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Research Article

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Mediation of Cytotoxic Effects of Streptococcal M Protein by Nontype-Specific Antibody in Human Sera

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ABSTRACT The cytotoxic moiety(ies) in highly purified streptococcal M protein has been shown to be distinct from the type-specific M determinant. This nontype-specific M-associated determinant(s) (NTSM) causes humoral and cellular immunotoxic responses in man. NTSM is common to M protein prepared from all streptococcal serotypes studied so far. In this study, immunoabsorbents prepared by entrapping purified M proteins in polyacrylamide gel were employed to identify, separate, and purify antibodies directed against NTSM as well as against the type-specific M (TSM) determinants.

We found that anti-NTSM present in human blood mediated cytotoxic platelet and leukocyte reactions in the presence of "M proteins" prepared from groups A, C, and G streptococci. Human sera that produced cytotoxic reactions and fixed complement in the presence of highly purified M protein but that contained no antibody to the homologous TSM determinant were used as a source of anti-NTSM. Anti-NTSM was absorbed with and eluted from M protein-polyacrylamide particles and identified as IgG. Antibodies to NTSM were present in most normal human sera and some primate sera (rhesus monkey and baboons) but not in the sera of other common laboratory animals. Further absorption studies showed NTSM to be a component not only of extractable M protein but also of protoplast membranes and of cell walls of avirulent streptococci that lacked extractable TSM antigen.

Preparation of antisera that can distinguish between the type-specific protective moiety and the closely associated immunotoxic components in purified M protein vaccines may help answer whether or not M-associated moieties play a role in pathogenesis of poststreptococcal diseases.

INTRODUCTION

Thus far, extraction and purification of group A streptococcal M protein has failed to yield a completely homogeneous type-specific antigen that reacts in human serum with its homologous-type antibody only. All of the purified fractions of M protein we have studied react either in the skin (1) or with the blood (2, 3) of human hosts whether or not they are type-specific immune to the homologous M serotype of streptococci. It became apparent, therefore, that even the most highly purified M proteins retain a nontype-specific antigen(s), hereafter called NTSM,[1] which has broad immunotoxicity. Intensive efforts by the conventional methods of gel filtration, ion-exchange chromatography, and polyacrylamide gel electrophoresis have failed to separate completely NTSM from the type-specific M (TSM) determinant in purified M protein fractions (2, 4–6).

In a previous report (2), the cytotoxic effect of M protein in human blood was shown to be mediated by thermostable and thermolabile serum components presumed to be antibody and complement. Further investigations were undertaken to identify more clearly the specificity of the antibodies involved in such cytotoxic reactions.

This report demonstrates the clear differentiation between antibodies in human sera to the NTSM and the TSM determinants of purified M protein by methods that we hope will clarify the immunology and possible

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[1] *Abbreviations used in this paper:* CF, complement fixation; HD, hemolytic doses; NTSM, nontype-specific M; PA, platelet aggregation; PMN, polymorphonuclear leukocyte; PRP, platelet-rich plasma; THB, Todd-Hewitt broth; TSM, type-specific M.

biologic significance of the NTSM moiety that up to now has been inadequately identified.

METHODS

Streptococci. The strains of streptococci used in this study included M types 6 (S43), 12 (SF42), and 30 (D24) which were obtained originally from Dr. Rebecca Lancefield, The Rockefeller University, New York, and have been repeatedly passed through mice for several years in our laboratories. In addition, an M24 strain isolated from a patient with acute rheumatic fever and an M-negative, type 1 (av) strain[2] were used. Stock cultures of each serotype were stored lyophilized or frozen (−70°C) in defibrinated sheep blood or in Todd-Hewitt Broth (THB) supplemented with 20% normal rabbit serum.

Extraction and purification of M protein. Streptococci were grown in 30–60-liter batches in THB, sedimented by continuous-flow centrifugation, washed in phosphate-buffered 0.9% NaCl, extracted with hot HCl, and purified by ribonuclease digestion, gel filtration, and column chromatography as previously described (1). The TSM antigen in the purified preparations was "titrated" with M type-specific rabbit antiserum by the capillary tube precipitin test (7) or by double-diffusion tests in agar gel, as previously described (1, 2). Antisera to whole streptococci were produced by immunization of rabbits according to the method of Lancefield (8).

