MULTIPLE MOUSE-PROTECTIVE ANTIBODIES DIRECTED AGAINST GROUP B STREPTOCOCCI

Special Reference to Antibodies Effective against Protein Antigens*

By REBECCA C. LANCEFIELD, MACLYN McCARTY, AND WILLIAM N. EVERLY

(From The Rockefeller University, New York 10021)

Streptococci for many years have been classified into a number of serological groups, many of which have also been subdivided serologically into specific types (1). It has been shown that these types, notably in Groups A and B, are related to immunity of the host on the basis of the composition of their specific antigens (2).

In the case of Group B streptococci, a surprisingly high proportion of strains isolated from both human and animal sources (references 3-12, and footnotes 1 and 2) could be classified into four types—Ia, Ib, II, and III—on the basis of serological precipitin tests with the specific capsular polysaccharides (Table I). Subsequently, a number of strains were encountered that reacted equally well with Type Ia and Type Ib type-specific antisera in the diagnostic precipitin test (13). By specific absorption experiments it was confirmed that these strains, provisionally designated as Type Ic, possess antigens in common, or closely related to, antigens that occur regularly in strains of Type Ia on one hand and Type Ib on the other (13). The polysaccharide component isolated from these newly recognized Type Ic strains was serologically indistinguishable from the hot HCl-extracted type-specific polysaccharide of Type Ia strains and is the basis for the Ic cross-reaction with Type Ia. A protein component common to Types Ib and Ic, designated Type Ibc protein antigen,[3] is responsible for the cross-reaction with Type Ib. No antigen with specificity solely related to Type Ic strain was found, although it is possible that one may yet be identified.

The immunology of the various Group B carbohydrate antigens has been the subject of earlier studies reported from this and other laboratories (2-4, 3-16, 18, 19), and some information has been obtained concerning the specific immunochemical determinants of certain of these antigens. However, a detailed chemical analysis of these polysaccharides with identification of each of the immunodominant components remains to be done. The present evidence indicates that more than one specific determinant occurs in each of the polysaccharides in its native form. Information concerning the protein antigens is meager. Here again, the available evidence indicates that the Ibc protein possesses more than one readily detectable specificity (13), but it has not been established whether this is referable to two separate protein antigens or two determinants on a single molecule.

The experiments presented in the present paper were designed to determine whether the protein antigens of Types Ib and Ic are significant in virulence and

* This study was supported in part by the National Institutes of Health grant no. HL 03919 and a grant from the New York Heart Association.

[1] Lancefield, R. C. Unpublished data on Group B, Strain A909, 1966.

[2] Type nomenclature different in Stableforth's classification (5).

[3] Formerly designated Ic protein (13).

protection. Mouse-protection tests involving antibodies to polysaccharide antigens have been previously reported (references 3, 4, 14, and 17 and footnote 1), and these were extended as a background for the study of protective antibodies directed specifically against the protein antigens. One of the interesting findings emerging from these studies is that antibodies directed to either the polysaccharide or the protein antigens of a single strain can be protective. Multiple mouse-protective antibodies have been found in the sera of immunized animals for all Group B types so far investigated. From one to three, or possibly four, distinct antibodies may be found in a serum raised against a single type. By absorption techniques, it can be shown that each antibody alone is capable of passively protecting mice against experimental infection.

Materials and Methods

Streptococcal Strains. Representative strains of the specific types of Group B streptococci employed previously (3) were used with the addition of a strain isolated from cultures of the umbilicus in a hospital nursery for newborn infants. The strains used in the present report were: 090, Type Ia; H36B, Type Ib; A909, Type Ic; 18RS21, Type II; D136C, Type III; and 090R used to prepare group-specific serum.

Mouse Passage. When a mouse-virulent derivative of a particular strain was required for the passive protection tests used for antigen and antibody analysis, the organisms were passed serially through mice.

The technique was as follows: 1 ml of fresh 16 h blood broth culture (or a larger dose if needed to kill the mouse) was inoculated intraperitoneally into a large adult mouse (26–28 g). If the animal was dead or moribund the next day, it was autopsied and the spleen removed aseptically to a small sterile mortar. The spleen was ground sterilely with a pestle and taken up in a minimal amount of broth. 1 ml of suspension was inoculated into a mouse in the morning. If the animal was sick by the end of the afternoon, it was etherized and the spleen removed for further passage in another mouse overnight. Smaller amounts of spleen suspension were passed twice daily into mice keeping the dose just large enough to kill the mice in these passages. Inoculated mice were held at refrigerator temperature overnight in order to reduce the danger of postmortem contamination of the spleen.

