four cell lines (numbers 1, 27, 31, and 49) had OD values equal to or greater than the OD value of the positive control after 30 minutes and 60 minutes. The supernatants of four other cell lines (numbers 2, 34, 58, and 60) had OD ratios of 0.75 to 1.0. The eight cell lines with OD ratios greater than or equal to 0.75 remained viable and continued to produce antibody. Therefore, a ratio of 0.75 or greater was considered a positive reaction indicating production of McAb to M. bovis.

Serial dilutions of supernatants from hybridoma colonies obtained from fusion 03 were evaluated using ELISA (Figure 3). A dilution of 1:10 was optimal for screening fusion supernatants. Results of evaluation on three supernatants representing high, intermediate, and low antibody activity to M. bovis activity (A, B, and C), a positive control (cell line 6), and a negative control (media control; no colony present) were compared. At a 1:10 dilution, antibody activity to M. bovis of supernatant C was similar to the negative control. The OD values of supernatant A and positive control supernatant also remained unchanged at this dilution. Supernatant C had intermediate antibody activity. Supernatants at serial dilution 1:10 were used in ELISA to screen fusion 06.

FIGURE 3. Results of enzyme-linked immunosorbent assays on three supernatants (fusion 03) as compared to positive and a negative control (A= high antibody activity to *M. bovis*, B= intermediate, and C= low antibody activity to *M. bovis*). Heat-killed *Mycobacterium bovis* (50 ug per well) was used as antigen. Serial dilutions (50 ul) of fusion supernatants and control supernatants were used. Antibody binding was detected using goat anti-mouse IgG (H+L) peroxidase labeled antibody. Optical density was recorded at 30 minutes


A
Pos Control 6
B
C
Neg Control
OPTICAL DENSITY (405 nm)
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
1
2
3
4
5
6
7
8
9
DILUTION (Log2)

DISCUSSION

Comparison of fusion experiments 01 and 02 indicates that use of mice further immunized with 5 mg heat killed M. bovis ATCC 19210 at day 55 and 15 mg at day 62 resulted in a greater number of supernatants with positive ELISA reactions. The use of an inoculum of 1 mg M. bovis may not have been adequate to immunize mice. The spleen of the mouse used in fusion 02 was larger than the spleen of the mouse used in fusion 01 suggesting improved immunization. Since about 90% of wet weight of bacterial cells is water, the number of remaining antigenic determinants may have been inadequate to stimulate a humoral response. The longer immunization period for fusion 02 may also have influenced the efficiency of McAb production. Although the number of hybridoma colonies obtained after fusion 01 was slightly greater than the number obtained after fusion 02, the percentage of hybridomas producing antibody with activity to M. bovis was greater after fusion 01. Antigen preparations used to produce McAb in other studies have included supernatant from sonicated M. bovis (50 ug),[9] M. tuberculosis whole cells ($2X10^7$ and $1X10^8$),[5] bacterial press extract from M. tuberculosis (100 ug),[11] and sonicated heat killed M. bovis (1 mg) in adjuvant[8].

Production of larger numbers of hybridoma colonies allows selection of McAb to different determinants. For fusion 01 and 02, 197 colonies out of 768 wells seeded and 142 out of 1152 wells represents

26% and 12% of the total number of wells seeded that contain colonies. Normal spleen cells and macrophages from a nonimmunized mouse were used as "feeder cells" in fusion 01 and 02. Approximately 90% of the seeded wells contained hybridoma colonies when FOX-NY myeloma cells where used as "feeder cells" in fusion 06. The increased number of hybridomas generated after fusion 06 may have resulted from improved cell culture conditions through release of cellular components from "feeder" myelomas that enhance growth. Additional myeloma cells may have allowed for further fusing of spleen cells possessing cell membranes affected by PEG. Cell cultures supplemented with myeloma cells showed improved growth. Fusion experiments using mice immunized with whole cells or sonicate of M. tuberculosis, sonicate of M. leprae, or a sonicate of M. bovis have resulted in 90% to 100% hybridomas.[5] These findings are simular to those reported herein. Efficient myeloma-spleen cell fusing techniques provide an increased population of hybridomas which is advantageous when producing McAb to numerous antigenic determinants.

The ELISA allows rapid screening of hybridoma colonies and selection of hybridomas producing antibody to target antigen. Optimizing parameters in ELISA provided for accurate detection of antibody activity to antigens of heat-killed M. bovis. This investigation indicates that conjugate antibody-substrate reaction time influences the distinction between antibody binding to M. bovis and nonspecific binding of mouse antibody and/or of conjugate. The number

of hybridoma supernatants with positive reactions (OD ratios equal to or greater than 2) did not increase after 30 minutes. A 30 minute time interval after initiation of enzyme-substrate reaction is considered adequate to allow detection of antibodies against M. bovis. High OD ratios indicate high levels of antibody, implying stable growth of hybridomas and antibody production. Hybridoma cell line 6 remained viable and supernatant containing VMB6 was used as a positive control in subsequent fusion experiments. The criteria of evaluating hybridoma supernatants by using VMB6 to calculate an OD ratio, with a positive reaction equal to or greater than 0.75, was considered acceptable. Ratios of OD values less than 0.75 indicate cell lines that loose the ability to produce antibody.

To minimize growth of fungi in prolonged cell culture, culture media was supplemented with amphotericin B. Amphotericin B did not affect hybridoma viability when used at 3 mg/ml in DMEM-AAT as indicated by survival of 12 of 15 cell lines. Death of hybridomas upon transfer from fusion cultures may be due to other factors such as selection of unstable hybridomas or unfused B-cells.

An attempt to improve efficiency of screening for McAb using ELISA was made to limit unnecessary selection and manipulation of numerous cell lines. The number of positive ELISA reactions were reduced by using a 1 to 10 dilution of supernatants from fusion cultures. Positive reactions, other than actual production of antibody from hybridomas, may result from initial production of antibody followed by

death of unstable hybridomas or residual antibody from unfused primed B-cells secreting antibody into the culture medium. In addition, newly formed hybridomas contain chromosomes from both parent cells and in early stages of hybridoma proliferation one complement of chromosomes can be lost[7,2]. Loss of light chain synthesis corresponding to antibody from the B-cell can result in viable hybridomas producing McAb that no longer exhibit binding activity for the target antigen. Dilution of fusion supernatant (1:10 with ELISA diluent) reduced residual antibodies to levels so that negative OD ratios were obtained as compared to positive values for control VMB6.

Hybridomas secreting monoclonal antibodies with anti-M. bovis activity were produced and detected using methods described herein. Monoclonal antibodies produced to M. bovis can be used to identify antigenic determinants, and corresponding antigens detected can be investigated further for the ability to stimulate immune responces or for use in detecting exposure to M. bovis.

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SECTION II. PRODUCTION AND PARTIAL CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO VIRULENT *Mycobacterium bovis*

SUMMARY

Monoclonal antibodies were produced to virulent M. bovis ATCC 19210 using a suspension of heat inactivated whole cells. Robertsonian (8.12) strain mice were given intraperitoneal injections at seven day intervals for six weeks. Hyperimmunized mice were boosted and spleen cells were fused with mouse FOX-NY myeloma cells. Hybridoma cell supernatants were screened for activity to the homologous strain of M. bovis using an enzyme-linked immunosorbent assay (ELISA). Heat-inactivated M. bovis cells were bound to microtiter plates by wet binding overnight at 4°C. Six monoclonal antibodies where chosen for characterization. Immunoglobulin isotypes were IgG1 for McAb VMB6, VMB73, and VMB93 and IgG2a for VMB31, VMB99, and VMB119 as determined by using isotype antisera. Monoclonal antibodies were examined for cross reactivity by using heat-inactivated M. tuberculosis, M. kansasii, M. fortuitum, M. paratuberculosis, M. avium serovars 1, 2, 4, 8, and 10, M. chelonei, M. phlei, M. scrofulaceum, M. smegmatis, N. asteroides, and, R. equi. The six McAb could be grouped on the basis of binding activity in ELISA and immunoblot where VMB6, VMB31, and VMB119 exhibited binding activity limited to M. bovis; VMB93 and VMB99 detected M. bovis and M. tuberculosis antigens, and VMB73 reacted with other mycobacterial species as well as with N. asteroides, and R. equi. Apparent molecular weight of antigens are 30 to 25 kilo (k) for VMB6, VMB31, and VMB119, and 63 k for VMB93 and VMB99, and ranged from >200

to 31 k for VMB73 as estimated by immunoblot techniques. Monoclonal antibody binding activity to 18 field isolates of M. bovis, obtained from different animal species across the United States, was evaluated in ELISA. Each of the 18 field isolates were detected using VMB6, VMB31, or VMB119, ten of the isolates were detected by VMB93/VMB99, and minimal binding activity to 14 field isolates was exhibited by VMB73. The McAb failed to detect antigens from M. bovis strain AN-5 in ELISA. Mycobacterium bovis strain AN-5 is used in production of reference M. bovis purified protein derivative (PPD) tuberculin utilized for testing cattle in the United States and several other countries.

INTRODUCTION

In past years, researchers have attempted to develop serological diagnostic tests for tuberculosis in humans[1] and in cattle.[20] Success in obtaining a reliable, sensitive, and convenient test system for cattle has been limited.[26] A common problem with serodiagnosis has been lack of specificity, occurring as negative results in infected animals or positive results in healthy animals.[5] Possible explanations for these observations could be the absence of humoral response at certain stages of infection or, in regards to the serological assay, lack of purified specific antigens and use of polyclonal antisera that can not identify individual antigenic determinants among complex common antigens of mycobacteria. Preparation of purified antigens from mycobacteria with specific antigenic determinants would improve skin testing and ELISA for diagnosis of bovine tuberculosis. Monoclonal antibodies (McAb) have the characteristic of identifying a single antigenic determinant[8]; therefore, a species specific McAb would allow detection of the corresponding antigen of M. bovis. Thus, McAb make it possible to identify and isolate an antigen for use in serodiagnosis.

Mycobacterium bovis is responsible for bovine tuberculosis[18,21] and virulent M. bovis ATCC 19210 is the neotype strain.[6] During the process of infection, antibodies are produced by the host animal and are directed against antigenic determinants of M. bovis.[22,23] This polyspecific antisera can be associated with the degree of exposure of

an animal to M. bovis. High antibody titers to M. bovis indicates exposure. Antigen with a specific determinant of M. bovis and the corresponding specific antibody can be used in assays to quantitate the level of antibodies in sera of animals in attempt to predict infection. This could result in greater sensitivity and an ability to detect antibodies in the early course of mycobacterial infection.

