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Production and Characterization of a Murine Monoclonal Antibody Recognizing a Shared Mycobacterial Polysaccharide[1]

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Arabinomannan (AM) is a complex polysaccharide widely distributed within the genus *Mycobacterium.* Its prominence in antigen preparations from mycobacteria (such as purified protein derivative or PPD) led to its designation as *Mycobacterium tuberculosis* Antigen 1 in the U.S.–Japan nomenclature ([2]). There are minor structural differences in the arabinomannans produced by different mycobacterial species ([8]) but extensive immunologic crossreactivity has been observed for all species tested, including *M. leprae* ([7]). As part of our research effort aimed at producing and characterizing a panel of murine monoclonal antibodies directed against defined protein, lipid, and carbohydrate antigens of *M. leprae,* we performed a murine-murine hybridoma fusion utilizing spleens from mice immunized with AM extracted from *M. smegmatis.* From several initial positive fusion events, one clone demonstrating sustained production of an IgM monoclonal antibody with high, specific affinity for AM was obtained and the antibody product characterized.

MATERIALS AND METHODS

Antigen preparation and immunization protocol. AM was extracted from *M. smegmatis,* purified, and characterized as previously reported ([7]). Purified AM (95% carbohydrate by weight) and a crude antigen preparation (obtained after the ethanol precipitation but prior to the phenol extraction, and which contained significant amounts of degraded protein) were used as immunogens. Twenty μg of the crude AM preparation was diluted in phosphate buffered saline (PBS), suspended in incomplete Freund's adjuvant (IFA) (total volume approximately 300 μl), and injected subcutaneously into ten-week-old BALB/c mice on day 0. On day 7, each mouse received 10 μg of the crude antigen, suspended in IFA, and injected subcutaneously. On day 28, the mice were boosted by intraperitoneal injection of 10 μg of purified AM preparation without adjuvant. A final boost of 25 μg of the purified AM was administered intravenously through the tail vein on day 92. Antibody response was measured by an en-

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zyme-linked immunosorbent assay (ELISA) on day 97, and the spleens harvested for fusion on day 98.

ELISA conditions. Serologic response in the immunized mice or specific antibody in the hybridoma supernatant or ascitic fluid was determined by an ELISA adapted from that previously reported ([7]). Purified AM was diluted in sodium carbonate buffer (0.05 M, pH 9.5) at a concentration of 10 μg/ml. Microtiter plates (96-well) were coated with 100 μl of this solution per well and stored at 4°C. On the day of the assay, the unadsorbed AM was removed and nonspecific binding sites were blocked with PBS containing 1% bovine serum albumin (PBS-BSA). One hundred μl of the test serum (diluted 1:50 in PBS-BSA), culture supernatant (diluted 1:1 in PBS-BSA), or ascitic fluid (various dilutions) was then added to each well and incubated at room temperature for 1 hr. All of the samples were assayed in duplicate. The plates were washed and peroxidase-conjugated goat anti-mouse immunoglobulin G, A, M (Miles Laboratories, Elkhart, Indiana, U.S.A.) diluted 1:4000 in PBS-BSA added. The plates were held for 1 hr at room temperature, washed, then 100 μl of substrate-dye solution (0.003% H_2O_2 and 0.1 mg/ml o-phenylenediamine) were added. After a 30 min incubation at 37°C, the reaction was halted by the addition of H_2SO_4 and the A_{492} measured using a Titertek Multiskan (Flow Labs, McLean, Virginia, U.S.A.).