Sometimes a mouse-virulent organism (by mutation or selection during residence in the mouse) was obtained in a few passages. Sometimes it was much more difficult or impossible to obtain such an organism. The development of virulence was measured by a standard test using intraperitoneal inoculation of serial dilutions of a broth subculture of the spleen suspension. Mouse passage was continued until the subculture killed mice in a dose of 10^{-6}–10^{-8} ml. The cultures were lyophilized or stored at −60°C in enough tubes to supply a fresh sample for each day's work. Virulence tests on cultures of related Type I types were performed in the manner shown in Table II.

Preparation of Antisera. New Zealand red or hare-brown rabbits weighing 8 to 10 lbs are used. Several different methods of treating the suspensions for immunizing with Group B streptococci have been employed:

FOR TYPE-SPECIFIC ANTISERA. (*a*) The sediment of an overnight broth culture is killed by adding 3% formalin to the 20 times concentrated suspension to give a final concentration of 0.3% formalin. The suspension is kept in the refrigerator at 4°C. Subcultures are usually sterile overnight.

Immunization schedule: A fresh 1:20 dilution in 0.85% NaCl solution is made each day, which restores the suspension to the volume of the original culture. Injections of this dilution are made intravenously into adult rabbits (8–10 lbs) as follows — 1st wk: 0.5 ml for 3 successive days and 1.0 ml for 2 successive days. 2nd wk: 1.0 ml for 5 successive days and 5 days after last injection, test bleeding; if unsatisfactory in precipitin test, continue injections. 3rd series: 1.0 ml for 5 successive days and 5 days later, test bleeding. 4th series (if necessary): 1.0 ml for 5 successive days. 5 days later, test bleeding. If unsatisfactory, rest animal for 1 mo.

Prepare fresh vaccine for booster series with 1:20 dilution for injection freshly prepared daily. 1st booster series: 0.5 ml for 3 successive days and 1.0 ml for 2 successive days; 5 days later, test bleeding. 2nd booster series: 1.0 ml for 5 successive days and 5 days later, test bleeding. If unsatisfactory, rabbits may be discarded or rested for a month or more.

Usually the serum is satisfactory much earlier. The final bleeding when the rabbit is exsanguinated must be individually tested for each rabbit to ascertain which of the possible antibodies have been formed.

(*b*) A higher concentration of formalin (up to 3%) was unsatisfactory for preparing vaccines for specific types of group B because type-specific polysaccharides are sometimes destroyed.

(*c*) Some rabbits were immunized with tryptic or peptic digests of heat-killed vaccines to destroy surface proteins which might interfere with the antigenicity of the polysaccharide type-specific antigens. The results were somewhat indeterminate. Heat-killed vaccines (56°C/30 min) without proteolytic digestion were also employed. Higher dosages of heat-killed organisms were used for immunization in the same dosage as regularly used for group A strains (17).

FOR GROUP-SPECIFIC ANTISERA. Strain 090R which contains little or no type-specific antigen was used either with 0.3% or with 3.0% formalin-killed cultures. This strain had been derived from the Type Ia type-specific strain by daily serial subculture in 10% type-specific antiserum in broth. Good group-specific antisera were obtained. The R strain (090R), i.e. devoid of type-specific antigens, tended to revert to the type-specific 090S form (containing type-specific antigens) under laboratory conditions. Cultures were lyophilized or kept at −60°C to prevent reversion. It was necessary to confirm frequently that the culture maintained the R form and to repeat subcultivation in type-specific immune serum when indicated.

Specific Absorption of Antisera. Absorption was carried out with whole bacterial cells centrifuged from broth cultures. These were usually heat-killed at 56°C for 30 minutes and used in a proportion of 3 parts of whole serum to 1 part of packed cells. Some absorptions were carried out with living cells. The preparations were mixed and incubated in a 37°C water bath for 0.5 h, then centrifuged and the serum removed and filtered through a millipore filter. The sera were tested by the precipitin and Ouchterlony techniques and in passive protection tests with mice.

This is essentially the immunological method previously used in the tests to separate Types Ia and Ib (4). The use of whole bacterial cells is a substitute for absorption with chemically purified antigens, some of which have not been isolated and characterized. Effective depletion of certain antibodies from whole serum required the undesirable procedure of repeated absorption. Better results could probably be obtained by absorbing diluted serum, but this would have precluded the use of precipitin and double diffusion tests as controls.

Protection Tests. The mouse was considered the most suitable experimental animal available for passive protection tests, because in previous work immunity to human infection with streptococci was well correlated with results of serum protection in mice.

In Group B, in contrast to Group A, protection obtained by simultaneous injection of culture and serum is equally as good as that obtained by injecting the serum a day in advance of the culture. In the studies with Group B streptococci, the titrations were carried out by injecting mixtures of the virulent organism and the antiserum simultaneously, and each serum dilution was tested in four mice with several dilutions of the broth culture. Large and small doses of each component were usually selected to cover the active ranges of both. In certain cases of doubt arising from erratic survival patterns presumably due to differences in unknown factors of host resistance, the test was repeated with 10 replicate mice for each dose of serum and culture, a total of 40 mice in each case.