Cell walls and protoplast membranes were prepared by rupturing whole streptococci with glass heads in a Braun MSK homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.) followed by differential centrifugation and washing as described by Bleiweis, Karakawa, and Krause (9). Cell wall mucopeptide was prepared by the hot formamide extraction method of Fuller (10).

Human blood. Fresh blood samples were obtained from healthy volunteers by venipuncture. Blood samples were immediately mixed with heparin (10 U/ml) to prevent clotting. All fresh blood samples were used in the various tests described below within 2 h after bleeding.

Streptococcal phagocytosis tests. Timed phagocytosis tests for TSM antibody were performed as previously described (2). Briefly, phagocytosis test mixtures consisted of 0.4 ml of fresh, heparinized human blood, 0.05 ml of a standardized suspension of phagocytosis-resistant streptococci (approximately 10 streptococcal U/leukocyte), and 0.05 ml rabbit antiserum against the homologous M type. The mixtures were incubated at 37°C in sterile, rubber-stoppered test tubes by tumbling end-over-end in a rotator apparatus at 8 rpm. The percentage of polymorphonuclear leukocytes (PMN) containing ingested streptococci (percent phagocytosis) was estimated by microscopic examination of stained smears prepared from a drop of test mixture at 15 and 30 min incubation.

The effect of purified M protein immunoabsorbents (see below) on the phagocytosis test was studied by incubating anti-M antiserum with homologous or heterologous M protein entrapped in polyacrylamide gel for 1 h in an ice-water bath. The immunoabsorbent was then removed by centrifugation and the supernatant antiserum added to the test mixture containing homologous-type streptococci as described above.

Preparation of M protein polyacrylamide immunoabsorbent. Insoluble immunoabsorbents were prepared by polymerizing macroporous acrylamide gels in the presence of purified M protein by the method of Carrel and Barandum (11). During polymerization the M protein becomes entrapped in the interstices of the polyacrylamide gels but its antigenic sites remain potentially free to react with antibodies. The monomer solution consisted of 7.5 g acrylamide and 2.5 g of N,N'-methylenebisacrylamide (BIS)/100 ml water. For entrapment, 5 ml of the monomer-BIS solution, 1 ml of M protein solution (5–20 mg/ml), and 0.2 ml of a riboflavin solution (3 mg/10 ml deionized water) were polymerized by photoactivation for 60–90 min at room temperature. The polymerized M protein-gel was mixed with 30 ml 0.1 Tris-NaCl buffer at pH 7.5 (Tris buffer) and mechanically dispersed into small particles by forcing through a syringe, once without a needle and a second time through a 20 gauge needle. The particles were washed three times in 30 ml Tris buffer then in 20 ml 0.1 M glycine-HCl buffer at pH 2.3 until the washes were free of protein. The gel particles were then equilibrated again in Tris buffer and stored at 4°C until used for absorption experiments. For absorption of TSM and NTSM antibody, 0.25 ml of washed, packed gel particles was suspended in 1 ml rabbit antiserum containing TSM antibody or human serum containing NTSM antibody. After stirring the mixture for 1 h at 0°C, the gel particles were removed by centrifugation and washed with glycine-HCl buffer, pH 2.3, as described above to remove the absorbed antibody. After resuspending in Tris buffer, the M protein-gel particles could be used again for absorption studies.

PMN migration tests. Tests of the migration of human blood PMN in the absence, and in the presence, of various M protein preparations were performed according to Bryant, DesPrez, VanWay, and Rogers (12) and as previously described (2), with the following modifications: Heparinized (10 U/ml) samples of fresh human serum were centrifuged at 1,000 *g* for 20 min to sediment the erythrocytes and leukocytes. The supernatant plasma was removed and the cells were washed three times with Tyrode's buffer (pH 7.4) supplemented with 0.25% gelatin and 10 U/ml of heparin. After the final wash, the cells were resuspended in fresh human serum that had previously been absorbed with various immunoabsorbents. All absorptions of the suspending sera were performed at 0°C for 1 h, a technique that removes antibody without removing significant quantities of complement. Test mixtures were then prepared by mixing 0.45 ml of the cells suspended in absorbed serum with 0.05 ml of M protein (1 mg/ml) dissolved in phosphate-buffered saline (PBS). Migration of PMN packed in glass capillary tubes was measured as previously described (2).