The objectives of this investigation were to a) produce McAb to virulent M. bovis ATCC 19210, b) determine isotype of McAb, c) obtain information on the binding activity of McAb by using different species of mycobacteria, d) estimate molecular mass of corresponding antigens, and e) use of McAb in comparing field isolates of M. bovis.

MATERIALS AND METHODS

Bacteria

Mycobacterium bovis ATCC 19210, M. tuberculosis ATCC 27294 (H37Rv), and M. paratuberculosis ATCC 19698 are neotype strains and were obtained from the American Type Culture Collection. Mycobacterium avium complex (serovars 1, 2, 4, 8, and 10), M. chelonei, M. fortuitum, M. kansasii, M. phlei, M. scrofulaceum, M. smegmatis, and 21 M. bovis field isolates were obtained from the National Veterinary Services Laboratories, U.S. Department of Agriculture, Ames, IA. The geographical location and species origin of the M. bovis isolates are listed in Table 1. Mycobacterium bovis strain AN-5, M. bovis BCG strain Pasteur, Nocardia asteroides, and Rhodococcus equi were obtained from the Department of Veterinary Microbiology and Preventive Medicine culture collection, College of Veterinary Medicine, Ames, IA. Organisms were subcultured on Middlebrook & Cohn 7H10 agar base medium[10] (BBL Microbiology Systems, Cockeysville, MD) with pyruvate (8.2 g/L), L-asparagine (5.0 g/L), L-glutamic acid (6.0 g/L), and dextrose (30 g/L) with incubation at 37°C.

Antigen Preparation

Cells were harvested after 6 to 8 weeks incubation and washed twice by resuspension in sterile, deionized, and distilled H_2O and by

TABLE 1. Isolate number, geographic location, and species of origin for 21 *M. bovis* field isolates

Isolate number	State	Species of Origin
1	TX	Antilocapra
2	NY	Wisent
3	MD	Antilocapra
4	TX	Bovine
5	NC	Bovine
6	NM	Bovine
7	HI	Porcine
8	HI	Porcine
9	KS	Bovine
10	LA	Bovine
11	TX	Bovine
12	IA	Bovine
13	VA	Bovine
14	NE	Bovine
15	TX	Bovine
16	CA	Bovine
17	CA	Bovine
18	NC	Bovine
19	HI	Porcine
20	OH	Bovine
21	SD	Bison

centrifugation at 10,000 rpm. The resulting pellet was weighed, resuspended in sterile phosphate buffered saline (PBS) to a cell suspension of 50 mg/ml (wet weight), and autoclaved for 10 minutes at 121°C. Antigens were prepared by the above method for all bacterial species, except *R. equi* which was not heat-killed, and stored at -20°C. The heat killed whole cell (HKWC) antigen was used for immunizing mice, ELISA evaluations, and in immunoblot procedures.

Immunization and Hybridoma Production

Female Robertsonian (8.12) 5 Bnr [Rb(8.12) mouse] mice[17] (Jackson Labs, Bar Harbor, MA) 8 to 12 weeks old were immunized on a schedule of 100 ul (5 mg) of HKWC *M. bovis* antigen given by intraperitoneal injection at one-week intervals for a total of 6 injections. Three days prior to fusion, mice where boosted with 200 ul (15 mg) HKWC *M. bovis* antigen adjusted to 75 mg/ml PBS.

Spleens from boosted mice were aseptically removed, rinsed in sterile PBS, transferred to a petri dish containing 10 ml of sterile PBS, and minced to obtain separate spleen cells. The spleen cell suspension was transferred to a centrifuged tube and FOX-NY myeloma cells (Hyclone Labs, Logan, UT) were mixed in at a ratio of 1:2, myeloma to spleen cell.[17] The myeloma/spleen cell suspension was centrifuged at 300 rpm for 10 minutes, supernate was discarded, and 0.5 ml of 45% polyethylene glycol 1540 (EM science, Gibbstown, NY) in sterile PBS was added. Fusion was allowed for 60 seconds at 37°C, and then the pellet was diluted in 20 ml of Dulbecco's Minimum Essential Medium[15] (Gibco Ltd., Grand Island, NY) (DMEM). To select for myeloma-B lymphocyte hybrids, the fused cell suspension was added to DMEM containing 7.5×10^{-5}M adenine, 8×10^{-7}M aminopterin, 1.6×10^{-5}M thymidine, streptomycin sulfate (100 mg/L), penicillin (1×10^{5} units/L), and 15% horse serum (DMEM-AAT), and distributed into 96-well tissue culture plates (Costar, Cambridge, MA). Hybridoma cell cultures were incubated in a humid, 5% CO_2 atmosphere at 37°C. After 8 to 10 days, hybridoma

supernatants were screened for the presence of antibody with activity to M. bovis HKWC using an ELISA. Hybridoma colonies producing antibody that detected M. bovis antigens were transferred to 24-well plates (Costar, Cambridge, MA), screened again for positive activity, and recloned twice by limiting dilution in 96-well plates using DMEM-AAT with amphotericin B added 3 mg/L (DMEM-AATF). Single colonies, corresponding to supernatants with strong positive ELISA reactions to M. bovis, were transferred to 24-well plates, then to 25 cm^2 tissue culture flasks (Costar, Cambridge, MA), and maintained in cell culture using DMEM-AATF. Aliquotes were cryopreserved at -70 C and in liquid nitrogen by using DMEM containing 15% horse serum and 10% dimethylsulfoxide (DMSO).

Enzyme-Linked Immunosorbent Assay

A modified ELISA was used to detect McAb in supernatant from hybridoma colonies.[19,20] Mycobacterium bovis ATCC 19210 HKWC antigen was used for hybridoma supernatant screening at a dilution of 100 ul (5 mg) in 5.0 ml 0.1 M Na_2CO_3, pH 9.6. The antigen solution (50 ul per well) followed by cyanamide 1 mg/ml 0.1 M Na_2CO_3, pH 9.6 (50 ul per well) were added to Immulon I microtiter plates (Dynatech, Alexandria, VA). Plates were incubated at 4^oC for 16 hours, and washed three times with PBS pH 7.2 prior to addition of 0.1 M NH_4Cl (100 ul per well) and incubation at room temperature (RT) for 30 minutes. The antigen-coated plates were then washed three times with phosphate buffer containing

0.5 M NaCl and 0.5% Tween 80, pH 7.5 (ELISA wash). Hybridoma supernatant and negative control samples at a serial dilution of 1:10 in phosphate buffer containing 0.5 M NaCl and 1.0% Tween 80, pH 7.5 (diluent) was added (50 ul per well) and incubated with shaking at RT for 1 hour. Negative controls included DMEM-AATF, conditioned medium from FOX-NY myeloma cell cultures (DMEM-CM), and horse serum diluted to 15% in diluent. The plates were then washed eight times with ELISA wash and goat anti-mouse IgG (H+L) horseradish peroxidase conjugate antibody (Kirkegaard & Perry Labs, Gaithersburg, MD), at a serial dilution of 1:500 in diluent, was added (50 ul per well) followed by incubation with shaking at RT for 1 hour. Plates were washed eight times with ELISA wash and briefly dried to remove excess fluid. Substrate/chromagen solution consisting of 0.4% H_2O_2, and 2,2'-azino-di-3-ethyl-benzthiazoline sulfonate (ABTS) in 0.05 M citric acid, pH 4.0 was added (100 ul per well) and absorbance at 405 nm was measured after 30 and 60 minutes.

Monoclonal antibody binding activity towards other mycobacteria, non-mycobacterial species, and field isolates of M. bovis were also evaluated by using ELISA. Each antigen preparation from mycobacterial and non-mycobacterial species was used to coat Immulon I microtiter plates. Supernatants from hybridomas producing antibody that detected M. bovis antigens were tested for specificity of antibody activity using the modified ELISA procedure as described above.

Isotype identification of McAb was determined using an ELISA

procedure as instructed by manufacturer in a Mouse Immunoglobulin Subtype Identification Kit (BMB, Indianapolis, IN). Briefly, M. bovis HKWC was used as antigen in microtiter plates followed by incubation with supernatant from VMB hybridoma cultures diluted 1 in 5 with ELISA diluent. Rabbit anti-mouse Ig of subclass specifies IgA (α), IgG_1 (γ 1), IgG_{2a} (γ2a), IgG_{2b} (γ2b), IgG_3 (γ3), and IgM (μ) were used to detect antigen-VMB complexes. Peroxidase-labeled goat anti-rabbit IgG was used to detect specific rabbit anti-mouse Ig antibody as indicated by reactions after adding a solution of ABTS.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblot

A SDS-PAGE procedure[11] was modified in which a 6 to 20% linear gradient resolving gel and 4% stacking gel was used with a discontinuous buffer system in SDS-PAGE. Samples were prepared for electrophoresis by resuspending a 5 mg pellet of HKWC antigen in 60 ul of 63 mM TRIS-HCl buffer, pH 6.8 containing SDS (3%), dithiothreitol (1%), and bromophenol blue (0.01%). Prestained molecular weight standards (BRL, Gaithersburg, MD) were also included. After heating the samples at 95°C for 20 minutes, insoluble material was removed by brief centrifugation at 14,000 rpm, and supernate was electrophoresed at 50 volts per gel constant voltage overnight. Polyacrylamide gels containing resolved components were transferred to nitrocellulose (NC) for use in immunoblotting experiments.

Resolved components were transferred to NC sheets[24] and detected by modified immunoblot techniques. Electrophoretic transfer from the polyacrylamide gel to NC was performed in 25 mM phosphate buffer, pH 7.5. Resolved components bound to NC were stained with colloidal gold total protein stain (Bio-Rad, Richman CA)[16] as described by manufacturers instructions or analyzed by McAb. The NC with bound resolved components was blocked using 0.01 M TRIS buffer, pH 7.4 containing Tween 20 (0.05%), 0.15 M NaCl, and non-fat dry milk[3] (5%) (TTS-MILK) for 1 hour. The blocked NC was incubated with McAb at a serial dilution of 1:20 or 1:100 in TTS-MILK buffer, washed in TTS-MILK buffer, and incubated with goat anti-mouse IgG (H+L) antibody conjugated with horseradish peroxidase diluted 1:1000 in TTS-MILK buffer. Detected antigen-antibody complexes bound to NC were visualized after washing in TTS-MILK buffer and development with 4-chloro-1-napthol (0.05%) in methanol-TRIS-saline buffer containing H_2O_2 (0.018%). Immunoblots were performed using M. bovis antigens and heterologous antigen preparations from other mycobacteria and non-mycobacterial species to estimate the molecular mass of antigenic components corresponding to McAb, and to detect cross reactions.