Most deaths of mice occurred in 1–2 days. The experiments were observed and deaths recorded for 1 wk, or for longer if any mice did not appear well. The death of animals receiving absorbed serum indicated the specific absorption of protective antibody. Survival of mice indicated that antibody was left in the serum.

Experimental

Serological analysis of Group B; Types Ia, Ib, and Ic strains

The analysis of representative strains and rabbit antisera revealed a complex pattern of antigenic composition reflected in corresponding antibodies. Previously the major type-specific polysaccharides which occur in Group B streptococci were referred to as "S" substances (soluble specific substances) in conformity with usage for pneumococcus types (3). This designation is now dropped for streptococci since other classes of substances have also been found

associated with specific type protection. By a combination of capillary precipitin, gel double diffusion, and mouse-protective tests, the following picture emerges of the major antigens involved in protection on which type designation is based:

Type Ia (Represented by Strain 090)

Ia, CARBOHYDRATE (*Ia CHO*)[4]. Ia CHO, the major polysaccharide antigen. (specific for Types Ia and Ic)

MINOR ANTIGEN. The minor antigen is probably a polysaccharide component and a part of Ia CHO, which is common to all of the Type I-related types. Designated as Iabc cross-reactive antigen.

Type Ib (Represented by Strain H36B)

Ib CHO. Ib CHO, a major type-specific polysaccharide antigen (specific for Type Ib).

Iabc CROSS-REACTIVE ANTIGEN. Iabc cross-reactive antigen is probably a minor determinant of Ib CHO.

A PROTEIN ANTIGENIC FRACTION. A protein antigenic fraction differentiated by trypsin and pepsin sensitivities (13). Designated Ibc protein and shared by Types Ib and Ic. This may be composed of two serologically distinct reactants.

Type Ic (Represented by Strain A909)1

Ia CHO. Ia CHO, a polysaccharide closely related to Ia CHO of Type Ia strains.

Iabc. Iabc, minor determinant of polysaccharide.

Ibc PROTEIN. Ibc protein, a fraction similar to that found in Type Ib. No strictly Ic-specific antigen has been found.

This summary is presented as a guide for the interpretation of the detailed experimental data which follow.

Mouse-Protective Antibodies Specific for the Proteins of Types Ib and Ic

Mouse protection with antibodies reactive against the specific polysaccharide immunodeterminants of Types Ia and Ib have been previously described. Special attention is given here to the protein antigens Ibc which are present together with the respective antigenic polysaccharides in Types Ib and Ic streptococci (Table I). A preliminary example of virulence tests of the strains used is given in Table II.

In the early studies of Group B streptococci, in contrast to the pattern of antigens in Group A, the antigenic structure seemed to be similar to that which was well known for pneumococcus. In pneumococcus, serologically specific polysaccharides were associated with protection for both man and mice.

The Group B streptococci were classified first by the precipitin test into types designated I, II, and III on the basis of the specific polysaccharides which they contained, with a certain number of strains unclassified. When several new sera were employed, it was found that two specific related types showing cross-protection in mice occurred among Type I strains. These were separated and designated Types Ia and Ib. By studying antisera against each strain, the occurrence was confirmed of specific types with varying amounts of cross-reactive antibodies in

[4] *Abbreviation used in this paper:* CHO, carbohydrate.

TABLE I

Summary of the Occurrence of Multiple Mouse-Protective Antibodies against Group B Streptococci

Type designation	Mouse-protective antibodies against:		
	Polysaccharides*		Antigenic protein determinants (Ibc) (cross-reactive)
	Major antigens (specific)	Common polysaccharide determinant (Iabc) (cross-reactive)	
Type Ia	Ia	Present	Absent
Type Ib	Ib	Present	Present
Type Ic	Ia	Present	Present
Type II	II	Absent	‡
Type III	III‡	Absent	‡

* The group-specific cell-wall polysaccharide does not give rise to protective antibodies.
‡ Mouse protection for these antigens not yet studied.

