HYBRIDOMA ANTIBODIES AGAINST PROTECTIVE AND NONPROTECTIVE ANTIGENIC DETERMINANTS OF A STRUCTURALLY DEFINED POLYPEPTIDE FRAGMENT OF STREPTOCOCCAL M PROTEIN*

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It has been known for a long time that the M protein on the surface of group A streptococci evokes protective immune responses against infections with the homologous M serotype of bacteria (1, 2). The finding that certain antigenic determinants, sometimes closely associated with the M protein, elicit antibodies that cross-react with host heart tissue has focused attention on defining precisely the minimal structure of the M protein molecule required to evoke protective immunity (3–5). Previous studies (6–8) have shown that protective immune responses can be evoked by native and synthetic peptide fragments that represent only limited regions of M protein, indicating that the remainder of the M protein molecule containing potentially tissue-cross-reactive antigenic determinants could be deleted from vaccine preparations. It has been difficult, however, to predict the location of limited protective regions with the use of conventional antisera because these antisera are polyclonal and contain antibodies to many different determinants (8, 9), some of which may not be exposed in the native state of the M protein on the bacterial surface, and others of which may cross-react with host tissue antigens.

To precisely define the protective determinants of M protein exposed on the surface of virulent streptococci, we have used hybridoma technology to produce a set of monoclonal antibodies against a purified polypeptide fragment of type 24 streptococcal M protein. In this paper we report evidence that some of the hybridoma antibodies are opsonic against the homologous type streptococci. Moreover, when injected into mice, the strongly opsonic monoclonal antibodies protect the animals against challenge infections with the homologous, but not a heterologous, serotype of bacteria. One of the hybridoma antibodies that reacted in high dilution (1:204,800) with the isolated M protein failed to opsonize the organisms or to protect mice against challenge infections. We present evidence to suggest that the antigenic determinant

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against which this antibody is directed is "buried" and inaccessible for antibody binding in the native state of the M protein on the surface of the streptococci. These studies indicate that a set of monoclonal antibodies may be used to identify the protective structural determinants of the M protein molecule exposed on the surface of group A streptococci.

Materials and Methods

Myeloma Cell Lines. Nonsecreting myeloma cell lines Sp2/O-Ag14 (Sp2 cells) and P3-X63-AG8.653 (653 cells) were maintained in Dulbecco's modification of Eagle's medium (DMEM)[1] containing horse serum (10%), calf serum (5%), nonessential amino acids (1×), L-glutamine (4 mM; Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), and gentamycin sulfate (100 μg/ml; Schering Corp., Kennilworth, NJ). All cells were maintained in a humidified incubator at 37°C with a gas phase of 5% CO_2 and 95% air. Periodically, the myeloma cells were treated with 8-azaguanine to insure against growth of revertant cells.

Extraction and Purification of M Protein. M protein from type 24 group A streptococci was purified from limited peptic digests as described (10, 11). The purified product, designated pep M24, was judged to be homogeneous by sodium dodecyl sulfate-gel electrophoresis and amino acid analysis (10). Pep M5 and pep M6 were purified and analyzed similarly from type 5 and type 6 streptococci.

Immunization of Mice. Pep M24 (50 μg) was dissolved in 0.1 ml 0.02 M phosphate/0.15 NaCl, pH 7.4 (PBS), emulsified with an equal volume of Freund's complete adjuvant, and injected subcutaneously into female BALB/c mice (15-g). The same dose emulsified in Freund's incomplete adjuvant was repeated 1 mo later. 1–3 mo later, mice were injected intraveneously with 50 μg pep M24 in PBS via the tail vein, and the animals were killed 2–3 d later.