RESULTS

Monoclonal Antibodies

The number of wells containing supernatant with antibody against M. bovis, as determined by ELISA, are listed in Table 2. Out of 1152 wells seeded after the fusion experiment, approximately 90% of the

TABLE 2. Antibody activity to Mycobacterium bovis HKWC 10 days after fusion

Number of Colonies[a]	Number of Antibody Positive Wells	%
1032	182	18

[a]Total number of wells seeded was 1152.

wells contained hybridoma colonies. Each hybridoma supernatant containing VMB6 and VMB31 (obtained in preliminary fusion experiments) having ELISA reactions to M. bovis and not other species tested were used as positive controls for screening supernatants from fusion cultures. The OD values for positive controls were 6.75 (VMB6) times greater and 9.0 (VMB31) times greater than negative control values. As compared to control VMB6, 587 positive ELISA reactions were observed, where as compared to control VMB31, 182 (18% of 1032) of the supernatants were positive for antibody activity to antigens of M. bovis. Hybridoma colonies of supernatants with positive ELISA

reactions as compared to positive control VMB31 were cloned and recloned. Antibody titration curves for supernatants from recloned hybridoma cell lines 6, 31, 73, 93, 99, and 119 are illustrated in Figure 1, and endpoint titers were 10, 100, 20, 10, 8, and 500 respectively. The immunoglobulin isotype of McAb VMB6, VMB73, and VMB93 were IgG_1 and VMB31, VMB99, and VMB119 were IgG_{2a}.

ELISA

Supernatant from recloned hybridoma cell cultures was tested for antibody activity to homologous M. bovis antigen and to heterologous HKWC antigens from various species of mycobacteria using an ELISA. Taxonomic binding patterns are summarized in Table 3. In using six McAb with antibody activity to M. bovis activity, VMB6, VMB31, and VMB119 detected M. bovis antigens, cross reactivity by VMB93 and VMB99 was limited to M. tuberculosis, and VMB73 exhibited non-specific binding activity. Binding activity of VMB73 was greater towards M. tuberculosis than towards the homologous antigen used in hybridoma production. Use of VMB73 also exhibited moderate binding activity towards M. kansasii, M. fortuitum, and N. asteroides and weak binding activity towards M. avium serovars 1 and 10, M. paratuberculosis, M. phlei, M. smegmatis, and R. equi.

Figure 1. Titration curves for each of 6 monoclonal antibodies as determined using an enzyme-linked immunosorbent assay. Mycobacterium bovis heat-killed whole antigen at 50 ug per well was used as antigen and monoclonal antibody binding was detected by goat anti-mouse IgG (H+L) peroxidase-labeled antibody. Optical density was recorded at 30 minutes of enzyme-substrate reaction time and optical density values are expressed as a mean of three determinations

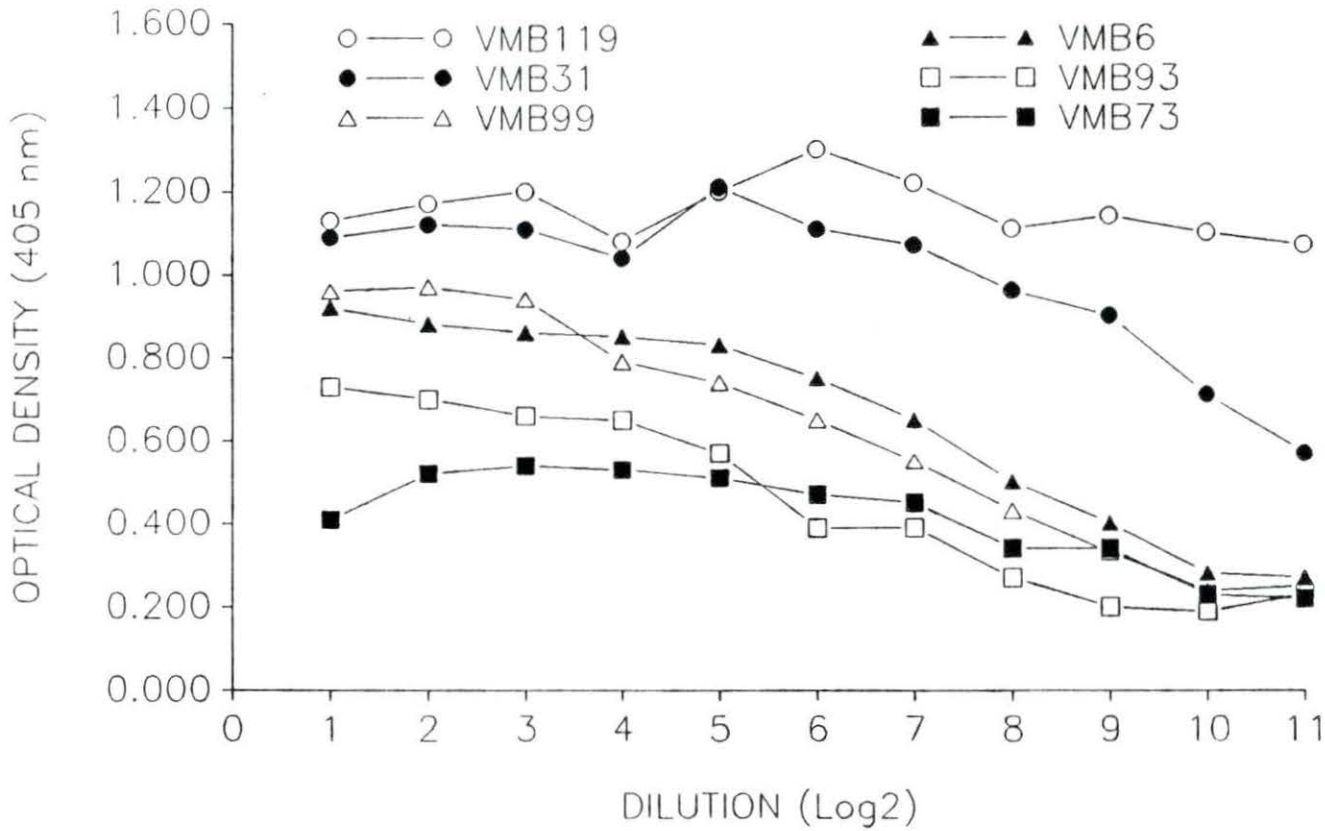

VMB119
VMB31
VMB99
VMB6
VMB93
VMB73
OPTICAL DENSITY (405 nm)
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
1
2
3
4
5
6
7
8
9
10
11
DILUTION (Log2)

Table 3. Enzyme-linked immunosorbent assay reactions of 6 monoclonal antibodies with 14 species of mycobacteria and with Nocardia asteroides or Rhodococcus equi

Species	Monoclonal Antibody					
	VMB6	VMB31	VMB73	VMB93	VMB99	VMB119
M. bovis	4.91[a]	6.22	2.53	6.45	6.53	6.10
M. tuberculosis	1.99	-	8.45	2.54	3.20	2.11
M. kansasii	-	-	4.31	-	-	-
M. fortuitum	-	-	4.02	-	-	-
M. paratuberculosis	-	-	2.78	-	-	-
M. avium serovar 1	-	-	1.86	-	-	-
M. avium serovar 2	-	-	-	-	-	-
M. avium serovar 4	-	-	-	-	-	-
M. avium serovar 8	-	-	-	-	-	-
M. avium serovar 10	-	-	2.14	-	-	-
M. chelonei	-	-	-	-	-	-
M. phlei	-	-	2.90	-	-	-
M. scrofulaceum	-	-	-	-	-	-
M. smegmatis	-	-	2.55	-	-	-
N. asteroides	-	-	4.70	-	-	-
R. equi	-	-	2.07	-	-	-

[a]Reactions are expressed as an optical density ratio of test sample optical density value to negative control optical density value. Optical density ratios equal to or less than 1.50 are indicated by -.

Supernatants containing McAb were also tested in ELISA using HKWC from 18 field isolates of M. bovis and three reference strains of M. bovis. Binding activity results are listed in Table 4. Use of VMB6, VMB31, and VMB119 exhibited positive binding activity towards each of 18 field isolates. Antigen from 10 of the isolates was detected using VMB93 and VMB99. Use of VMB73 detected antigen from 14 isolates with a

binding pattern similar to VMB93 and VMB99 binding patterns, although overall binding activity was weaker. None of these six McAb detected antigen from M. bovis strain AN-5 and only VMB73 had minimal binding activity to M. bovis BCG strain Pasteur.

Table 4. Enzyme-linked immunosorbent assay reactions of 6 monoclonal antibodies with 18 field isolates of Mycobacterium bovis and three reference strains

M. bovis	Monoclonal Antibody					
	VMB6	VMB31	VMB119	VMB93	VMB99	VMB73
ATCC 19210	4.91[a]	6.22	6.10	6.45	6.53	2.53
#1	6.91	8.45	8.64	4.92	5.75	3.50
#2	8.02	8.47	12.98	2.47	3.02	3.75
#3	4.92	7.30	7.16	-	-	2.11
#4	5.80	8.11	8.13	1.69	2.28	3.03
#5	6.42	5.47	6.01	-	-	-
#6	3.62	5.00	5.23	-	-	-
#7	8.19	9.88	7.90	7.28	9.85	2.89
#8	7.82	9.19	7.79	3.80	4.83	2.20
#9	5.63	8.69	6.71	-	-	2.62
#11	3.82	5.07	3.88	-	-	1.86
#12	7.14	8.41	6.62	7.09	8.19	2.64
#13	2.83	3.93	2.41	-	-	-
#14	4.21	6.38	5.40	-	-	1.74
#15	2.74	3.44	4.15	-	-	-
#17	5.36	5.49	7.13	1.74	2.15	2.13
#19	3.98	3.99	4.42	3.06	3.83	2.57
#20	4.77	4.88	6.86	3.76	6.01	2.46
#21	7.90	8.18	9.22	5.35	7.14	2.4
AN-5	-	-	-	-	-	-
Pasteur	-	-	-	-	-	1.95

[a]Reactions are expressed as an optical density ratio of test sample optical density value to negative control optical density value. Optical density ratios less than 1.50 are indicated by -.

SDS-PAGE and Immunoblot

Mycobacterial HKWC antigens and non-mycobacterial antigens were heated in the presence of SDS under reducing conditions, separated by polyacrylamide gel electrophoresis, and transferred to NC sheets as described above. Bacterial components bound to NC were stained for total protein (Figure 2). Molecular weight standards consisted of myosin (H-chain), phosphorylase B, bovine serum albumin, ovalbumin, a-chymotrypsinogen, B-lactoglobulin and lysozyme with apparent molecular weight 197,000, 104,000, 72,200, 44,800, 28,400, 18,000, and 14,600, respectively.