TABLE II

Example of Virulence Tests with Streptococcal Strains: Mouse-Passage Cultures of Group B; Types Ia, Ib, and Ic

Virulence titration* (no serum employed; 0.2 ml culture dilutions)	Strain 090/14, Type Ia		Strain H36B/60, Type Ib		Strain A909/14, Type Ic	
	No. of colonies	Deaths	No. of colonies	Deaths	No. of colonies	Deaths
$1:10^{-8}$	11	D2‡	9	D2	7	S§
$1:10^{-7}$	57	D1	38	D4	31	D2
$1:10^{-6}$	247	D1	239	D1	217	D1
$1:10^{-3}$		D1		D1		D1

The number of streptococcal chains in the test culture was obtained by counting the numbers of colonies in pour blood agar plates from each culture dilution employed.
* No. of mouse passages indicated after slanted line (/), e.g., 090/14.
‡ D, followed by a numeral indicates day of death.
§ S, indicates survival of a mouse for at least 1 week.

the antisera prepared. The cross-reacting antigen could not be separated from the type-specific polysaccharide determinants. It appeared to be a nonprotein serological determinant (Table I) common to the Ia and Ib strains (and later to Type Ic strains.[5] The unabsorbed Ia and Ib antisera were often completely type specific and devoid of cross-reactivity. Type-specific antisera could be prepared by absorbing with any one of the three Type I-related strains in the appropriate combination of serum and strain.

The third Type I-related type, designated Ic, contained the same polysaccha-

[5] Designated Iabc polysaccharide (Table I).

ride antigen present in Type Ia, together with antigenic proteins not present in Type Ia but closely similar to those in Ib. This protein fraction was designated Ibc to indicate the types in which it was found.

Table I is a summary of the information obtained in this and earlier work on the occurrence of the antigens of Group B streptococci that induce mouse-protective antibodies. The experimental data which follow were selected from a large number of mouse protection experiments using a variety of strains, antisera, and reciprocal absorptions. The representative experiments presented (Table III) serve to illustrate the finding that led to the current picture of mouse-protective antibodies in the subtypes of Group B, Type I streptococci. This summary is presented as a further guide for interpretation of the experimental data that follow.

TABLE III

Guide for Sera Used in Analyzing the Occurrence of Multiple Mouse-Protective Antibodies against Group B Streptococci

Serum designation			Strains used for immunization	Mouse-protective antibodies against involved antigens			
Type	Code	Rabbit		Ia major CHO (specific)	Ib major CHO (specific)	Iabc common CHO determinant	Ibc antigenic protein determinant
Type Ia	26 (Table V)	W3178	Strain 090 (Ia), formalin killed	Present	Absent	Absent	Absent
Type Ib	52 (Table IV and IV A)	W3192	Strain C12B (Ib), formalin killed	Absent	Present	Absent	Present
Type Ib	13 (Table VI)	W3835	Strain H36B (Ib), tryptic digest vaccine	Absent	Present	Absent	Present
Type Ib	20 (Table VII)	W3181	Strain H36B (Ib), formalin killed	Absent*	Present	Present	Present
Common CHO (Iabc)	22* (Table VIII)	W3224	Strain A A909 (Ic)	Absent*	Absent	Present	Absent*

For virulence of streptococcal strains used in these studies, see Table II.

* This serum was selected as an example of antiserum having protective antibodies directed only to the Iabc determinant. Antisera to strain A909 (Ic) usually contain antibodies to both the Ia CHO and Ibc Protein.

Mouse Protection with Antisera Containing Ibc Antibodies

A Type Ib antiserum (no. 52) was selected which did not show cross-protection with Type Ia streptococci in order to avoid the complication of the Iabc cross-reaction (Tables I and III). Response of individual rabbits to the same antigen varies. This rabbit (W3192) had been immunized with a Type Ib strain (C12B) and represented one of the variations in response of rabbits to immunization with the same vaccine. The unabsorbed serum (no. 52, Table IV) contained streptococcal antibodies Ibc corresponding to antigens in both Type Ib and Type Ic organisms. After four repeated absorptions with living or heat-killed culture of either Ib or Ic streptococci, the mouse-protective antibodies referable to the immunodeterminants Ibc were absorbed by either strain. This was shown even when the absorbed serum samples were tested with 10 replicate mice for each dose of absorbed serum (Table IV A, samples no. 52R or no. 52N). It was concluded that the Ibc antibodies had been absorbed specifically by strain A909

(sample no. 52N), and also by strain H36B (sample no. 52R) which contained Ibc protein antigen as well as Ib type-specific polysaccharide. Serum sample no. 52N, however, still contained mouse-protective antibodies effective against the type-specific Ib polysaccharide determinant. This protective property was removed only if a Ib strain (H36B) containing the Ib CHO determinant were the absorbing agent (sample no. 52R). When, however, strain A909, the Ic strain which did not contain the Ib CHO was the absorbing agent (sample no. 52N), the antibodies to the Ib polysaccharide remained in the serum and protected mice against strain H36B. Only those to the Ibc proteins were absorbed from this sample by strain A909, thus showing their relationship to the Ic strain A909. Table IV shows that absorption of serum no. 52 with Ib strain H36B removed antibodies protective for both Types Ib and Ic (no. 52R). Strain A909 (Type Ic) absorbed Type Ic protective antibodies (no. 52N) but the same absorbed serum still protected against infection with strain H36B (Type Ib).