Cell Fusion. Spleen cells were prepared by teasing the spleen apart with forceps and pressing the pieces through a stainless steel wire mesh. The cells were washed three times with serum-free DMEM buffered with 10 mM Hepes and collected by centrifugation at 400 *g* for 5 min. Approximately 8×10^7 spleen cells were obtained from immunized animals. Myeloma cells were harvested, washed with serum-free DMEM three times, and combined with spleen cells at a ratio of 5:1 (spleen/myeloma). The cells were fused using polyethylene glycol (PEG), according to the method of Galfre et al. (12). Briefly, after centrifugation the supernatant was removed, and 1 ml warm 50% PEG (Carbowax 1500; Fisher Scientific Co., Pittsburgh, PA) was added with gentle stirring of the cell pellet. After 1 min, ~20 ml of serum-free DMEM was added dropwise to dilute the PEG without osmotically damaging the fused cells. The cells were centrifuged at 400 *g*, diluted to 100 ml with complete growth medium, and seeded into four 24-well plates (Costar Data Packaging, Cambridge, MA). After 24 h, 1 ml growth medium containing hypoxanthine (100 μM), aminopterin (0.3 μM), and thymidine (16 μM) (HAT) was added. This was repeated three times at 24-h intervals, removing 1 ml medium from each well before the addition. Thereafter the medium was changed at 3–4-d intervals. After ~2 wk in HAT, the aminopterin was removed, and after another week the hypoxanthine and thymidine were also removed.

Detection of Antibody-secreting Hybridomas. Antibody-secreting hybridomas were detected using an enzyme-linked immunosorbent assay (ELISA; 13) of the culture medium supernatants. The assay was performed as previously described (14) using pep M24, pep M5, and pep M6 (5 μg/ml) adsorbed to polystyrene tubes as the solid-phase antigen.

In addition, to determine which antibodies were able to recognize immunodeterminants in their native state on the surface of *Streptococcus pyogenes*, ELISA were performed using whole streptococci as particle phase antigens. In a series of test tubes, a suspension of log-phase streptococci (0.1 ml) adjusted to an absorbency of 0.2 at a wavelength of 530 nm was mixed with 0.1 ml of serially diluted ascites fluid and incubated for 15 min at 37°C and 15 min at 0°C. The cells were washed three times with PBS supplemented with 0.05% Tween 20 (PBS-

[1] *Abbreviations used in this paper:* CFU, colony-forming unit; DMEM, Dulbecco's modification of Eagle's medium; ELISA, enzyme-linked immunosorbent assay; HAT, hypoxanthine, aminopterin, and thymidine; PBS, 0.02 M phosphate, 0.15 M NaCl, pH 7.2; PEG, polyethylene glycol; pep M5, M6, and M24, purified type 5 M, 6 M, and 24 M proteins extracted from streptococci with pepsin; PMN, neutrophilic leukocyte.

Tween), resuspended in 0.2 ml of a 1:2,000 dilution of peroxidase-conjugated goat anti-mouse IgG (N. L. Cappel Laboratories Inc., Cochranville, PA), incubated for 30 min at 37°C, washed as before, and resuspended in 0.2 ml PBS-Tween. 0.15 ml of the suspension was then added to 0.3 ml of substrate (5 amino-salicylic acid) in a cuvette, incubated at ambient temperature for 30 min, and the endpoint determined by measuring the absorbency at 450 nm in an automatic spectrophotometer (EIA PR50 Gilford Instrument Laboratories Inc., Oberlin, OH).

Cloning Procedures. Cells from antibody-producing wells were cloned in medium containing 0.8% methylcellulose (Methocel A4M Premium, Dow Chemical Co. Indianapolis, IN). Similar procedures have been used previously for cloning of drug-resistant cell lines (15). The methylcellulose was suspended in hot water at a concentration of 4% and autoclaved. The suspension was dissolved by stirring overnight at 4°C and then diluted to 2% with 2× DMEM. The cells from positive wells were diluted to ~50 cells/ml in medium containing 0.8% methylcellulose and other supplements of the standard growth medium. Approximately 4×10^5 normal spleen cells were included to act as a "feeder layer". Cloning efficiencies using this technique were ~50%. Within 1 wk, colonies were of a size appropriate for harvesting (~100 cells). Isolated colonies were picked at random and seeded into 24-well plates containing 2 ml growth medium with or without 4×10^5 normal spleenocytes. Culture medium was assayed within 1 wk. Cells were grown to mass cultures for production of ascites fluid, re-cloned, or resuspended in growth medium containing an extra 20% serum and 10% dimethylsulfoxide and frozen in liquid nitrogen. All antibody-producing hybridoma clones described in this report have been re-cloned at least once. In all re-clones the percentages of antibody-producing clones have generally been >90%.