The NC bound M. bovis antigens were analyzed using VMB6, VMB31, VMB73, VMB93, VMB99, and VMB119 (Figure 3). VMB6 detected a major antigenic component with an apparent molecular weight in the range approximately 30 to 25 kilo (k) and faint smears were also visible at approximately 54 k and 17 k. Similarly, VMB31 and VMB119 detected antigenic components in a range of 30 k to 25 k. Smearing was also visible at 55 k and 18 k after detection with VMB31 and at 55 k and 17 k with VMB119. One antigenic component was detected by each VMB93 and VMB99 having molecular weight of 63 k. Weak smearing was visible at approximately 200 k to 31 k when VMB73 was used to detect NC bound antigens.

Nitrocellulose bound components from mycobacteria other than M. bovis were also analyzed using each McAb. Use of VMB6, VMB31, and

FIGURE 2. Protein analysis of bacterial components.
Bacterial components from heat-killed whole cells were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Protein was detected by colloidal gold total protein stain.
Lanes: A, prestained molecular weight standards;
B, *M. bovis*;
C, *M. tuberculosis*;
D, *M. kansasii*;
E, *M. fortuitum*;
F, *M. paratuberculosis*;
G, *M. avium* serovar 1;
H, *M. avium* serovar 2;
I, *M. avium* serovar 4;
J, *M. avium* serovar 8;
K, *M. avium* serovar 10;
L, *M. chelonei*;
M, *M. phlei*;
N, *M. scrofulaceum*;
O, *M. scrofulaceum*;
P, *M. scrofulaceum*;
Q, *M. smegmatis*;
R, *N. asteroides*;
S, *R. equi*

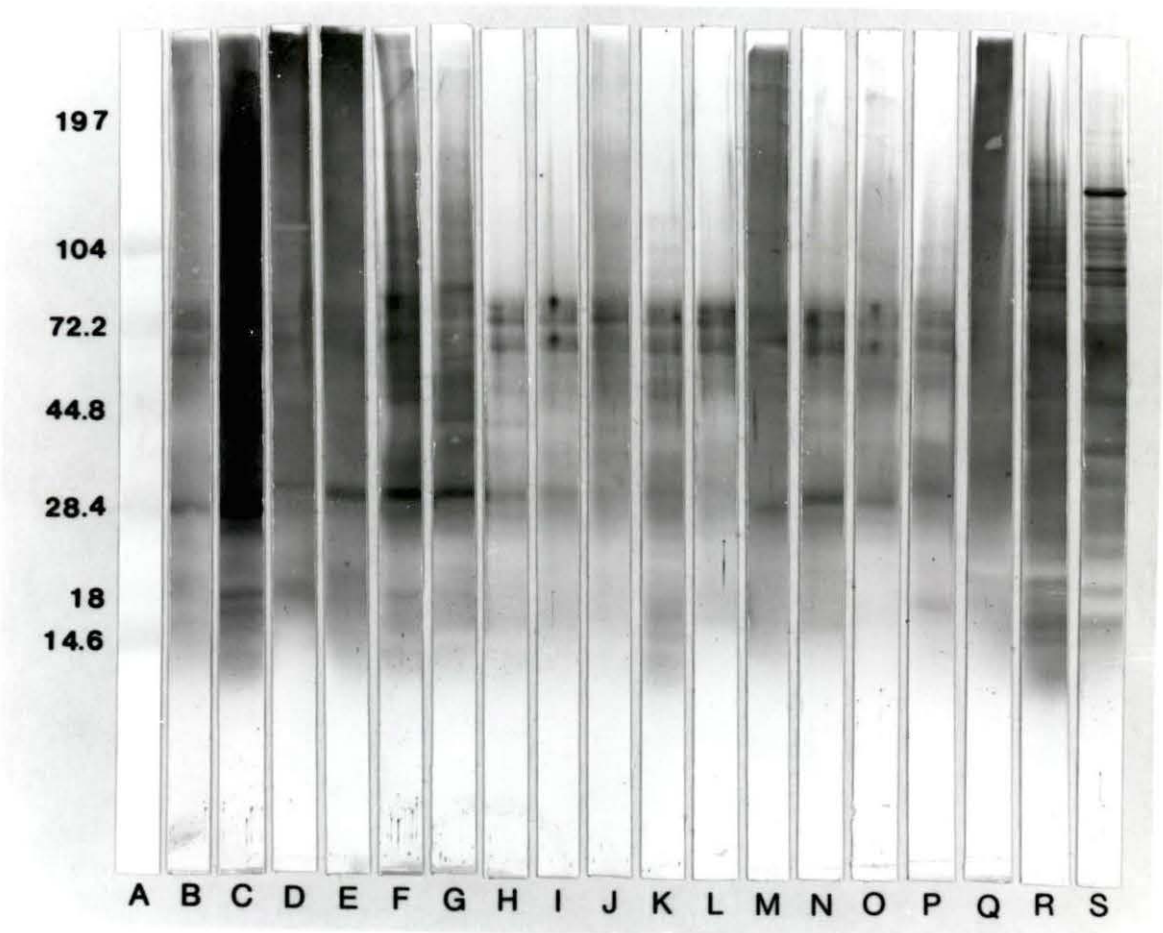
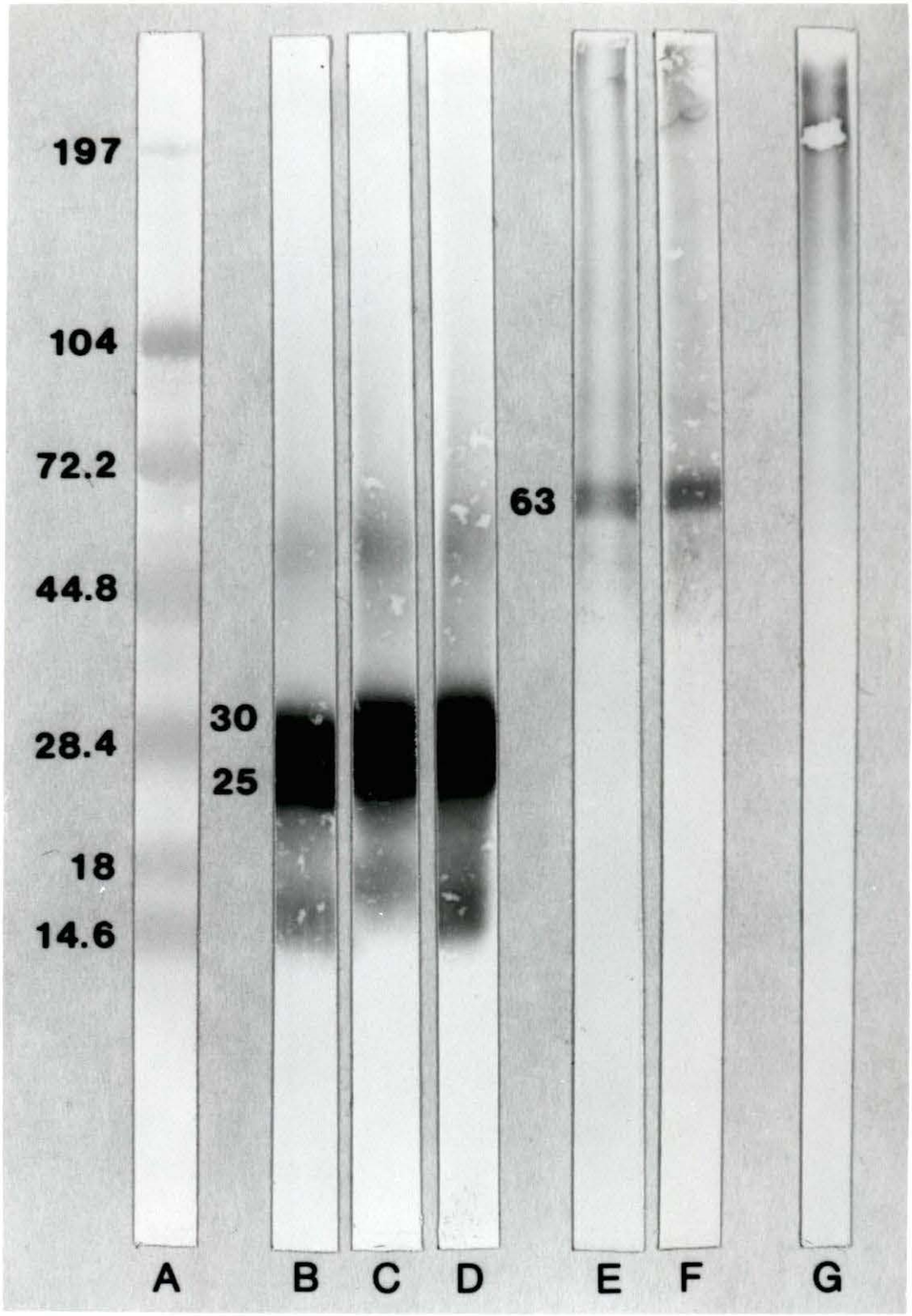

197
104
72.2
44.8
28.4
18
14.6
A B C D E F G H I J K L M N O P Q R S

FIGURE 3. Immunoblot for each of six monoclonal antibodies using Mycobacterium bovis heat-killed whole cell antigen. Culture supernatants containing monoclonal antibody were used and antibody reactivity was detected using goat anti-mouse IgG (H+L) peroxidase-labeled antibody.
Lanes: A, prestained molecular weight standards;
B, VMB6 (1:100);
C, VMB31 (1:100);
D, VMB119 (1:100);
E, VMB93 (1:20);
F, VMB99 (1:20);
G, VMB73 (1:20)


197
104
72.2
44.8
28.4
18
14.6
30
25
63
A
B
C
D
E
F
G

VMB119 detected antigens from M. kansasii (Figures 4, 5, and 6), and VMB93 and VMB99 detected M. tuberculosis antigens (Figures 7 and 8). VMB73 detected antigens from M. tuberculosis, M. kansasii, M. fortuitum, M. paratuberculosis, M. avium serovars 1 and 10, M. smegmatis, N. asteroides, and R. equi (Figure 9). Apparent molecular weight of antigenic components detected by use of McAb with binding activity to M. bovis are summarized in Table 4. VMB6, VMB31, and VMB119 faintly detected M. kansasii components with molecular weight 34 k, 32 k, and 31 k respectively. VMB93 and VMB99 each detected a component from M. tuberculosis having molecular weight 58 k with smearing faintly visible at approximately 49 k. Molecular weight of antigens detected by use of VMB73 were greater than 200 k, 65 k, 49 k, 43 k, 38 k, 32 k, 29 k, 25 k and 18 k from M. tuberculosis, and 65 k and 22 k from M. kansasii. VMB73 also detected M. fortuitum antigens with molecular weight 65 k, 53 k, 48 k, 42 k, 37 k, 25k, and 15 k. A 72 k antigen from M. paratuberculosis and M. avium serovar 1 was detected by use of VMB73 as well as a 19 k component from M. avium serovar 10. Components from M. smegmatis, detected by use of VMB73, had molecular weight of 25 k, 17 k, and 13 k. VMB73 also detected numerous antigenic components from N. asteroides with molecular weight ranging from 65 k to 10 k, and a single R. equi component of 65 k.