Fusion. The fusion protocol and cell culture methods were adapted from techniques previously reported ([4, 10]). Spleens were harvested from the two mice with the strongest antibody responses to AM, and a single cell suspension of splenocytes in RPMI 1640 (GIBCO Laboratories, Grand Island, New York, U.S.A.), supplemented with penicillin and streptomycin, was prepared by extensive mincing of the tissue and passage through nylon mesh. The cells were washed three times with RPMI and viability was assessed. NSI/1 myeloma cells were maintained in log-phase growth for three days prior to the fusion. The splenocytes were combined with washed NSI/1 cells in a 4:1 ratio in a single 50 ml conical tube and pelleted by centrifugation at 200 × *g* for 5 min. The supernatant was removed and 1.0 ml of 40% polyethylene glycol 1450 (Kodak Laboratories, Rochester, New York, U.S.A.), pH 7.8, was added with gentle dispersion of the pellet. The cell mixture was pelleted at 350 × *g* for 10 min, washed with RPMI 1640, and then resuspended in RPMI supplemented with penicillin, streptomycin, 15% fetal calf sera (FCS), 1 mM sodium pyruvate, and 2 mM L-glutamine ("complete media") plus 0.1 mM hypoxanthine, 0.4 mM aminopterin and 0.015 mM thymidine. The cells were dispersed into 96-well microtiter plates at a density of 5×10^5 cells/well (200 μl volume). A feeder-cell layer was not used. Antibody activity in the supernatant was assayed on day 13 and day 14. Each strongly positive well was diluted in complete media with hypoxanthine and aminopterin and replated onto one half of a microtiter plate (48 wells) at a density of 5 cells per well ("minicloning"). These wells were screened after 14 days of growth, and 4 positive wells per miniclone were formally cloned at a density of 1 cell per well onto a 96-well plate, diluted in complete media without hypoxanthine, aminopterin, or thymidine. Wells containing discrete, single clones (visually assessed) were coalesced onto a single plate and were again tested for antibody activity. Strongly positive clones were selected and amplified by injection into the peritoneums of pristane-primed BALB/c mice for production of antibody-enriched ascites.

Monoclonal characterization. The class and subclass of the monoclonal antibodies produced were determined by immunodiffusion precipitation with antibodies specific for the various murine immunoglobulin species (Miles Laboratories). Discrete monoclonal bands were detected using electrophoresis on cellulose acetate (Microzone System, Beckman Instruments, Inc., Fullerton, California, U.S.A.). Inhibition of monoclonal binding by various monosaccharides and charides was assayed by an ELISA. Serial dilutions of the sugars were prepared in PBS-BSA containing a 1:16,000 dilution of antibody-containing ascites. After incubation for 90 min at 37°C, the mixture was transferred to microtiter plates precoated with purified AM and the ELISA was performed as described above, with the exception that peroxidase-conjugated goat anti-mouse IgM, μ-chain specific conjugate (Cappel Laboratories, Cochranville, Penn-

sylvania, U.S.A.), diluted 1:1000, was used in place of the nonspecific conjugate. The ability of the monoclonal antibodies to recognize the arabinomannans of various mycobacterial species was assessed by an ELISA using microtiter plates pre-coated with soluble and insoluble antigen preparations from sonicated mycobacterial stock cultures ([4]). These antigen preparations were standardized by dilution in sodium carbonate buffer, pH 9.5, to a protein concentration of approximately 100 μg/ml. The ascitic fluid containing the monoclonal antibody was diluted 1:1000 in PBS-BSA, and the μ-specific conjugate was used.

The immunologic specificity of the monoclonal antibodies was determined by gel-immunoradioassay (GIRA) ([9]). Samples diluted in a buffer containing 1% sodium dodecyl sulfate (v/v) were applied to 3 mm thick discontinuous (5% stacking, 10% separating) polyacrylamide gel. Electrophoresis was carried out at 50 mA constant current, and the gel was removed after the buffer front had migrated 4 cm into the separating gel. The portion of the gel containing the samples was excised and applied onto brass cryostate mounts using a filter paper spacer and O.C.T. embedding compound (Tissue-Tek II®, Lab-Tek Products Division of Miles Laboratories). The specimen was frozen using liquid nitrogen and 50 micron thick longitudinal slices were obtained using a microtome-cryostat (Damon/IEC, Needham Heights, Massachusetts, U.S.A.). Approximately 40 thin sections were obtained from each gel and they were stored in 80% ethanol at −20°C until needed. The assay was performed as follows: The slices were rehydrated in PBS for 10 min, and were then incubated overnight at 4°C in 1.5 ml conical tubes containing 500 μl of PBS with 0.2% Triton X-100 and 0.5% BSA (reaction buffer) and monoclonal ascites diluted 1:25. The following day, the slices were washed twice in plastic Petri dishes containing 30–40 ml of PBS with 0.5% Triton X-100 (wash buffer) for 30 min per wash, and then placed in clean 1.5 ml tubes containing 500 μl reaction buffer and approximately 10^6 counts per minute (CPM) of ^{125}I-labeled sheep anti-mouse immunoglobulin. This labeled antibody was prepared by iodinating the IgG fraction from sheep anti-mouse immunoglobulin with ^{125}I as described by Greenwood, *et al.* ([5]). The gel : radiolabel mixtures were incubated for 1 hr at room temperature. The gels were then placed into Petri dishes and washed extensively (8–9 washes, each consisting of 30–40 ml of wash buffer and lasting 30 min). After the final wash, the gels were mounted onto Gel Bond film (Marine Colloids Division, Bioproducts, Rockland, Maryland, U.S.A.), allowed to slowly air dry at room temperature, and then autoradiographed.