In serum sample no. 52R absorbed with the Ib strain H36B it was observed that the strain containing the major type-specific polysaccharide (H36B) was slower in removing Ibc antibody than the Ic strain A909, possibly due to steric hindrances between the polysaccharide and protein antigenic determinants in Type Ic or the competitive absorption of antibody to antigens in the cell surface. Precipitin tests both in capillary tube and agar gel techniques supported the conclusions of the serological similarity of these protein determinants in the two types.

It was concluded from the results of this and similar experiments that separate antibodies to specific polysaccharides and antigenic protein immunodeterminants existed in the same serum after immunization with a single strain. Mouse protection was brought about by either kind of antibody specificity, Ibc protein as well as specificity due to the polysaccharides Ia or Ib, in response to strains containing the corresponding antigenic determinant.

Re-Examination of Mouse Protection with Antibodies to Specific Polysaccharides of Individual Types

Types Ia and Ic: The Major Specific Polysaccharide, Ia CHO. To furnish a background for the studies described of mouse protection with Ibc antibodies against Group B streptococci containing Ibc protein antigens, protective antibodies directed against polysaccharide immunodeterminants have been reexamined for several specific types within Group B. These polysaccharides appear to be the major type-specific antigens (Tables I and III). They are characterized by a high degree of immunological activity. Passive protection of mice by means of dilutions of antiserum to 1:10,000 or more is not unusual. A dilution of 1:80,000 has been effective with some sera. An example (Table V) is shown of a Type Ia serum which protects both Ia and Ic strains. Both strains contain the Ia polysaccharide and mice are protected against either strain at a serum dilution of at least 1:4,000. This serum affords no protection to a strain of heterologous Type Ib (H36B) which does not contain Ia type-specific polysaccharide, and none to other heterologous types (Type II).

Either strain 090 (Type Ia) or strain A909 (Ic) absorbs all protective antibody from this serum in suitable absorption experiments which have been performed but are not recorded here.

TABLE IV

Absorption of Type Ib Antiserum: Example to Show Mouse Protection with Antibodies Directed to Ibc Proteins

Serum no. 52 (against Ib strain) absorption record		Challenge strains (1 mouse challenged with each dose of serum and culture; culture dilution 10^{-3})*			Interpretation of content of relevant antibodies before and after absorption of no. 52 serum sample resulting in protection of mice
Serum sample treatment and designation	Serum dilution	090/14, Type Ia	H36B/60, Type Ib	A909/14, Type Ic	
Unabsorbed*; no. 52	1:50	D1	S	S	Unabsorbed antiserum contains antibodies to (*a*) type-specific Ib CHO, and (*b*) protein Ibc. See Table III.
	1:1,000	D1	S	S	
Interpretation of protection			Protected by anti-Ib CHO and by anti-Ibc protein	Protected by anti-Ibc protein	
Absorbed 4 times with Type Ib, Strain H36B; no. 52R	1:50	D1	D1	S	No significant antibodies left in no. 52R after absorption with Ib strain. See Table IV A
	1:1,000	D1	D1	D2	
Interpretation of protection				Slight or none	
Absorbed 4 times with Type Ic Strain A909; no. 52N	1:50	D1	S	D1	Antitype-specific Ib CHO left in no. 52N after absorption with Ic strain. Protects homologous type strain
	1:1,000	D1	S	D2	
Interpretation of protection			Protected by anti-Ib CHO		

0.2 ml of serum dilutions plus 0.2 cc of 10^{-3} dilution of culture (10^{-6} of culture dilution tested but not plotted here). See Table IV A for continuation of record with certain critical combinations of serum and culture challenged with 10 mice each for confirmatory tests of absorption of antibodies. Absorption of serum no. 52 with Type Ia (strain 090) was performed as above. Results were not tabulated here. Four repeated absorptions did not remove the Ibc antibodies since Ia strains do not contain the protein Ibc antigen.

* See Materials and Methods and Tables I, II, and III.

Type Ib: Major Specific Polysaccharide, Ib CHO. Using an example of Type Ib antiserum (Table VI) which does not have cross-reactivity due to the common polysaccharide determinant Iabc, specific protection directed to the Ib polysaccharide can be demonstrated. This has been confirmed in appropriate cross-absorption experiments (Tables IV, IV A, and VII). A complication is encountered because antibody against the Ibc protein has also been induced in this serum (serum no. 13, Table VI) by the Ib strain, as is customary with rabbits immunized with strains of this type. Table VI illustrates protection obtained when this Ib serum is tested unabsorbed against the three strains in question. It does not protect mice against infection with the Type Ia strain because no Iabc antibody is present. However, this serum protects mice against the homologous Ib strain with one or both Ib antibodies which are present, anti-Ib polysaccharide or anti-Ibc protein. The unabsorbed Ib antiserum also protects mice against the heterologous strain A909 because of the anti-Ibc antibodies present. Similar selective absorption of this serum, not recorded here, confirms this.