FIGURE 4. Immunoblot analysis of bacterial components using VMB6. Bacterial components from heat-killed whole cells were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Culture supernatant containing monoclonal antibody was used and antibody reactivity was detected using goat anti-mouse IgG (H+L) peroxidase-labeled antibody. Molecular weight (k) of antigenic components are indicated in lane space.

Lanes: A, prestained molecular weight standards;
B, *M. bovis*;
C, *M. tuberculosis*;
D, *M. kansasii*;
E, *M. fortuitum*;
F, *M. paratuberculosis*;
G, *M. avium* serovar 1;
H, *M. avium* serovar 2;
I, *M. avium* serovar 4;
J, *M. avium* serovar 8;
K, *M. avium* serovar 10;
L, *M. chelonei*;
M, *M. phlei*;
N, *M. scrofulaceum*;
O, *M. scrofulaceum*;
P, *M. scrofulaceum*;
Q, *M. smegmatis*;
R, *N. asteroides*;
S, *R. equi*

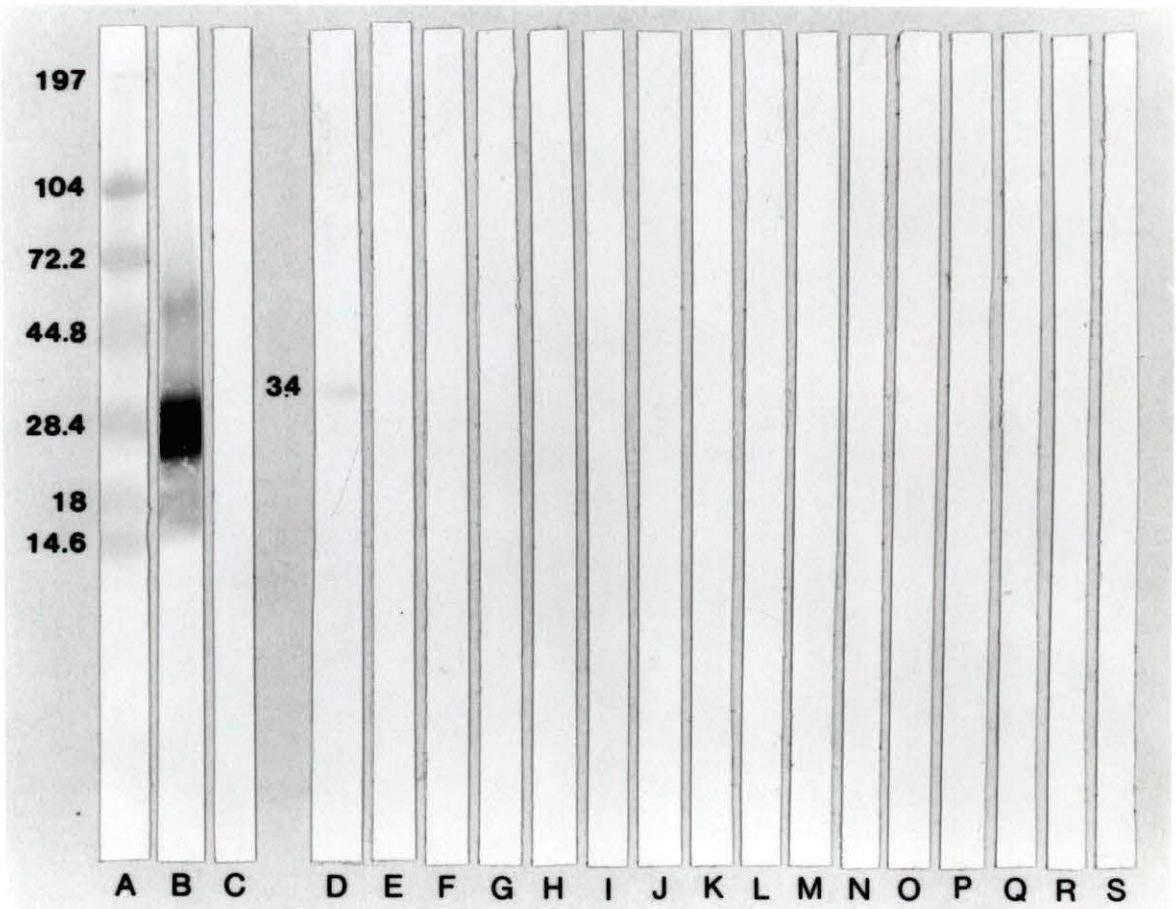

197
104
72.2
44.8
34
28.4
18
14.6
A B C D E F G H I J K L M N O P Q R S

FIGURE 5. Immunoblot analysis of bacterial components using VMB31. Bacterial components from heat-killed whole cells were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Culture supernatant containing monoclonal antibody was used and antibody reactivity was detected using goat anti-mouse IgG (H+L) peroxidase-labeled antibody. Molecular weight (k) of antigenic components are indicated in lane space.

Lanes: A, prestained molecular weight standards;
B, *M. bovis*;
C, *M. tuberculosis*;
D, *M. kansasii*;
E, *M. fortuitum*;
F, *M. paratuberculosis*;
G, *M. avium* serovar 1;
H, *M. avium* serovar 2;
I, *M. avium* serovar 4;
J, *M. avium* serovar 8;
K, *M. avium* serovar 10;
L, *M. chelonei*;
M, *M. phlei*;
N, *M. scrofulaceum*;
O, *M. scrofulaceum*;
P, *M. scrofulaceum*;
Q, *M. smegmatis*;
R, *N. asteroides*;
S, *R. equi*

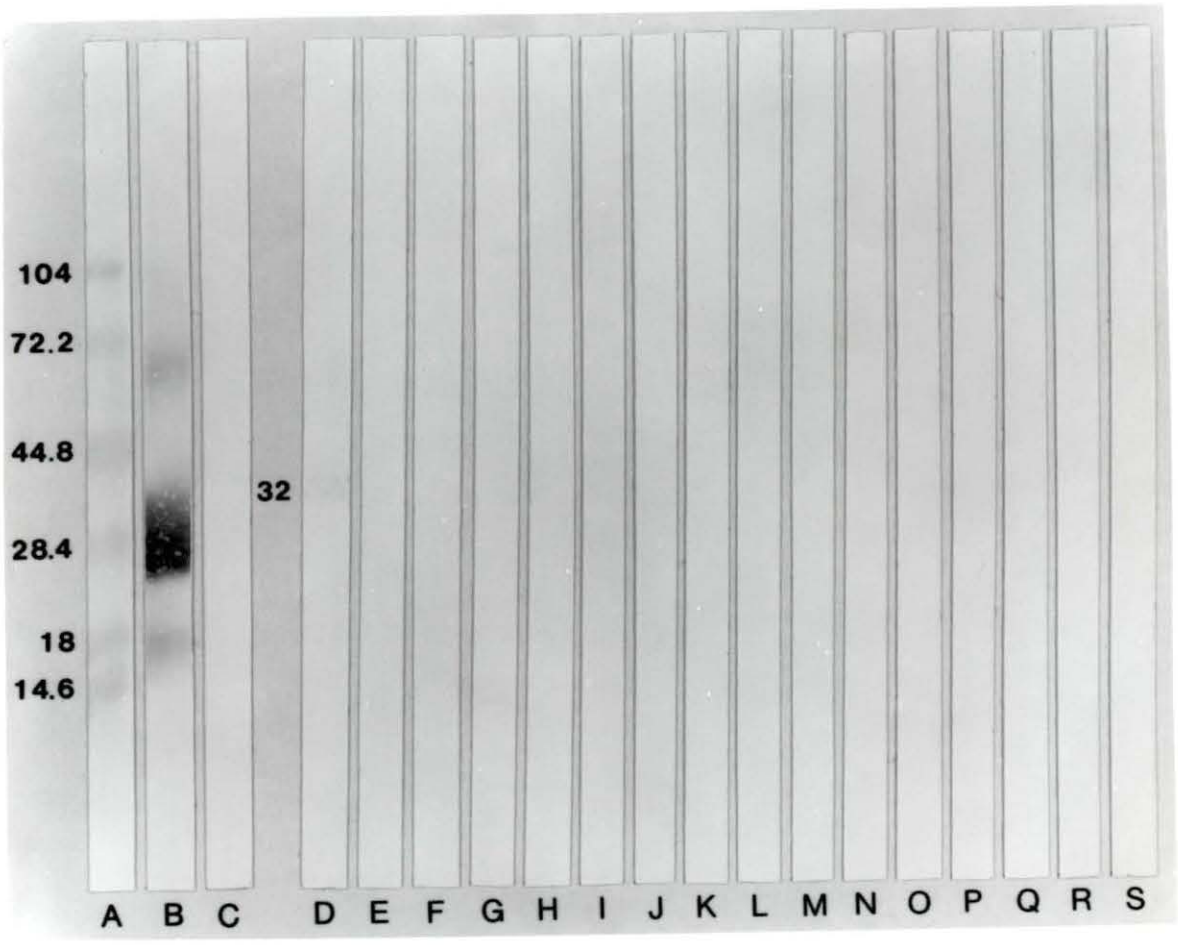

104
72.2
44.8
28.4
18
14.6
32
A B C D E F G H I J K L M N O P Q R S

FIGURE 6. Immunoblot analysis of bacterial components using VMB119. Bacterial components from heat-killed whole cells were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Culture supernatant containing monoclonal antibody was used and antibody reactivity was detected using goat anti-mouse IgG (H+L) peroxidase-labeled antibody. Molecular weight (k) of antigenic components are indicated in lane space.