RESULTS

The cells from the hybridoma fusion were distributed over 384 microtiter wells. Of these 384 wells, 16 (4.2%) were positive on both the day 13 and day 14 screenings and the ten most strongly positive were minicloned. Three of these ten failed to grow or ceased production of antibody, leaving cells originating from only seven (1.8%) of the original wells to be cloned. Formal cloning of cells from these 7 miniclones resulted in no growth in 2 instances, poor growth in 2 others, and excellent growth in the remaining 3 cases. The five clones with growth were injected into the peritoneal cavities of primed mice. Two of these failed to grow, one (#41) exhibited vigorous cell growth and good ascites production but produced no detectable antibody by cellulose acetate electrophoresis. The two remaining clones each grew well, and the resulting ascites produced strong monoclonal antibody spikes. These two clones (AM31-3a and AM8-2c) each secreted IgM class monoclonal antibody.

Standard curves for these two monoclonals were generated by testing sequential twofold dilutions of monoclonal ascites in an ELISA with plates coated with AM. Eighty percent of maximal binding was observed at a dilution of approximately 1: 16,000 for AM31-3a and at 1:20,000 for AM8-2c. Subsequent work with monoclonal AM31-3a proved difficult because of the high backgrounds produced by nonspecific adherence to plastic surfaces. This made it difficult to document antibody specificity by either GIRA or inhibition of binding by purified AM. Subsequent studies were conducted using only monoclonal AM8-2c.

Specific inhibition of binding by monoclonal AM8-2c to AM-coated plates is

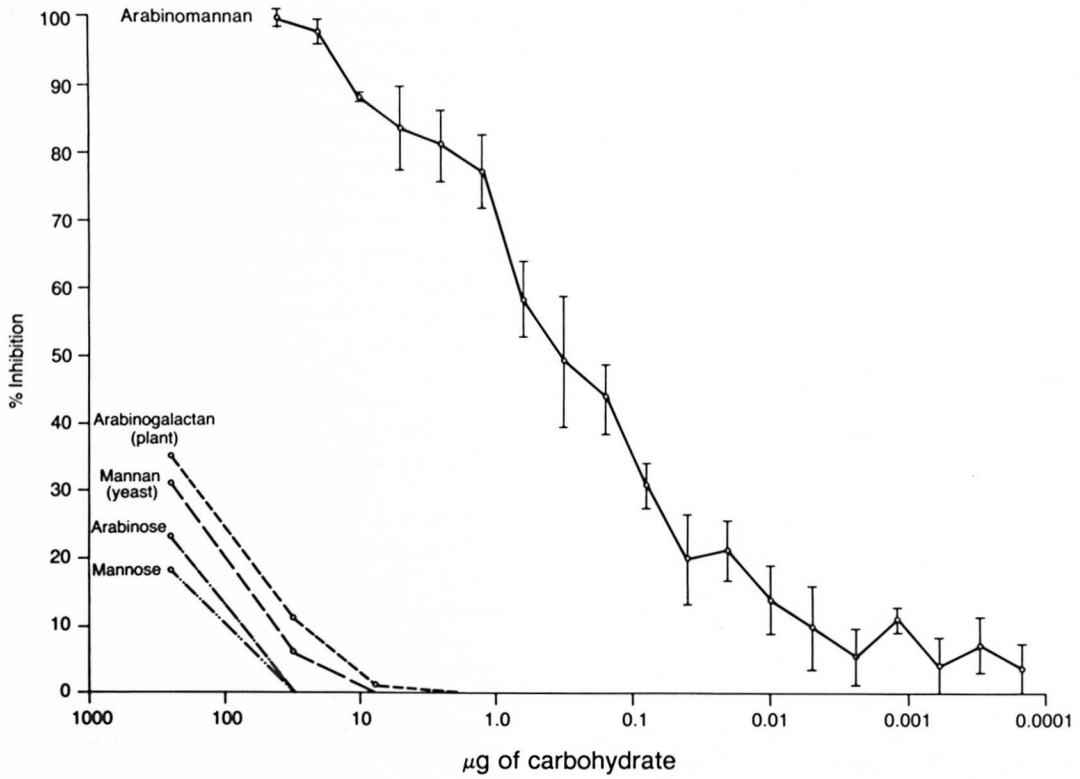

FIG. 1. Inhibition of binding of AM8-2c to arabinomannan-coated plates by various monosaccharides and polysaccharides.

shown in Figure 1. Fifty percent inhibition of binding was observed with 275 ng of AM. Minimal inhibition of binding was observed with the free monosaccharides, arabinose and mannose, even when tested at concentrations as high as 500 μg/well (0.5% solution). Unrelated polysaccharides containing mannose (yeast mannan) or arabinose (arabinogalactan from larchwood) were also unable to inhibit binding.