Another of the cross-absorption experiments to confirm the specific protection

TABLE IV A

Confirmatory tests of absorption of Ibc antibodies

Serum no. 52 (against Ib strain) absorption record		Challenge strains (10 mice challenged with each dose of serum and culture; culture dilution 10^{-3})				Comment on relevant antigens in strains used
		H36B/60, Type Ib		A909/14, Type Ic		
Treatment and designation	Serum dilution	Mice survived	Mice died	Mice survived	Mice died	
Absorbed 4 times with Type Ib, Strain H36B; no. 52R	1:50	4	6	4	6	Strain H36B contains type-specific antigen Ib CHO and protein antigen Ibc (probably covered by major antigen Ib CHO)
	1:1,000	1	9	0	10	
Interpretation of protection		Partial absorption of Anti-Ib CHO		Partial absorption of Anti-Ibc protein		
Absorbed 4 times with Type Ic, Strain A909; no. 52N	1:50	10	0	0	10	Strain A909 contains protein antigen Ibc but not polysaccharide antigen Ib CHO
	1:1,000	10	0	1	9	
Interpretation of protection		Protected by anti-Ib CHO		Probably complete absorption of anti-Ibc (possibly trace of anti-Iabc)		

See Materials and Methods. 0.2 ml of serum dilutions plus 0.2 ml of 10^{-3} dilution of culture injected into each mouse. 10^{-6} dilution of culture tested with same results.

directed against the Type Ib-type polysaccharide is shown in Table VII. This serum (no. 20) contains three separate protective antibodies demonstrable by selective absorption (see guide for sera analyzed, Table III). Suffice it to say that examination of the data recorded in Table VII shows that after suitable selective absorption the serum (no. 20d) protects specifically against Type Ib, and not against Types Ia and Ic. The same result is seen throughout Group B experiments but is not tabulated here. Type-specific protection can be obtained by absorbing the Type Ib unabsorbed serum shown in Table VI. Absorption with strain A909 (Ic) removes antibody directed against the Ibc antigen but does not absorb the protective antibody against Ib CHO. Strain H36B (Ib) is still protected.

Chemical studies of the responsible type-specific polysaccharides, Ia CHO and Ib CHO, were previously reported (4, 13, 19) and have not been repeated in these experiments.

Mouse-Protective Antibody with Specificity for the Polysaccharide Determinant (Iabc) Common to Three Types; Types Ia, Ib, and Ic

Another protective antibody can be demonstrated which is common to Types Ia, Ib, and Ic (3). The antigen is designated temporarily Iabc to indicate the types involved. Antibodies have been induced with representatives of all three of these types, and sera have been obtained in each case in which the protective properties appear the same for all of them. An example is shown in Table VIII (serum no. 22). Any one of the three Type I-related strains absorbs all antibody for the common polysaccharide determinant. The nature of this common

TABLE V

Type Ia Unabsorbed Antiserum: Mouse-Protection Tests in Related Types Ia, Ib, Ic; Example of Serum with Major Ia CHO Antibody

Serum no. 26 (against strain 090), Type Ia serum dilutions	Challenge strains, culture dilutions 10^{-3}		
	090/14, Type Ia	H36B/33, Type Ib	A909/14, Type Ic
1:1	S	D1	S
1:10	S	D1	S
1:1,000	S	D1	S
1:2,000	S	D1	S
1:4,000	S	D1	S
1:10,000	D1	D1	D1
1:20,000	D1	D1	D1
Interpretation of protection	Protected by anti-Ia CHO		Protected by anti-Ia CHO

See Materials and Methods and Table III. 0.2 ml of serum dilution plus 0.2 ml of 10^{-3} dilution of culture injected into each mouse. No cross reaction with Types II and III. This serum does not contain antibodies to Iabc common determinant. All anti-Ia CHO antibodies can be absorbed with either Ia or Ic strains.

TABLE VI

Type Ib Unabsorbed Antiserum Mouse Protection Tests in Related Types Ia, Ib, and Ic; Example of Serum with Ib Antibodies (Anti-Ib CHO, and anti-Ibc Protein)

Serum no. 13 (against strain H36B), Type Ib serum dilutions	Challenge strains											
	090/14, Type Ia				H36B33, Type Ib				A909/14, Type Ic			
	Culture dilutions											
	10^{-2}	10^{-3}	10^{-6}	10^{-7}	10^{-2}	10^{-3}	10^{-6}	10^{-7}	10^{-2}	10^{-3}	10^{-6}	10^{-7}
1:1	D1	D2	D2	S	S	S	S	S	S	S	S	S
1:10	D1	D1	D2	D2	S	S	S	S	S	S	S	S
1:100	D1	D6	D6	D1	S	S	S	S	D1	S	D6	S
1:1,000	D1	D1	D1	D1	D2	S	S	S	D1	D1	S	S
1:10,000	D1	D1	D1	D2	D2	D2	S	D6	D1	D1	D2	S
Interpretation of protection	No protection				Protected by anti-Ib CHO and anti-Ibc protein				Protected by anti-Ibc protein			