Lanes: A, prestained molecular weight standards;
B, *M. bovis*;
C, *M. tuberculosis*;
D, *M. kansasii*;
E, *M. fortuitum*;
F, *M. paratuberculosis*;
G, *M. avium* serovar 1;
H, *M. avium* serovar 2;
I, *M. avium* serovar 4;
J, *M. avium* serovar 8;
K, *M. avium* serovar 10;
L, *M. chelonei*;
M, *M. phlei*;
N, *M. scrofulaceum*;
O, *M. scrofulaceum*;
P, *M. scrofulaceum*;
Q, *M. smegmatis*;
R, *N. asteroides*;
S, *R. equi*

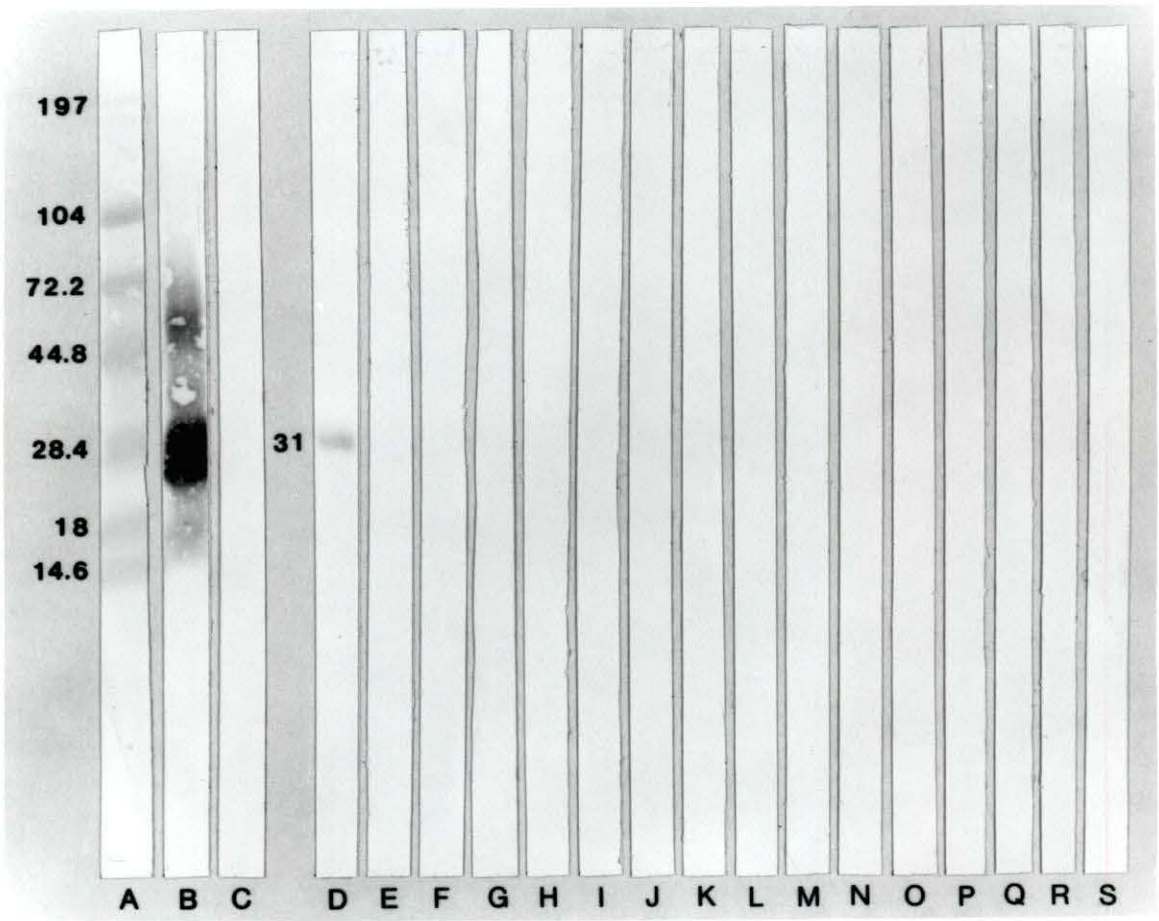

197
104
72.2
44.8
28.4
18
14.6
31
A B C D E F G H I J K L M N O P Q R S

FIGURE 7. Immunoblot analysis of bacterial components using VMB93. Bacterial components from heat-killed whole cells were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Culture supernatant containing monoclonal antibody was used and antibody reactivity was detected using goat anti-mouse IgG (H+L) peroxidase-labeled antibody. Molecular weight (k) of antigenic components are indicated in lane space.

Lanes: A, prestained molecular weight standards;
B, *M. bovis*;
C, *M. tuberculosis*;
D, *M. kansasii*;
E, *M. fortuitum*;
F, *M. paratuberculosis*;
G, *M. avium* serovar 1;
H, *M. avium* serovar 2;
I, *M. avium* serovar 4;
J, *M. avium* serovar 8;
K, *M. avium* serovar 10;
L, *M. chelonei*;
M, *M. phlei*;
N, *M. scrofulaceum*;
O, *M. scrofulaceum*;
P, *M. scrofulaceum*;
Q, *M. smegmatis*;
R, *N. asteroides*;
S, *R. equi*

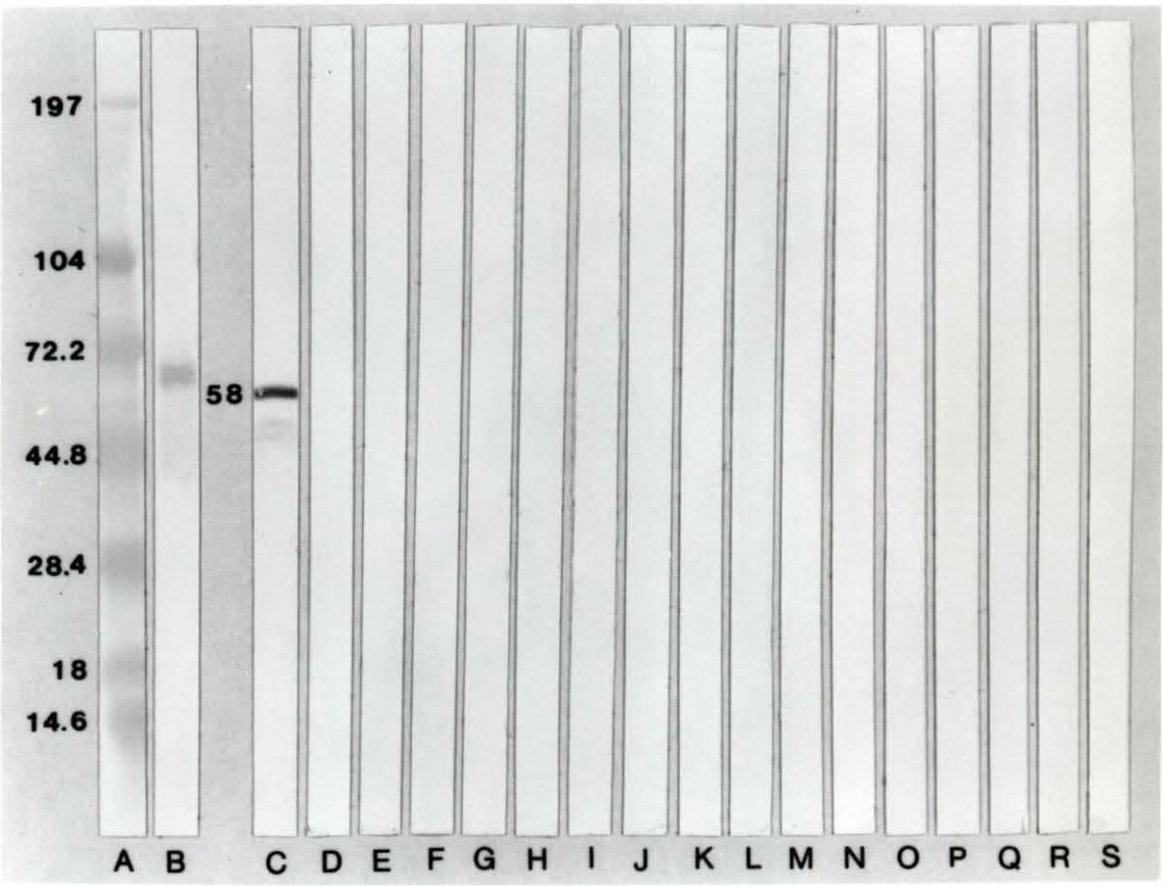

197
104
72.2
58
44.8
28.4
18
14.6
A B C D E F G H I J K L M N O P Q R S

FIGURE 8. Immunoblot analysis of bacterial components using VMB99. Bacterial components from heat-killed whole cells were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Culture supernatant containing monoclonal antibody was used and antibody reactivity was detected using goat anti-mouse IgG (H+L) peroxidase-labeled antibody. Molecular weight (k) of antigenic components are indicated in lane space.

Lanes: A, prestained molecular weight standards;
B, *M. bovis*;
C, *M. tuberculosis*;
D, *M. kansasii*;
E, *M. fortuitum*;
F, *M. paratuberculosis*;
G, *M. avium* serovar 1;
H, *M. avium* serovar 2;
I, *M. avium* serovar 4;
J, *M. avium* serovar 8;
K, *M. avium* serovar 10;
L, *M. chelonei*;
M, *M. phlei*;
N, *M. scrofulaceum*;
O, *M. scrofulaceum*;
P, *M. scrofulaceum*;
Q, *M. smegmatis*;
R, *N. asteroides*;
S, *R. equi*