Ascites Fluid. To produce large quantities of antibody, 5×10^6 antibody-producing cells were injected into the peritoneal cavities of BALB/c mice injected 1 wk–2 mo previously with 0.3 ml 2,6,10,14-tetramethylpentadecane (Pristane, Aldrich Chemical Co. Inc., Milwaukee, WI). 1–2 wk later ascites fluid was removed, clarified by centrifugation and sterilized by membrane filtration. In some cases the hybridoma cells in the pellet were transferred to the peritoneal cavities of new mice. The IgG isotype of each hybridoma antibody was determined by double immunodiffusion in agar gel of 1:200 dilutions of each ascites fluid against mouse IgG subclass specific antisera to IgG1, IgG2a, IgG2b, and IgG3 (Miles Laboratories Inc., Elkhart, IN).

Preparation of Metaphase Chromosomes. Chromosome spreads of myeloma and hybridoma cells were performed using standard procedures (16). Cells were suspended in 0.075 M KCl for 11–12 min, centrifuged, and fixed in glacial acetic acid/methanol (1:3, vol/vol) for 20 min. Drops of the cell suspension were placed onto a cold, wet slide, dried, and stained with Giemsa at pH 6.8.

Tests for Type-specific Antibodies. Opsonization by the hybridoma antibodies was tested by timed, in vitro phagocytosis tests as previously described (10, 11). The test mixtures consisted of 0.4 ml of fresh, heparinized (10 U/ml) human blood, 0.5 ml of a standard suspension of type 24, type 6, or type 5 streptococci, and 0.05 ml of control or hybridoma ascites fluids. The ratio of streptococcal units per leukocyte was ~10:1. The percentage of neutrophilic leukocytes (PMN) that had ingested one or more bacteria was estimated by microscopic examination of stained smears prepared from an aliquot of the test mixtures taken after 30 min of rotation at 37°C.

Indirect bactericidal tests were performed as described (17) to confirm results of the opsonization assay. 0.4 ml of heparinized (10 U/ml) human blood was mixed with 0.05 ml control or hybridoma ascites fluid and 0.05 ml of a suspension of streptococci containing the number of colony-forming units (CFU) indicated. The mixtures were rotated for 3 h at 37°C, after which pour-plates were prepared by adding the test mixtures to 20 ml of 5% sheep blood agar.

Mouse-protection tests were performed as described (6). White Swiss mice (15-g) were injected intraperitoneally with 0.1 ml of control or hybridoma ascites fluid and challenged 24 h later by the same route with various doses (ranging from 50 to 3×10^5 CFU) of type 24 or type 6 streptococci. Survival was recorded over a 7-d period.

Tests for Heart Cross-Reactive Antibodies. Frozen sections (4-μm thick) of human heart tissue were reacted at ambient temperature for 30 min with serial dilutions of each of the hybridoma ascites fluids. After washing three times for 5 min in PBS, the antibody-treated sections were

treated for 30 min with a 1:40 dilution of fluorescein labeled goat anti-mouse IgG, again washed three times in PBS, and mounted with a drop of 1% Gelvatol, pH 7.0, and a coverslip. The sections were then examined using a Leitz fluorescence microscope (E. Leitz, Inc., Rockleigh, NJ).

Results

Cell Fusions. BALB/c mice were hyperimmunized with a purified peptic extract of pep M24, and cells obtained from their spleens within 3 d of a final intravenous boost were fused with either Sp2 or 653 myeloma cells. The parental 653 cells were shown by analysis of metaphase chromosomes to contain ~57 chromosomes, whereas the number of chromosomes of antibody-secreting hybridomas ranged from 80 to 88. Sp2 cells and hybrids were not analyzed for chromosome numbers.