Platelet aggregation tests. Platelet aggregation was studied by the turbidometric method of Born (13) and as previously described (2). Platelet-rich plasma (PRP) was obtained by centrifuging fresh heparinized (10 U/ml) or citrated (1 part 3.8% sodium citrate: 9 parts fresh blood) human blood at 226 *g* for 10 min at room temperature. The supernatant PRP was transferred in 0.5-ml amounts into 6 × 45-mm glass cuvettes. A small siliconized iron bar was added to the cuvette and with continuous magnetic stirring, light transmission was recorded in a Payton aggregometer module.[3] Each substance to be tested was added in 0.025- to 0.05-ml amounts. An increase of less than 10% in light transmission was read as negative for aggregation 15 min after addition of the test substance.

[2] Kindly supplied by Dr. Roger Cole, National Institutes of Health.

[3] Model 300A, Payton Associates Ltd., Buffalo, N. Y.

NTSM platelet aggregating (PA) antibody in human sera was titrated using PA antibody-deficient PRP obtained from individuals whose PRP was previously shown not to aggregate in the presence of M protein. Serial twofold dilutions of test serum were added to 0.5 ml of the deficient PRP in 0.05-ml amounts. After establishing a stable base-line recording for 3–5 min, purified type 30 M protein was added as described above. The highest dilution of serum that restored PA activity multiplied by the dilution in PRP was designated the PA antibody titer.

In some experiments the test serum was absorbed with various immunoabsorbents before it was added to the deficient PRP.

Complement fixation tests. The tests were performed by the microtechnique employed at the Communicable Disease Center, Atlanta, Ga. (14). 0.05 ml of guinea pig complement, diluted to contain eight hemolytic doses (HD), 0.025 ml of various antigen dilutions, and 0.025 ml of serum dilutions were mixed and incubated overnight at 4°C. Sheep erythrocytes (2.4%), sensitized with antibody, were added in 0.025-ml amounts to each cell. The reaction mixture was then incubated at 37°C for 30 min and the complement-fixation (CF) titers recorded as described (14). All human sera were diluted 1:10 and then heat-inactivated at 56°C after addition of five HD of guinea pig complement to prevent anticomplementary effects encountered in some human sera.

RESULTS

Comparison of PMN migration, PA, and CF tests for anti-NTSM in human blood. Surveys of human bloods were made for their nontype-specific reactivity with M protein employing PA, PMN migration inhibition, and CF tests. The results of this systematic study will be reported elsewhere (15) but from preliminary observations it became apparent that PMN migration and PA tests parallel each other in detecting the nontype-specific cytotoxic effects of streptococcal M protein in human blood (Table I). Moreover, the tests of cytotoxic serum activity in PA tests paralleled CF titers of antibody against purified type 30 M protein (Table II). None of the sera contained type-specific opsonic antibody against type 30 streptococci, indicating that the CF antibody was nontype-specific.

Approximately 75% of the adult human sera tested with M protein reacted in these systems. Furthermore, the greatest anti-NTSM activity by all three tests was observed in sera obtained from patients with acute rheumatic fever (15) which is in agreement with the findings recently reported by Widdowson, Maxted, and Pinney (5).

Sera were selected, therefore, for absorption studies with M protein, cell walls, and protoplast membranes in order to identify more clearly the specificity of the antibodies involved in these reactions. These absorption studies required an insoluble M protein preparation that could be removed from the serum after absorption. Insoluble immunoabsorbents were prepared, therefore, by mechanically entrapping M protein in macroporous polyacrylamide gel (see Methods), a method that leaves both type-specific and nontype-specific antigenic sites potentially exposed so that they can react with their respective antibodies.