See Methods and Table III. 0.2 ml of serum dilution plus 0.2 ml of the culture dilution injected into each mouse.

antigenic determinant is not known, but the evidence indicates that it occurs in the capsular polysaccharide. It is characterized as a minor determinant because it appears to induce antibody less commonly and in smaller amount than the major determinants of the two polysaccharides (Ia and Ib). Detailed immuno-

TABLE VII

Absorption of Type Ib Antiserum Mouse Protection Tests; Example of Type-Specific Antibody (Anti-Ib CHO not Absorbed by Ibc Protein in Absorbent)

Serum no. 20 (against strain H36B), Type Ib absorption record		Challenge strains					
Serum sample treatment and designation	Serum dilution	090/14, Type Ia		H36B/60, Type Ib		A909/14, Type Ic	
		Culture dilutions					
		10^{-3}	10^{-6}	10^{-3}	10^{-6}	10^{-3}	10^{-6}
Unabsorbed*; no. 20	1:50	S	S	S	S	S	S
	1:1,000	D1	S	S	S	S	S
Interpretation of protection		Protected by anti-Iabc		Protected by anti-Ib CHO, anti-Iabc, and anti-Ibc		Protected by anti-Ibc	
Absorbed 1 time with Type Ia, 090/14; no. 20a	1:50	D1	D1	S	S	S	S
	1:1,000	D1	D1	S	S	S	S
Interpretation of protection		No protection (anti-Iabc absorbed)		Protected by anti-Ib CHO and anti-Ibc		Protected by anti-Ibc	
Absorbed 1 time with Type Ic, A909/14; no. 20c	1:50	D1	D2	S	S	S	S
	1:1,000	D1	D1	S	S	S	S
Interpretation of protection		No protection (anti-Iabc absorbed)		Protected by anti-Ib CHO with or without anti-Ibc		Protected by anti-Ibc, absorption incomplete	
(Sample no. 20c absorbed 1 time with Type Ib, H36B)	1:50	D1	D1	S	S	D1	D2
No. 20d	1:1,000	D1	D1	S	D2‡	D1	D1
Interpretation of protection		No protection		Complete absorption of anti-Ibc			
				Incomplete absorption of anti-Ib CHO which protects		No protection	

0.2 ml of serum dilution plus 0.2 ml of culture dilution injected into each mouse. This serum is also illustrative of immunology of Iabc and Ibc antigens and antibodies. Sample no. 20d illustrates complete absorption of anti-Ibc from Ib antiserum without removing type-specific anti-Ib CHO. Compare with titration of these antibodies in Table VI.

* See Methobs and Tables III and VI.

‡ Probably a variation of host resistance in single mouse.

chemical studies of these antigens are needed for precise identification of the determinants.

It is obvious that these results merely begin to uncover the intricacies of this problem. It is strongly indicated from these experiments that separable protective antibodies to several bacterial determinants, both polysaccharide and protein, exist and are reflected in a single serum with cross-relationships between strains and types. Any one of these diverse antibodies appears capable alone of protecting challenged mice from becoming infected with a strain containing any one of the corresponding antigens which induce the antibody specific for that antigen.

Further work on the results of quantitative comparisons is indicated to evaluate this complicated series of immunological reactions. The solution of these in-

TABLE VIII

Type Ic Unabsorbed Antiserum Mouse-Protection Tests in Related Types Ia, Ib, and Ic; Example of Serum with Iabc Antibodies

Serum no. 22 (against strain A909), Type Ic serum dilutions	Challenge strains, culture dilutions 10^{-3}		
	090/14, Type Ia	H36B/60, Type Ib	A909/14, Type Ic
1:50	S	S	S
1:1,000	S	S	S
1:2,000	D6	S	S
1:4,000	D4	S	S
1:8,000	D5	D1	S
1:16,000	D5	D1	D5
Interpretation of protection	Protected by anti-Iabc	Protected by anti-Iabc	Protected by anti-Iabc

See Methods and Table III. 0.2 ml of serum dilution plus 0.2 ml of culture dilution injected into each mouse. Type Ic antisera-containing antibodies (anti-Ia, anti-Iabc, and anti-Ibc) in all various combinations occur and have been analyzed: no strictly Ic-specific antigen has been found. All antibodies (Iabc) can be absorbed from this serum with any one of the three challenge strains.

terrelationships would aid an understanding of the infective properties of these organisms.