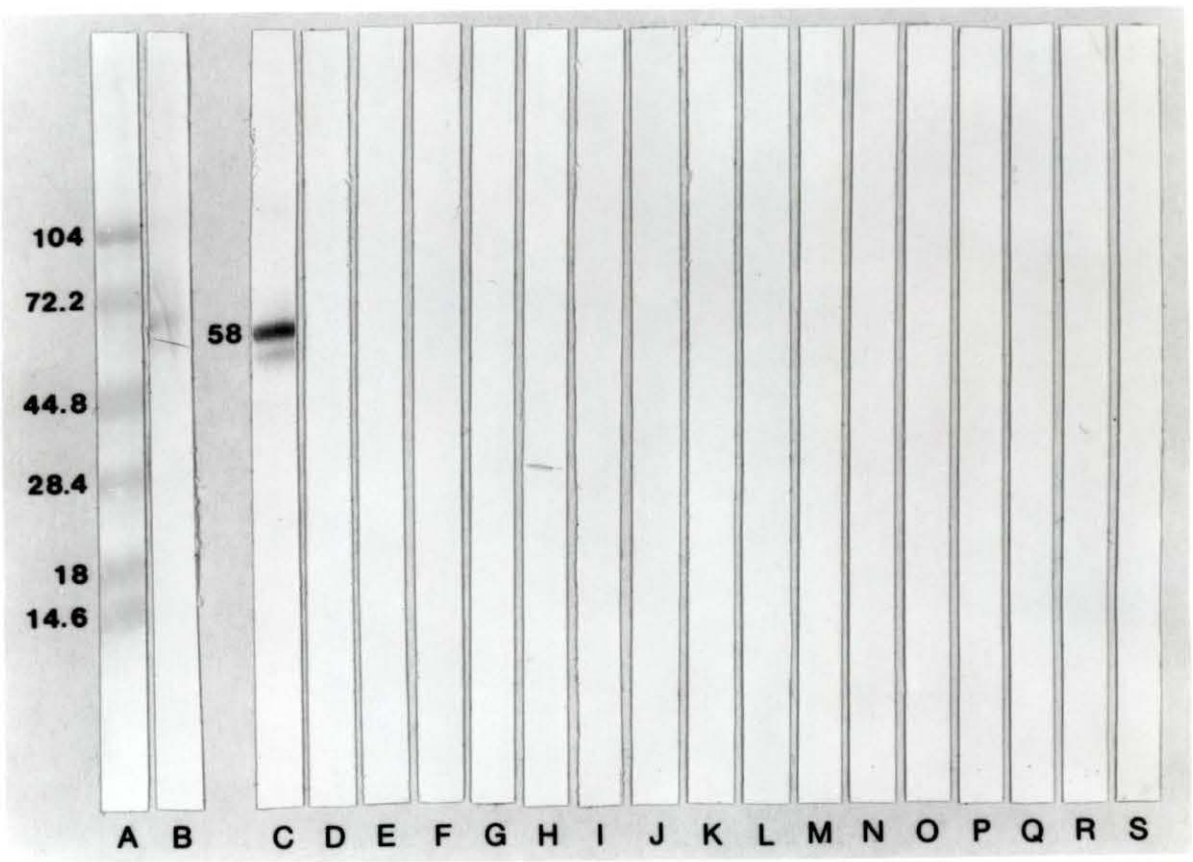
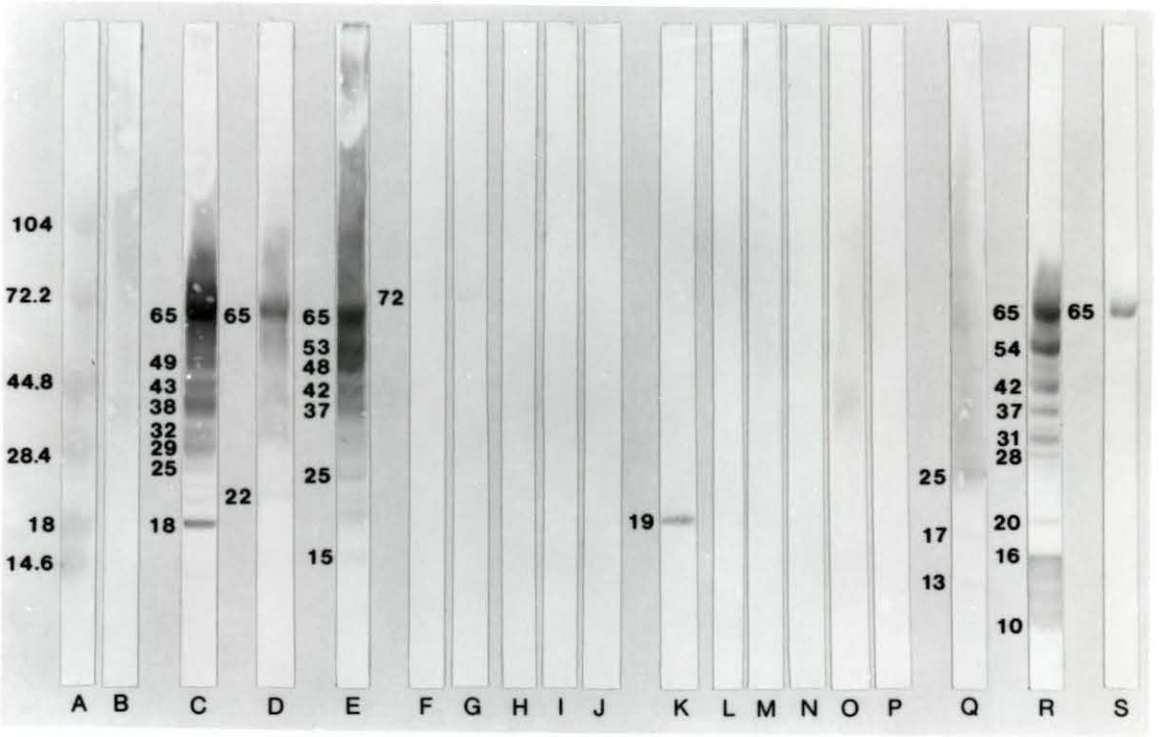

104
72.2
58
44.8
28.4
18
14.6
A B C D E F G H I J K L M N O P Q R S

FIGURE 9. Immunoblot analysis of bacterial components using VMB73. Bacterial components from heat-killed whole cells were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Culture supernatant containing monoclonal antibody was used and antibody reactivity was detected using goat anti-mouse IgG (H+L) peroxidase-labeled antibody. Molecular weight (k) of antigenic components are indicated in lane space.

Lanes: A, prestained molecular weight standards;
B, *M. bovis*;
C, *M. tuberculosis*;
D, *M. kansasii*;
E, *M. fortuitum*;
F, *M. paratuberculosis*;
G, *M. avium* serovar 1;
H, *M. avium* serovar 2;
I, *M. avium* serovar 4;
J, *M. avium* serovar 8;
K, *M. avium* serovar 10;
L, *M. chelonei*;
M, *M. phlei*;
N, *M. scrofulaceum*;
O, *M. scrofulaceum*;
P, *M. scrofulaceum*;
Q, *M. smegmatis*;
R, *N. asteroides*;
S, *R. equi*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44.8
28.4
18
14.6
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65
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43
38
32
29
25
22
18
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48
42
37
25
15
72
19
25
17
13
65
65
54
42
37
31
28
20
16
10
A B C D E F G H I J K L M N O P Q R S

TABLE 5. Estimated molecular weight of corresponding antigenic components for each of six *Mycobacterium bovis* monoclonal antibodies

Antigen	Monoclonal Antibody					
	VMB6	VMB31	VMB119	VMB93	VMB99	VMB73
M. bovis	30-25[a]	30-26	30-25	63	63	>200-31(smear)
M. tuberculosis				58	58	>200,65,49,43,38,32,29,25,18
M. kansasii	34	32	31			65,22
M. fortuitum						65,53,48,42,37,25,15
M. paratuberculosis						72
M. avium serovar 1						72
M. avium serovar 10						19
M. smegmatis						25,17,13
N. asteroides						65,54,42,37,31,28,20
						16-10(multiple bands)
R. equi						65

[a]Molecular weight values expressed in kilo.

DISCUSSION

Stringent criteria in designating positive hybridomas was adopted as elevated ELISA OD values may be considered a measure of anti-*M. bovis* activity implying stable cell line growth and antibody production. Supernatants from hybridoma cell lines 73, 93, 99, and 119 had the greatest activity in ELISA. VMB73, VMB93, VMB99, and VMB119 along with VMB6 and VMB31 were characterized by identifying immunoglobulin isotype, by determining cross reactivity to other bacteria using ELISA and immunoblot, and by estimating apparent molecular mass of corresponding antigens.

Stock supernatants containing VMB6, VMB31, VMB73, VMB93, VMB99, and VMB119 were diluted to end point titers in an attempt to adjust McAb to equal concentrations with respect to *M. bovis* binding activity in ELISA. The six McAb were placed in three groups based on results of binding activity in ELISA and immunoblot. In ELISA, VMB6, VMB31, and VMB119 exhibited binding activity limited to *M. bovis*, VMB93 and VMB99 detected antigens of *M. tuberculosis* and *M. bovis*, where as VMB73 cross reacted with other mycobacterial species, *N. asteroides*, and *R. equi*. In addition to antigens of *M. bovis*, antigen from *M. kansasii* was detected in minor amount by VMB6, VMB31, and VMB119, as indicated by a faint band on immunoblots. Detection of antigens of *M. kansasii* by VMB6, VMB31, and VMB119 in immunoblot analysis and not in ELISA may be related to increased sensitivity in detecting NC bound antigens, steric

hindrance due to antigen binding to polystyrene plates, and/or unmasking effects of the determinant by SDS in western blotting. Antigens of M. kansasii detected by VMB6, VMB31, and VMB119 appeared to have an apparent molecular weight of 32 k. Other McAb produced with binding activity to M. tuberculosis have also detected antigens of M. bovis and M. kansasii.[2,9,13] The finding that VMB6, VMB31, and VMB119 identify antigens of M. kansasii provides additional evidence that shared determinants exist between M. kansasii and M. tuberculosis complex.

The apparent MW of antigenic component(s) from M. bovis as visualized in immunoblots using VMB6, VMB31, and VMB119 revealed antigens of 25 k and 30 k or a single 27 k antigen. Broad staining bands and smearing within lanes after immunoblotting indicates non-protein components. Although VMB6, VMB31, VMB119 identify M. bovis antigens having the same apparent molecular weight, the same determinant may not be recognized. Since VMB6, VMB31, and VMB119 detect similar antigens and were produced in three separate fusions, the 30-25 k component(s) may be immunodominant. High antibody activity of VMB6 and VMB31 in ELISA could reflect a greater concentration of this antigen in M. bovis and McAb with intermediate activity should be considered for future characterization.

Immunoblot analysis using VMB93 and VMB99 identified antigens of M. bovis and of M. tuberculosis, similar findings were obtained in ELISA. Existing McAb with specificity to the M. tuberculosis complex[25]

have been used to isolate corresponding antigens with MW 38 k[4,28] and 14 k.[7] Detection of a 63 k antigen from M. bovis and a 58 k antigen from M. tuberculosis by VMB93/VMB99 identifies an antigenic component of M. tuberculosis complex which has not been reported previously.