Absorption of TSM antibody from hyperimmune rabbit antisera. Since most of the normal human sera we studied lacked TSM antibodies against the serotypes of M protein used, absorption experiments were performed with hyperimmune rabbit sera to study the capacity of each M protein-gel to remove type-specific opsonic antibodies. Each M preparation was capable of absorbing homologous, but not heterologous, type-specific streptococcal opsonins (Table III). Control gel particles containing bovine gamma globulin did not absorb opsonic antibodies.

TABLE I

Comparison of PA and Inhibition of PMN Migration Tests for Anti-NTSM Activity in Normal Human Blood

Blood donor	Inhibition of PMN migration* in presence of:			PA‡ in presence of:		
	M12	M24	M30	M12	M24	M30
E. B.	+§	+	+	+	+	+
S. S.	+	+	+	+	+	+
C. L.	−	−	−	−	−	−
C. C.	ND	+	+	+	+	+
P. B.	+	+	ND	ND	+	+
T. P.	+	+	+	+	+	+
M. C.	−	−	−	−	−	−
R. R.	ND	+	+	ND	ND	+

* Each M protein (1 mg/ml) was added in 0.05-ml amounts to 0.45-ml washed blood cells reconstituted in fresh donor serum.

‡ M protein solutions (1 mg/ml) were added in 0.05-ml amounts to 0.5 ml PRP and PA recorded in an aggregation module. See also Methods.

§ +, positive reaction; −, negative reaction; ND, not done.

TABLE II

Comparison of CF and PA Tests for Anti-NTSM in Human Sera Against Streptococcal M30 Protein

Serum	NTSM antibody titer: CF	PA
J. E.	80	40
D. W.	80	40
E. H. B.	40	20
S1123	40	20
P. M. C.	<10	<10
C. L. S.	<10	<1

* See Methods.

TABLE III

Type-Specific Absorption of Streptococcal Opsonic Antibody by M Protein-Polyacrylamide Particles

Rabbit antiserum absorbed with:	% Phagocytosis of Streptococci in absorbed antisera	
	Type 12, opsonic system*	Type 24, opsonic system
M12-P‡	*0*	96
M24-P	92	*4*
Gamma globulin-P§	99	98

* Opsonic systems consisted of a mixture of human blood, type-specific antiserum, and homologous type streptococci.
‡ P, polyacrylamide.
§ Bovine gamma globulin.

Absorption of anti-NTSM from normal human serum with M protein immunoabsorbent. In addition to their ability to absorb type-specific antibody, each of the M protein immunoabsorbents tested was capable of absorbing anti-NTSM, as measured by the loss of M protein's leukotoxic effect in human serum absorbed with M protein-gel particles. Both M24 and M30 inhibited migration of PMN in unabsorbed serum that did not contain detectable type-specific opsonic or precipitating antibodies. In contrast, both M preparations lost their ability to inhibit PMN migration in serum absorbed with M24-polyacrylamide particles but not in serum absorbed with the control bovine serum albumin (BSA)-gel particles (Table IV).

Thus, the M protein entrapped in the gel particles appeared to have at least two distinct antigenic determinants, one of which was capable of absorbing type-specific opsonic antibody and one or more of which were capable of absorbing nontype-specific antibody. Further evidence that these antibodies are distinct was obtained in an experiment in which NTSM antibody was independently absorbed from a human serum that

TABLE IV

Loss of M Protein's Leukotoxic Effect in Serum Absorbed with M Protein-Polyacrylamide Particles

esh human serum absorbed with:	% Migration of PMN suspended in absorbed fresh serum in presence of:	
	M24	M30
M24-P	*93*	*94*
BSA*-P	36	47
Unabsorbed	49	53

* BSA, bovine serum albumin.