Culture supernatants were screened by ELISA using pep M24, pep M5, and pep M6 as antigens. Within 10 d after the fusions, type-specific antibody against the immunizing antigen was detected. Cells from antibody-producing colonies were cloned in 0.8% methylcellulose medium, expanded by culture in vitro, and injected into the peritoneal cavities of Pristane-primed BALB/c mice. 5 to 10 ml of ascites fluid accumulated over a period of 1–3 wk. The ascites fluids produced by six different hybridomas were selected for the following studies.

IgG Class, Immunoprecipitating Activity, and ELISA Titers of Hybridoma Antibodies. All of the hybridoma antibodies were of the IgG1 isotype except clone IIA6.6, which was IgG2b. ELISA titers against pep M24 ranged from 102,400 to 819,200 (Table I); antibodies against pep M5 or pep M6 were not detected. Four of the six hybridoma antibodies precipitated pep M24 in double diffusion tests in agar gel (Table I). None of the cloned antibodies precipitated heterologous types 5 or 6 pep M proteins. These results suggest that the four precipitating antibodies are directed against repeating antigenic determinants, whereas the two nonprecipitating antibodies are directed against nonrepeating determinants of the pep M24 molecule. None of the monoclonal antibodies reacted in immunofluorescence tests with human heart tissue.

TABLE I

Type-specific Opsonic Activities of Monoclonal Antibodies against Pep M24*

Ascites fluids used to opsonize streptococci	Anti-pep M24 ELISA titers‡	Immunoprecipitation with pep M24	Percentage of PMN with associated streptococci		
			Type 24	Type 5	Type 6
Controls§	<200	—	6	6	6
IC1.3‖	102,400	Yes	26	6	2
IIC3.7‖	819,200	Yes	78	10	2
IIC4.6‖	409,600	No	86	8	2
IIA6.8¶	102,400	Yes	58	8	6
IIA6.6¶	204,800	No	26	8	2
IIB4.1¶	204,800	Yes	4	6	2

* All monoclonal antibodies reported are of the IgG1 isotype except IIA6.6, which is IgG2b.

‡ Titers of all ascites fluids against pep M5 and pep M6 were <200.

§ Control ascites fluid was produced by injecting 5×10^6 Sp2 or 653 cells into the peritoneal cavity of mice injected 2–4 wk previously with 0.3 ml Pristane.

‖ Produced using the 653 myeloma line.

¶ Produced using the SP2 myeloma line.

Type-specific Opsonic and Bactericidal Activities of Hybridoma Antibodies. To determine whether hybridoma antibodies would effectively opsonize homologous M-type bacteria, types 24, 6, or 5 streptococci were incubated in fresh heparinized human blood in the presence of the hybridoma ascites fluids. Five of the six ascites fluids opsonized type 24 streptococci to various degrees, whereas none opsonized types 5 or 6 organisms (Table I). The opsonic effects were confirmed by indirect bactericidal tests (Table II). Those hybridoma fluids showing the strongest opsonization (IIC3.7, IIC4.6, and IIA6.8) were also the strongest in the bactericidal tests, completely eradicating all viable streptococci in the test mixtures. The antibodies showing intermediate opsonic effects gave intermediate values in the bactericidal test, whereas the nonopsonic antibodies totally lacked bactericidal activity. It should be noted that no relationship could be seen between the ELISA titers and the bactericidal capacities of the hybridoma antibodies. These results suggested that monoclonal antibodies directed against only certain antigenic determinants of the isolated M protein molecule are opsonic and protective.