Although VMB73 was produced by immunizing mice with M. bovis and hybridoma production, VMB73 exhibited greater binding activity, in ELISA, to M. tuberculosis than to M. bovis, indicating a higher concentration of corresponding epitope in M. tuberculosis. Cross reactivity of VMB73 was confirmed by immunoblot analysis. Multiple components detected by VMB73 could result from presence of the determinant on numerous antigenic components, or by fragmentation of a component containing the determinant. Antigen preparations were stored at -70 C to minimize protein degradation. The cross-reactive determinant identified by VMB73 may be responsible in part for cross reaction due to mycobacteria other than those in the M. tuberculosis complex. The antigen detected by VMB73 resembles a previously described 65 k antigen.[27] The 65 k antigen is common to numerous mycobacteria and other bacteria including N. asteroides.[27] Immunoblots using McAb that detect multiple band patterns may result from proteolytic degradation of the 65 k protein antigen.[27]

Each of the six McAb were tested in ELISA to evaluate binding to 18 field isolates of M. bovis and reference strains AN-5 and M. bovis BCG strain Pasteur. Unexpectedly, some McAb did not exhibit binding activity to some of the M. bovis field isolates tested and the six McAb

could again be grouped based on three different types of ELISA reactions. VMB6, VMB31, and VMB119 had binding activity to M. bovis field isolates but not to strains AN-5 and BCG Pasteur, VMB93 and VMB99 detected antigen in some isolates but not in strains AN-5 and BCG Pasteur, and VMB73 exhibited minimal binding activity to some field isolates, similar to VMB93 and VMB99, and BCG Pasteur but not to strain AN-5. Monoclonal antibody MB5[14] produced against a virulent field isolate of M. bovis detected a 29.8 k antigen in 44 field isolates of M. bovis and in strains Vallee and AN-5 but not in M. bovis BCG strain Glaxo. Monoclonal antibody MB5 may be similar to VMB6, VMB31, and VMB119. Failure of VMB93 and VMB99 to detect antigen in M. bovis field isolates tested may reflect variation of antigen composition in isolates of M. bovis or possibly differences in antigen availability in assay conditions. The determinant of the M. tuberculosis complex detected by VMB93 and VMB99 may be present in low amounts or absent in some M. bovis isolates. Failure to exhibit binding activity to M. bovis strain AN-5 is of practical importance considering use of this strain in PPD production. The cross reactive McAb TB23, TB68, TB77, TB78, ML30, and ML34 were used in a radioimmunoassay in attempt to differentiate between M. bovis isolated from cattle and badgers.[13] These McAb detected each of 22 M. bovis isolates from both cattle and badgers, except TB23 which failed to detect one isolate, but failed to detect strain AN-5 when grown on Sautons medium. Differences in OD values between McAb detecting similar antigenic components could result

from variation in McAb binding avidity. The variation in detecting antigens in isolates of M. bovis by VMB93/VMB99 and VMB73 was not associated with geographic location or the animal of origin.

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APPENDIX

Table A1. Optical density values of enzyme-linked immunosorbent assay for supernatants from fusion 01

Cell Line	Time (minutes)					
	5	10	20	30	40	60
Negative control	0.058	0.075	0.099	0.112	0.120	0.117
1	0.668	0.773	1.081	1.234	1.237	1.166
2	0.122	0.119	0.165	0.199	0.205	0.203
3	0.066	0.070	0.107	0.141	0.148	0.146
4	0.068	0.132	0.137	0.170	0.177	0.175
5	0.113	0.146	0.219	0.253	0.270	0.272
6	0.227	0.300	0.512	0.657	0.690	0.698
7	0.105	0.125	0.184	0.226	0.239	0.233
8	0.090	0.107	0.159	0.190	0.201	0.203
9	0.111	0.122	0.174	0.210	0.219	0.218
10	0.112	0.122	0.157	0.194	0.205	0.203
11	0.061	0.082	0.111	0.148	0.155	0.152
12	0.111	0.153	0.232	0.275	0.298	0.302
13	0.088	0.115	0.147	0.178	0.186	0.189
14	0.083	0.101	0.127	0.161	0.169	0.170
15	0.097	0.096	0.116	0.150	0.157	0.158
16	0.066	0.082	0.105	0.140	0.149	0.150
17	0.064	0.083	0.101	0.133	0.140	0.139
18	0.074	0.092	0.125	0.169	0.180	0.181
19	0.055	0.065	0.091	0.123	0.130	0.128
20	0.120	0.185	0.328	0.409	0.434	0.442
21	0.076	0.107	0.166	0.206	0.222	0.222
22	0.105	0.175	0.265	0.339	0.360	0.361
23	0.104	0.142	0.243	0.319	0.342	0.351
24	0.105	0.143	0.197	0.237	0.252	0.253
25	0.104	0.146	0.219	0.278	0.299	0.302
26	0.123	0.161	0.225	0.292	0.312	0.315
negative control	0.140	0.225	0.377	0.489	0.574	0.642
27	0.203	0.351	0.760	0.996	1.207	1.382
28	0.216	0.380	0.760	0.964	1.157	1.329

Table A2. Optical density values of enzyme-linked immunosorbent assay for supernatants from fusion 02

Cell Line	Time (minutes)		
	10	30	60
Negative Control	0.031	0.067	0.104
1	0.678	1.427	2.000
2	0.559	1.182	1.697
3	0.061	0.144	0.235
4	0.078	0.187	0.301
5	0.125	0.171	0.224
6	0.116	0.255	0.440
7	0.129	0.250	0.401
8	0.129	0.284	0.487
9	0.115	0.236	0.359
10	0.090	0.171	0.265
11	0.121	0.282	0.440
12	0.087	0.202	0.323
13	0.146	0.243	0.357
14	0.217	0.482	0.725
15	0.128	0.293	0.474
16	0.067	0.154	0.245
17	0.068	0.159	0.249
18	0.150	0.339	0.544
19	0.098	0.233	0.377
20	0.048	0.113	0.179
21	0.165	0.244	0.336
22	0.218	0.525	0.859
23	0.190	0.382	0.615
24	0.173	0.393	0.595
25	0.220	0.511	0.813
26	0.101	0.179	0.252
27	0.525	1.251	1.799
28	0.163	0.356	0.560
29	0.137	0.181	0.214
30	0.038	0.090	0.132
31	0.674	1.438	1.925
32	0.070	0.164	0.257
Positive Control 1	0.533	1.307	1.990
Positive Control 6	0.624	1.261	1.764
Negative Control	0.078	0.087	0.113
33	0.131	0.155	0.212

Table A2. (Continued)

Cell Line	Time (minutes)		
	10	30	60
34	0.423	1.063	1.604
35	0.088	0.123	0.185
36	0.182	0.350	0.563
37	0.217	0.438	0.686
38	0.085	0.124	0.178
39	0.097	0.166	0.266
40	0.029	0.069	0.116
41	0.131	0.150	0.187
42	0.035	0.066	0.112
43	0.050	0.118	0.196
44	0.064	0.142	0.230
45	0.040	0.099	0.163
46	0.080	0.191	0.302
47	0.091	0.217	0.331
Positive Control 1	0.633	1.352	1.782
Positive Control 6	0.667	1.353	1.813
Negative Control	0.054	0.066	0.078
48	0.002	0.023	0.042
49	0.493	1.040	1.405
50	0.022	0.062	0.095
51	0.118	0.153	0.182
52	0.046	0.075	0.098
53	0.061	0.108	0.151
54	0.056	0.090	0.112
55	0.061	0.091	0.110
56	0.057	0.177	0.112
57	0.072	0.136	0.188
58	0.196	0.513	0.739
59	0.119	0.164	0.192
60	0.210	0.508	0.678
61	0.126	0.339	0.493
62	0.088	0.228	0.329
63	0.141	0.366	0.516
64	0.130	0.340	0.489
Postive Control 6	0.245	0.628	0.851

Table A3. Optical density values of supernatants for heterologous enzyme-linked immunosorbent assay[a]

Species		Monoclonal Antibodies					
	NC	VMB6	VMB31	VMB73	VMB93	VMB99	VMB119
M. bovis	0.170	0.835	1.058	0.431	1.096	1.109	1.037
M. tuberculosis	0.142	0.283	0.103	1.200	0.360	0.454	0.300
M. avium serovar 1	0.195	0.112	0.066	0.363	0.148	0.092	0.248
M. avium serovar 2	0.155	0.150	0.113	0.212	0.091	0.117	0.175
M. avium serovar 4	0.190	0.057	0.075	0.144	0.077	0.092	0.080
M. avium serovar 8	0.184	0.130	0.129	0.036	0.044	0.107	0.130
M. avium serovar 10	0.239	0.240	0.121	0.511	0.142	0.132	0.075
M. chelonei	0.252	0.125	0.126	0.344	0.133	0.097	0.120
M. fortuitum	0.333	0.450	0.317	1.328	0.456	0.289	0.466
M. kansasii	0.301	0.380	0.227	1.312	0.235	0.166	0.391
M. paratuberculosis	0.093	0.099	0.121	0.259	0.073	0.127	0.140
M. phlei	0.157	0.034	0.055	0.456	0.236	0.068	0.062
M. scrofulaceum	0.149	0.061	0.068	0.205	0.123	0.116	0.133
M. smegmatis	0.362	0.439	0.391	0.924	0.391	0.372	0.512
N. asteroides	0.210	0.147	0.230	0.988	0.147	0.178	0.240
R. equi	0.139	0.066	0.065	0.288	0.056	0.074	0.083

[a]Average of six evaluations using antigen of M. bovis and an average of evaluations using antigen of other species.

Table A4. Optical density values of enzyme-linked immunosorbent assay using monoclonal antibodies and 18 field isolates of Mycobacterium bovis and three reference strains

M. bovis	Monoclonal Antibody						
	Negative Control	VMB6	VMB31	VMB73	VMB93	VMB99	VMB119
ATCC 19210	0.170	0.835	1.058	0.431	1.096	1.109	1.037
#1	0.170	1.174	1.436	0.595	0.837	0.978	1.468
#2	0.063	0.505	0.534	0.236	0.156	0.190	0.818
#3	0.170	0.836	1.242	0.359	0.226	0.142	1.217
#4	0.170	0.986	1.379	0.515	0.287	0.387	1.382
#5	0.170	1.091	0.930	0.224	0.162	0.224	1.027
#6	0.170	0.616	0.851	0.199	0.109	0.105	0.890
#7	0.170	1.392	1.680	0.492	1.238	1.674	1.343
#8	0.170	1.329	1.562	0.375	0.646	0.822	1.325
#9	0.170	0.957	1.478	0.445	0.126	0.131	1.141
#11	0.170	0.650	0.863	0.317	0.110	0.118	0.659
#12	0.170	1.214	1.429	0.450	1.205	1.392	1.125
#13	0.170	0.481	0.668	0.186	0.068	0.108	0.410
#14	0.170	0.716	1.085	0.296	0.117	0.150	0.918
#15	0.170	0.465	0.585	0.219	0.017	0.125	0.706
#17	0.165	0.887	0.908	0.353	0.288	0.357	1.176
#19	0.313	1.245	1.248	0.802	0.957	1.197	1.382
#20	0.179	0.854	0.873	0.441	0.673	1.076	1.227
#21	0.093	0.735	0.760	0.228	0.497	0.664	0.857
AN-5	0.140	0.155	0.042	0.204	0.173	0.163	0.136
Pasteur	0.190	0.175	0.079	0.370	0.209	0.247	0.281

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