TABLE V

Differential Absorption of Anti-NTSM and Anti-TSM (Type 12) from Human Serum with M Protein-Polyacrylamide

Human type 12 antiserum absorbed with:	Reactions of human type 12 antiserum		
	PA		Opsonization, type 12 strep.
	M12	M30	
BSA*-P	+‡	+	+
M30-P	0	0	+
M12-P	0	0	0

* BSA, bovine serum albumin.
‡ +, positive test; 0, negative test.

also contained type-specific opsonic antibody against type 12 streptococci. The human serum was absorbed as before with M12-, M30-, or BSA-polyacrylamide. The absorbed serum was then tested for its capacity to opsonize type 12 streptococci and to mediate PA in the presence of M protein when added to the PRP of an individual whose serum lacked anti-NTSM activity (Table V). Absorption with the M12-gel particles removed the serum's capacity both to opsonize type 12 streptococci and to mediate M protein-induced PA. In contrast, the M30-gel removed PA activity but had no effect on type 12 opsonic activity.

It should be noted that the type 12 opsonic antibody reactions occurred in absorbed serum at levels apparently inadequate to produce PA by the homologous type 12 M protein. This result was apparently due to the low titer ($<1:2$) of M12-specific antibody in the human serum used in this study; M antibody in hyperimmune rabbit sera was capable of mediating type-specific PA (Table VI). In any case, it appeared that most, if not all of the PA antibodies in the human sera studied was due to anti-NTSM.

TABLE VI

Mediation of Type-Specific PA by Rabbit Anti-M Antisera

Rabbit serum added to human PRP*	PA in presence of:		
	M24	M30	PBS‡
Normal	0§	0	0
Anti-24	++++	0	0
Anti-30	+	++++	0

* PRP obtained from a donor who lacks anti-NTSM and anti-TSM. Rabbit sera were added in 0.05-ml amounts to 0.5 ml of PRP. After 5 min of constant stirring, 0.025 ml of M protein (1 mg·ml) was added.
‡ PBS, phosphate-buffered 0.9% NaCl pH 7.4.
§ No PA 15 min after addition of M protein or PBS, 0; aggregation within 3 min, ++++; 3–6 min, +++; 6–9 min, ++; 9–15 min, +.

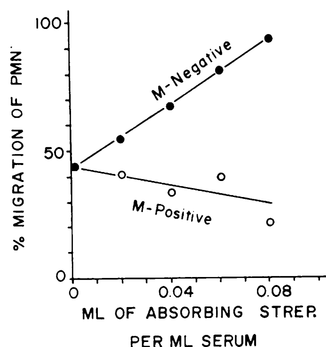

FIGURE 1 Effect of dose of whole M-negative (Type 1 av) or M-positive (type 30 D24) streptococci on absorption of NTSM antibodies from fresh human serum. After absorption for 1 h at 0°C, the fresh serum was mixed with washed PMN and M24. Migration was measured as described in Methods.

Absorption of anti-NTSM by M-negative streptococci. Our previous studies (2) had demonstrated that M-rich, phagocytosis-resistant streptococci had no apparent toxic effects on platelets and PMN in fresh human blood whereas M-negative streptococci, which were highly susceptible to phagocytosis, clumped platelets and PMN in a manner similar to that produced by purified M protein. This suggested that the extraction and purification process may have uncovered common antigenic determinant(s) buried just beneath the TSM in M-rich streptococci and that such common NTSM determinants may remain exposed on the surface of M-negative organisms that lack a fully formed TSM structure. To test this hypothesis, fresh human serum was absorbed with various amounts of washed, M-rich, or M-negative whole streptococci. As predicted, M-negative type 1 streptococci removed the anti-NTSM in a dose-related fashion (Fig. 1). In contrast, absorption with the same amounts of M-rich, type 30 streptococci did not affect the inhibition of migration in the absorbed serum when M protein was added. Similar results were obtained with other M-negative or M-rich streptococci, respectively. In each case, the M-negative organisms removed anti-NTSM and restored PMN migration in the presence of M whereas all strains of M-rich streptococci had no effect. A weak, M-positive strain of type 24 streptococci had an intermediate effect (Table VII).

These experiments, therefore, supported our hypothesis that NTSM is buried beneath the surface of M-rich streptococci but is exposed in dissociated variants that have lost their resistance to phagocytosis.