Protection by Hybridoma Antibodies of Mice Against Challenge S. pyogenes Infection. Passive mouse protection tests were performed to determine the in vivo bactericidal activity of two hybridoma antibodies that were bactericidal in vitro (clones IIC3.7 and IIC4.6) and one that was not bactericidal in vitro (IIB4.1). Mice were injected intraperitoneally either with control ascites fluid or with ascites fluid produced by one of the hybridoma clones and 24 h later were challenged with either type 24 or type 6 streptococci. The two strongly opsonic hybridoma antibodies were clearly protective (Table III). The nonopsonic antibody (IIB4.1) lacked significant protective activity. Thus, although the latter clone produced antibodies that reacted in high dilution (1:204,000) with the isolated pep M24 in ELISA, it did not produce antibodies capable of opsonizing or protecting against the intact streptococci.

Reaction of Monoclonal Antibodies with Native M Protein on the Surface of Intact Streptococci. Since clone IIB4.1 produced antibodies that reacted in high dilution with the isolated pep M protein molecule, and yet failed to opsonize streptococci or to protect mice against challenge infections, we investigated the accessibility on the intact surface of the parent streptococci of the antigenic determinant against which these antibodies were directed. In these experiments, whole log-phase streptococci were utilized as the solid phase M protein antigen in ELISA. As can be seen in Table IV, each of the monoclonal antibodies except IIB4.1 reacted at a high dilution against the

TABLE II

Indirect Bactericidal Tests of Hybridoma Ascites Fluids against Type 24 S. pyogenes

Ascites fluid	Number of colonies of type 24 streptococci after 3-h growth in test mixtures	
	Inoculum 51	Inoculum 14
Control	>2,000	1,016
IC1.3	77	62
IIC3.7	0	0
IIC4.6	0	0
IIA6.8	0	0
IIA6.6	420	159
IIB4.1	>2,000	>2,000

TABLE III

Type-specific Protection by Hybridoma Antibodies IIC3.7 and IIC4.6 of Mice Challenged with Type 24 or Type 6 Streptococci

Ascites fluid used to passively immunize mice	LD_{50} in mice challenged with:	
	Type 24 streptococci	Type 6 streptococci
Control, nonimmune	<50 (0/15)*	<130 (0/15)
Clone IIC3.7	300,000 (12/15)	<130 (1/15)
Clone IIC4.6	300,000 (12/15)	130 (2/15)
Clone IIB4.1	600 (5/15)	ND‡

* The numbers of survivors per number of mice challenged in each group are shown in parentheses.

‡ Not done.

TABLE IV

ELISA Titers of Hybridoma Antibodies against Intact Type 24 Streptococci

Ascites fluid	ELISA titers against type 24 streptococci
Control ascites	<200
IIC4.6	409,600
IIC3.7	819,200
IIA6.8	12,800
IC1.3	51,200
IIA6.6	51,200
IIB4.1	<200

whole streptococci. Thus, although the ascites fluid produced by clone IIB4.1 reacted at a dilution of 1:204,000 against the isolated pep M24 (see Table I), this fluid failed to react with the intact type 24 streptococci at a dilution as low as 1:200, indicating that the antigenic determinant is not available for antibody binding in the native state of the M protein. These results, therefore, are consistent with the findings that the antibody produced by clone IIB4.1 is devoid of opsonic or mouse protective activity.

Discussion

Hybridoma technology (18) provides the cell biologist with methodology that renders practical the production of highly specific probes for analysis of structure-function relationships of complex macromolecules. The data presented here clearly demonstrate the type-specific opsonic and protective properties of several distinctly different hybridoma antibodies raised against a structurally defined fragment of type 24 streptococcal M protein, a complex bacterial cell surface macromolecule that renders these microorganisms resistant to phagocytosis in the nonimmune host.

Because hybridoma antibodies are directed against a single distinct immunodeterminant, it is assumed that the antibodies that precipitated pep M24 in double immunodiffusion tests are directed against an immunodeterminant that is repeated at least once in the molecule to enable lattice formation between antigen and antibody. The concept of repeating antigenic determinants is consistent with the repeating covalent structure of streptococcal M protein established in our laboratories

(7, 10) as well as by Manjula and Fischetti (19, 20). Our studies do not exclude the possibility, however, that the nonprecipitating hybridoma antibodies may be directed against a determinant repeated in another fragment of M protein that may not have been included in the fragment extracted with pepsin during the purification process; recent studies (21, 22) suggest that the native M protein is considerably larger than the pep M protein fragment.