Final confirmation of the antigenic specificity of monoclonal AM8-2c was accomplished by GIRA (Fig. 2). A slice from a polyacrylamide gel containing purified AM was incubated with the monoclonal. Subsequent exposure to radiolabeled anti-murine IgM demonstrated binding in two broad bands, one in the region corresponding to proteins of approximately 30–40,000 molecular weight and the other in the 80–90,000 molecular weight range. Two bands in corresponding positions were seen when immune sera were reacted with the purified AM in a Western Blot assay ([6]).

Reactivity of monoclonal AM8-2c with antigen mixtures prepared from 18 species of mycobacteria is shown in The Table. When standardized for protein concentration in the antigen preparations, all 18 species showed strong recognition (3+ or 4+) by the specific monoclonal with the exception of *M. tuberculosis* which had only 1+ binding. The antigen mixtures containing insoluble or minimally soluble antigens showed uniformly low reactivity for the six species tested.

DISCUSSION

Polysaccharides constitute an important class of antigenic compounds in the genus *Mycobacterium*. Arabinomannan, in particular, figures prominently in both the clinical and theoretical aspects of mycobacterial disease. Given its solubility and preservation of antigenicity when heated, it is not surprising that it is one of the most readily identifiable and quantitatively important antigenic constitutents in PPD ([2]). Its role

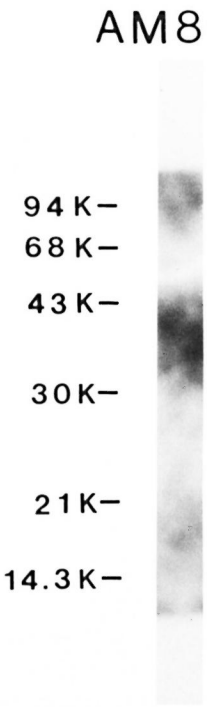

FIG. 2. Gel immunoradioassay demonstrating binding of AM8-2c to two broad bands. Molecular weight standards are represented on the left.

in the delayed hypersensitivity response to PPD is unsettled ([1]), but the strong antibody response elicited by AM ([7]) makes it likely that a significant fraction of the antibody directed against antigens in PPD is directed at AM rather than at the protein components in the mixture (many of which have likely suffered significant antigenic alteration from heat-killing of the cultures). Other investigators have attributed immunosuppressive properties to AM, and postulated that it may play an important role in the pathogenesis of mycobacterial infections ([3]). These features, and its broad distribution within the genus, led us to attempt production of a monoclonal antibody specific for AM.

The scheduling of the immunization protocol was designed to maximize the probability of generating hybridoma lines producing immunoglobulins other than IgM. Unfortunately, the cell lines that survived through the cloning step all produced IgM monoclonals, suggesting that the arabinomannan antigen in a purified form is predominantly thymus independent and preferentially stimulates IgM production. If so,

THE TABLE. *Protein and carbohydrate (CHO) concentrations and intensity of monoclonal AM8-2c binding for soluble and insoluble antigen preparations from mycobacterial species.*

Mycobacterial species	Antigen prep.	Protein conc. (μg/ml)	CHO conc. (μg/ml)	Monoclonal binding (AM8-2c)
M. leprae	Sol	125	41	4+
M. leprae	Insol	88	374	1+
M. smegmatis	Sol	98	133	4+
M. smegmatis	Insol	116	44	1+
M. bovis	Sol	96	184	4+
M. bovis	Insol	50	<1	1+
M. flavescens	Sol	110	8	3+
M. flavescens	Insol	90	21	1+
M. vaccae	Sol	75	18	4+
M. vaccae	Insol	100	17	2+
M. kansasii	Sol	97	482	3+
M. kansasii	Insol	94	393	1+
M. tuberculosis	Sol	110	8	1+
M. marinum	Sol	75	<1	4+
M. peregrinum	Sol	112	151	4+
M. gordonae	Sol	92	81	4+
M. phlei	Sol	95	3	4+
M. intracellulare	Sol	108	138	4+
M. fortuitum	Sol	100	444	4+
M. nonchromogenicum	Sol	95	10	4+
M. thamnopheos	Sol	104	44	4+
M. duvalii	Sol	102	546	3+
M. diernhoferi	Sol	102	658	4+
M. triviale	Sol	100	18	4+

one might expect higher yields for fusions timed to coincide with the period of maximal IgM synthesis.