Absorption of anti-NTSM with streptococcal protoplast membranes. To determine whether the NTSM antigen(s) represented a deeper structure of the cell wall or, perhaps, an extension of the underlying protoplast membrane through the cell wall, fresh human serum, which contained NTSM antibody, was absorbed with 2 mg /ml of cell walls, purified cell wall mucopeptide, or protoplast membranes (Table VIII). The cell walls, but not mucopeptide, were capable of removing anti-NTSM. The protoplast membrane preparation (obtained from type 12 streptococci) however, was even more efficient in absorbing anti-NTSM. The presence of large amounts of protoplast membranes in purified cell walls (16) probably accounts for the capacity of M-rich cell wall preparations to absorb anti-NTSM in contrast to the surface of the intact M-rich streptococci.

TABLE VII

Absorption of NTSM Antibody from Fresh Human Serum with Whole Group A Streptococcal Cells

Streptococci used to absorb fresh human serum*	% Migration of PMN in presence of M protein and absorbed fresh serum:
M-negative	
Type 1 (av)	86
Type 2/44/19	100
M-positive (Strong)	
Type 30 (D24)	24
Type 24 (Vaughn)	51
M-positive (Weak)	
Type 24 (dissociated)‡	62
Unabsorbed serum	44

* 1 ml of fresh human serum was absorbed with 0.08 ml washed packed streptococci for 1 h at 0°C, centrifuged to remove streptococci, passed through 0.45 membrane filter, and mixed with an equal volume of washed blood cells.

‡ Type 24 streptococci that lost their resistance to phagocytosis after multiple subcultures in laboratory media.

TABLE VIII

Loss of M Protein's Leukotoxic Effect in Serum Absorbed with Streptococcal Cell Walls, Mucopeptide, or Protoplast Membranes

Fresh human serum absorbed* at 0°C with:	Percent migration of PMN suspended in absorbed fresh serum in presence of:	
	M24	M30
Unabsorbed	38	39
Protoplast membranes	100	100
Type 6 cell walls	66	ND
Mucopeptide	42	34

* 0.25-ml samples of fresh serum were absorbed with 1-mg amounts of the lyophilized streptococcal products.

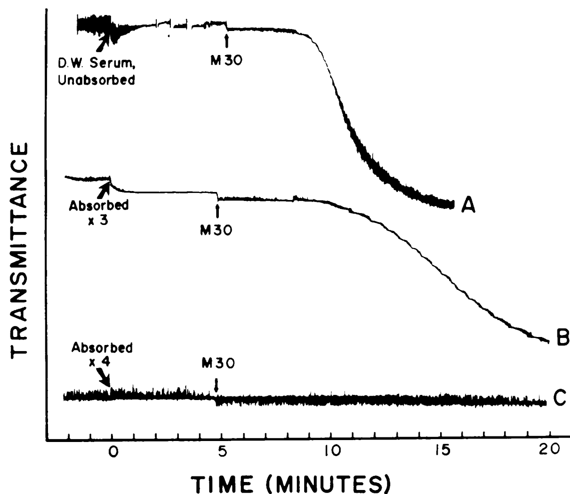

FIGURE 2 Absorption of anti-NTSM platelet-aggregating antibodies in human serum by streptococcal protoplast membranes. D. W. serum was added in 0.035-ml amounts to PRP deficient in anti-NTSM. After 5 min, M30 was added to induce aggregation. In experiment A, the unabsorbed serum was added. In B and C the serum was absorbed three and four times, respectively, with 0.3 mg/ml of type 24 protoplast membranes.

PA experiments confirmed the ability of protoplast membranes to absorb NTSM antibody from human serum. Fresh PRP of an individual who lacked anti-NTSM activity was selected as a source of thermolabile factors that are necessary to mediate PA. To this, heat-inactivated (56°C for 30 min) serum from a donor known to contain anti-NTSM was added. When M30 protein was added to the mixture, platelets aggregated promptly (Fig. 2A). After absorbing the serum three times with type 24 protoplast membranes (0.3 mg/ml serum), the M30-induced aggregation time was prolonged, suggesting a reduction in NTSM antibody (Fig. 2B). After a fourth absorption, the serum completely lost its power to mediate M30-induced aggregation (Fig. 2C).