Neither the ability to precipitate pep M24 nor the ELISA titer of hybridoma antibodies correlated with opsonic, bactericidal, or protective activity. In fact, one of the nonprecipitating antibodies, IIC4.6, was equal in opsonic, bactericidal, and protective activity to the strongest opsonin among the precipitating antibodies, IIC3.7. One precipitating hybridoma antibody (IIB4.1), which had a high ELISA titer against pep M24, was neither opsonic nor protective.

Several alternative explanations could have accounted for the lack of protective activity of antibodies produced by clone IIB4.1. First, the antibody may have been directed toward a determinant not involved in the antiphagocytic activity of the M protein. Second, the antibody may have been able to bind to the M protein determinant, but because of an internal location on the streptococcal surface may not have been recognized by complement or phagocytic cells. This latter situation has been suggested to be responsible for the antiphagocytic effect of the capsules of certain strains of *Staphylococcus aureus* (23). Third, a new antigenic determinant may have been created on the isolated M protein fragment during the pepsin extraction procedure. Finally, the antigenic determinant may have been buried in the surface M protein in its native conformation either by other surface structures or within molecular folds on the M protein molecule itself. The last hypothesis is consistent with our previous observation that certain immunodeterminants exposed on the surface of one serotype of M protein may be buried in the molecular folds of another (14). Our data, obtained by using intact streptococci as the solid phase antigen in ELISA, support the idea that the antigenic determinants against which the antibodies in clone IIB4.1 are directed are inaccessible in the native state of M protein on the intact streptococcal surface. Recent studies (E. H. Beachey et al., manuscript in preparation) show that this antibody binds to peptide fragments of pep M24, indicating that the antibody is directed against antigenic determinants of the M protein molecule and not against a contaminating antigen.

Thus, not all of the immunodeterminants of type 24 M protein are exposed on the surface of the intact bacteria. This may explain the disparity reported by several investigators between the opsonizing and immunoprecipitating activities of certain antisera directed against streptococcal M protein (24–27). If the immunoprecipitating antisera contained a predominance of antibodies directed only at determinants buried on the surface of intact organisms, one would expect an inability of the antisera to opsonize the organisms. On the other hand, those sera having a predominance of antibodies directed against a nonrepeating antigenic determinant exposed on the surface of the bacteria would be opsonic, but would fail to precipitate isolated M protein.

We have presented data in previous studies (7, 8) demonstrating that the whole pep M24 molecule is not needed for the development of protective immunity. Evidence presented here suggests the possibility that a single antigenic determinant could evoke type-specific protective immunity if presented appropriately to the host.

Further studies to determine precise antibody-combining sites will be useful in determining the minimum peptide sequence necessary to evoke protective immunity against homologous streptococci. Small protective determinants from several different serotypes of M protein could then be included in a multivalent vaccine which would be free of nonessential structures that may give rise to toxic or tissue cross-reactive responses.

Summary

Hybridoma technology was used to produce a set of monoclonal antibodies against a purified polypeptide fragment of type 24 streptococcal M protein to delineate the protective determinants of M protein exposed on the surface of the virulent streptococci. Several hybridoma antibodies were found to be opsonic against the homologous type streptococci. At least two of these antibodies (IIC3.7 and IIC4.6) protected mice against challenge infections with the homologous, but not a heterologous, serotype of bacteria. One of the hybridoma antibodies that reacted in high dilution (1:204,800) with the isolated M protein failed to react with the M protein on the surface of type 24 streptococci, and thus did not opsonize the homologous organisms or protect mice against challenge infections. Because hybridoma antibodies are directed against a single distinct immunodeterminant, these results indicate that protective immunity may be directed at any one of several distinct antigenic determinants of M protein exposed on the surface of virulent group A streptococci.

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