The failure of the monosaccharides to inhibit binding, even when present at concentrations 10,000-fold greater than the concentration of AM that produced significant inhibition, implies that the binding site of the monoclonal antibody encompasses more than a single sugar residue. The negative results produced by the unrelated polysaccharides are further evidence that the specificity of the antigenic site requires a specific sequence and linkage of monosaccharides. The structures of arabinomannans from several mycobacterial species have been elucidated by Misaki, *et al.* ([8]), and appear to consist of a common central core of arabinose and mannose residues with an arabinose-containing oligosaccharide major side chain. Other side chains consist primarily of mannose and exhibit much more species-to-species variation. From the lack of species specificity exhibited by monoclonal AM8-2c, we postulate that it is directed at an epitope(s) on the core and/or major side chain. In this respect, the poor binding to the *M. tuberculosis* antigen preparation becomes interesting. The AM from *M. tuberculosis* H37Rv has been described as similar to that of *M. bovis* ([8]), and we have shown that patients with tuberculosis manifest a strong serologic response to purified AM from *M. smegmatis* ([7]). The strain used for our antigen preparation was an avirulent strain, H37Ra. The possibilities exist that either the attenuated, avirulent strain produces reduced amounts of AM compared with virulent strains, or that the specific epitope recognized by monoclonal AM8-2c is present in reduced quantities (or is shielded from antibody binding) in *M. tuberculosis.* When paired soluble and insoluble antigen preparations from six species of mycobacteria were compared, AM was found almost exclusively in the soluble fraction, an observation consistent with the strongly hydrophilic nature of the molecule.

Monoclonal AM8-2c is one of the first murine monoclonal antibodies characterized as specific for a mycobacterial carbohydrate antigen. Our laboratory has also produced monoclonal antibodies to the trisaccharide moiety of the phenolic glycolipid of *M. leprae,* all of which are also of the IgM class ([11]). These monoclonal antibodies are of potential value in the elucidation of the role of AM and the phenolic glycolipid in the pathogenesis of tuberculosis, leprosy, and perhaps other mycobacterial infections, and may prove applicable to inhibition-based serologic tests such as that reported by Hewitt, *et al.* ([6]).

SUMMARY

An IgM monoclonal antibody specific for mycobacterial arabinomannan was produced by the fusion of splenocytes from BALB/c mice immunized with purified arabinomannan with NSI/1 myeloma cells. Specificity was demonstrated by gel-radioimmunoassay, and by inhibition of binding using the purified polysaccharide. The monoclonal antibody recognized the arabinomannans from all 18 species of mycobacteria tested, including *Mycobacterium leprae.* This antibody expands the number of defined mycobacterial antigens against which monoclonal antibodies have been produced, and has potential application in studies concerning the pathogenesis of mycobacterial disease.

RESUMEN

Se produjo un anticuerpo monoclonal IgM, específico para arabinomanana micobacteriana, por fusion de esplenocitos de ratones BALB/c inmunizados con arabinomanana purificada con células de mieloma NSI/1. La especificidad del anticuerpo se demostró por radioinmunoensayo en gel, y por inhibición del enlazamiento usando el polisacárido purificado. El anticuerpo monoclonal reconoció a las arabinomananas de todas las 18 especies micobacterianas probadas, incluyendo al *Mycobacterium leprae.* Este anticuerpo expande el número de antígenos micobacterianos definidos contra los que se han producido anticuerpos monoclonales, y tienen una aplicación potencial en estudios sobre la patogénesis de enfermedadas micobacterianas.

RÉSUMÉ

On a isolé un anticorps monoclonal IgM spécifique pour l'arabinomannan par la fusion de splénocytes de souris BALB/c immunisées par de l'arabinomannan purifié avec des cellules de myélomes NSI/1. La spécificité de cet anticorps a été confirmé par des épreuves radio-immunologiques sur gel, et par une épreuve d'inhibition par le polysaccharide purifié. L'anticorps monoclonal identifiait les arabinomannans des 18 espèces de mycobactéries qui ont été étudiées, y compris *Mycobacterium leprae.* Cet anticorps élargit ainsi le nombre des antigènes mycobactériens bien définis

contre lesquels des anticorps monoclonaux ont été produits. Ceci présente des possibilités d'application pour les études relatives à la pathogenèse de la maladie mycobactérienne.

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