TABLE IX
Mediation of PA by Anti-NTSM Eluted from M Protein Immunoabsorbents

Addition to PRP*	PA in presence of M30
Human serum absorbed with:	
M12-P‡	0
M30-P	0
BSA-P	+
pH 2.3 eluate from:	
M12-P	+
M30-P	+
BSA-P	0

* See footnote Table VI and Methods.
‡ P, polyacrylamide.

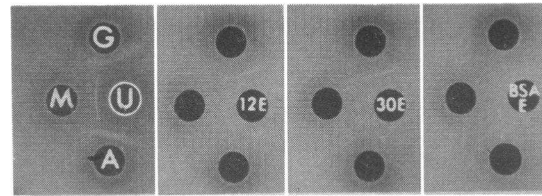

FIGURE 3 Agar gel-diffusion tests of unabsorbed human serum (U) and pH 2.3 eluates from M12(12E), M30(30E), and BSA(BSAE) immunoabsorbents with antihuman IgG(G), IgM(M), and IgA(A).

Elution of absorbed TSM and NTSM antibody from M protein-polyacrylamide gels. The preceeding experiments demonstrated that the M protein-polyacrylamide particles and protoplast membrane preparations were capable of absorbing the serum factor(s), presumed to be antibodies, that mediates M protein-induced PA and inhibition of PMN migration. Attempts were made, therefore, to purify the cytotoxic anti-NTSM factor. Human sera showing anti-NTSM activity were absorbed with M12-, M30-, or BSA-polyacrylamide. The adsorbed proteins were then eluted at pH 2.3 (see Methods), concentrated by ultrafiltration, and tested for anti-NTSM activity. Human anti-NTSM cytotoxic activity was eluted from both M12- and M30-polyacrylamide gels. Eluates from BSA-polyacrylamide gels did not contain anti-NTSM activity (Table IX). Double diffusion tests in agar gel with antisera to specific human serum fractions demonstrated that the eluted proteins contained precipitable amounts of IgG, but not IgM or IgA (Fig. 3).

Bacterial species specificity of NTSM. The following experiments were undertaken to determine whether or not NTSM antigen is unique to group A streptococci. The toxic effects of partially purified hot HCl extracts of a number of different bacterial species were studied in PA-tests. The bacteria studied other than group A streptococcal strains, included serogroups B, C, D, and G *streptococci, Streptomyces viridans, Diplococcus pneumoniae, Streptococcus fecalis, Staphylococcus aureus, Proteus mirabilis,* and *Pseudomonas aeruginosa.* Only the "M proteins" prepared from groups A, C, and G streptococci and one strain of *Proteus mirabilis* were capable of aggregating platelets in fresh human PRP. NTSM activity could not be extracted by hot acid from M-negative group A streptococci although whole cells of these same strains absorbed anti-NTSM. Staphylococcal protein A and *Escherichia coli* lipopolysaccharide were also without effect in PA or inhibition of PMN migration tests in human sera tested. This is in contrast to the reported effects of protein A in PRP of rabbits (17).

Distribution of NTSM antibody among animal species. A preliminary survey of various animal species for naturally acquired NTSM antibody using PA tests demonstrated such antibodies only in primates. Purified preparations of M24 and M30 did not aggregate platelets in the plasma of dogs, rats, rabbits, or guinea pigs. The PRP of one baboon and two rhesus monkeys reacted with both preparations in a manner similar to that observed with human PRP containing anti-NTSM.

DISCUSSION

In previous studies (1, 2) we were unable to separate physically the type-specific from the nontype-specific antigenic determinants in highly purified preparations of M protein. Therefore, we shifted our attack on the immunology of NTSM to the identification and isolation of the antibodies which we believe are the mediators of the cytotoxic effects of M protein vaccines in human bloods. The use of immunoabsorbents has enabled us to establish the separate identity of anti-NTSM and anti-TSM and offers now a valuable method for removal of cross-reactions which should provide human, as well as animal, antisera of exquisite type specificity. Furthermore, this procedure should provide antisera that will help resolve the question of the number and variety of NTSM antigens.

After immunization of rabbits with streptococcal vaccines, type-specific antisera can be prepared relatively readily by cross-absorption with heterologous M serotypes of streptococci because anti-NTSM is not naturally present in rabbit antisera and, as we have shown, is often not induced by artificial immunization with whole streptococcal cells. Studies of human sera for type-specific antibodies however, have been plagued by broad cross-reactivity of such sera with various serotypes of M protein and it has always been difficult to absorb selectively these cross-reactive antibodies from human sera.

The nature of the antibodies to M protein has created confusion and controversy as to their significance, especially in studies of the immune response in man to M vaccines. Antibodies induced by such vaccines often can be detected only by highly sensitive, but not completely type-specific, methods such as passive hemagglutination (6, 18) or CF (5, 19).

The polyacrylamide gel-M protein immunoabsorbents should help to prepare human type-specific antisera that can be employed in systems more sensitive and less cumbersome than the bactericidal test (i.e., passive hemagglutination, CF, radioimmunoassay). Such methods would help to prove the effectiveness of M protein vaccines in stimulating anti-TSM in man.

The immunology and identity of NTSM are of special interest both with regard to the potential use of streptococcal M protein as a human vaccine and with regard to the role NTSM may play in the pathogenesis of poststreptococcal diseases. Because the NTSM determinants appear to be attached to cell walls and protoplast membranes, and the latter have been implicated in immunological cross-reactions with human host tissue, notably sarcolemma of the heart muscle, further understanding of the biological properties of these antigens is mandatory if immunization of streptococcal vaccines containing these moieties is to be pursued. Our preliminary studies (3) of the relationship between anti-NTSM and antibodies against heart tissue demonstrated that protoplast membranes and purified M protein share antigen(s) that cross-reacts with heart tissue. Our findings are in agreement with both the studies of Kaplan (20) who believes that streptococcal antigens cross-reactive with heart are closely associated with antigens in M protein, and with Zabriskie (14, 21) who believes such cross-reactions are associated with protoplast membrane antigens. The NTSM of purified M protein preparations we have been studying may represent the antigen that is common to both.

Another aspect of the significance of NTSM is its biologic role in host resistance to streptococcal infections. Antibodies to NTSM transmitted through the placenta and rapidly acquired in early life by infection with ubiquitous streptococcal strains appear to confer on the human and animal host considerable "natural immunity" against those streptococci whose surface is not fully covered by the TSM antigen (2, 22). The masking of the highly reactive NTSM antigen of the cell wall is the basis of resistance to phagocytosis by M protein-rich strains in bloods that lack homologous type-specific antibody (23).

We conceive of M protein as projections from the streptococcal protoplast membrane that protrude through the cell wall glycopeptide, polysaccharide, and into the hyaluronate capsule. These fimbriae-like structures (3, 24) appear to carry the unique TSM antigenic determinant that is exposed on the surface of fully "virulent" organisms. The deeper NTSM-associated part of the fimbriae may arise at the protoplast membrane and may be continuous with, but lie proximal to, the TSM.

Although M-negative streptococci absorbed anti-NTSM, we were unable to extract the antigens from the whole organisms with hot HCl. Further studies are necessary, however, to determine whether or not NTSM, presumably trapped in the cell wall, may be extracted by isolation and rupture of cell wall preparations of M-negative strains.

Previous preparations of glycopeptide "endotoxins" from cell walls have involved deproteinization procedures. These deproteinized preparations were believed to contain the major toxicity of the cell wall (25–28). It would appear that protein endotoxins, such as NTSM,

have received inadequate attention as agents inducing tissue damage.

Our preliminary studies (15) and those of Widdowson, Maxted, and Pinney (5) already show (by CF tests) that antibodies to NTSM are readily stimulated by streptococcal infection and are particularly high in acute rheumatic fever. It should now be possible to determine whether these antibodies are related to the antiheart antibodies demonstrated by immunofluorescence in the sera of patients with acute rheumatic fever (21). So far, the latter have been only vaguely defined in terms of the specific antigens to which they are directed.

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