Discussion

The investigation of the immunological importance of the protein antigen Ibc led to re-examination of other type-related antigens in Group B found previously to induce specific mouse-protective antibodies. In the study of any newly identified antigen in bacterial species connected with immunity, it is essential to establish its composition and structure, and to ascertain its relationship to infection and resistance. In investigations focused on human or other animal infections, it is not enough to show that antigens fulfill the usual criteria of inducing and reacting with antibodies. A biological animal test of immunity is requisite. Procedures for defining identity or partial relationship of strains or individual antigens have included reciprocal absorption of sera with whole or treated bacteria and subsequent testing of each supernatent by mouse protection. Usually in immunological studies of protective antibodies, specific polysaccharides or proteins have been the basis for establishing individual types among the gram-positive cocci which could then be confirmed by mouse protection. The protective antibodies for types have usually been limited in these bacteria to a single antigenic component among the many occurring in the specific types investigated. The specific type designation has corresponded to this antigen. In the present example more than one specific antigen capable of inducing mouse protective antibodies has been encountered in several types.

In a study of the immunological importance of an antigen, it is preferable to use chemically purified antigens derived from the type strains, with the results evaluated by means of protection tests in experiments paralleling the quantitative precipitin analysis. Since purified preparations were not available for most of

the Group B streptococcal antigens, the earlier standard immunological absorption techniques with whole bacteria were employed to demonstrate the importance of the newly encountered Ibc proteins in infection and immunity. It was much more difficult to interpret the value of the antigenic components in the present experiments where Type Ic was involved than in the earlier studies with Types Ia and Ib. This may have been due to the larger number and varying concentrations of antigen-antibody systems involved, to the chemical or spatial arrangements of the antigens in the cell, or to other unknown factors, such as stability of the antigens or competitive binding of antibodies to the surface of cells. These factors remain to be elucidated.

A provisional type designation depending upon differences in specific mouse-protection tests was made for Type Ic with a view to accounting for cross-protection results. The new type, Ic, was clearly different by the mouse-protection test from either of the established types, Ia or Ib, both of which cross-reacted strongly with Type Ic in protection experiments. Studies of this type have so far not demonstrated a specific antigen to correspond solely to Type Ic either by serological or by other biological-testing methods.

It was thought that a combination of the cross-protective antibodies found might be sufficient to account for all the type-related protection of Type Ic which gave cross-reactions with both Ia and Ib. The mouse-protection experiments with Ic antisera absorbed with various combinations of the cross-reacting strains of these types have, however, been indecisive in some experiments as to whether there remained a protective antibody after complete absorption of Ic antiserum with all of the known antigens involved. No suggestion has been found of the nature of such a hypothetical unknown antigen, although it may be recalled that a type-specific sialic acid immunodeterminant was demonstrated in previous studies with Type II after absorption was complete with other type-specific antigens. The uncertainty about any additional antigen in Ic may be due to the chemical lability of such a substance or to confusion caused by the high dilutions in which several known Group B type-related antibodies are protective. Alternatively, no such antigen may exist.

The antibodies in Ic antisera which are known to be mouse-protective are summarized in Table I. Those specific for the Ibc proteins were demonstrably mouse-protective whether induced in response to Ib or Ic strains, provided the immunizing strains carried the Ibc protein determinants. These results demonstrated that in the absence of the known polysaccharide-type antigens the protein antigenic determinants, Ibc, were capable of eliciting protective antibodies against passive infection in mice with strains containing this protein. The necessity and value of further studies of this component of Group B streptococci were therefore evident.

In previous work on the protective antigen-antibody systems of Type II, dual immunodeterminant antigens had been found occurring in the type-specific substance in the same strain. This finding led to the discovery of two distinct antibodies both directed specifically against Type II. Chemically these antigens were shown to consist of a heat- and acid-stable polysaccharide separable from the native whole antigenic polysaccharide which does contain a labile immunodeterminant, an antigenic sialic acid (18). Both these components contrib-

uted to the whole antigen. The possible existence of similar specific constituents of the other known types requires further study. Complete mouse protection studies have not so far been made with Type III.

Summary

The data presented in this paper establish the finding that multiple specific protective antibodies exist in rabbits in response to immunization with Group B streptococci. The summary in Table I indicates the serological types into which Group B streptococci have been divided on the basis of their antigenic composition. This classification is dependent upon passive protection of mice with antibodies directed against the specific antigens, and types are defined in these terms.

Heretofore, it was thought that type-specific polysaccharides accounted for all such protection in Group B streptococci. Certain exceptions of cross-protection between types due to minor polysaccharide determinants soon appeared; cross-protection reactions based on protein determinants in at least two types were also discovered. The present experiments show that specific antibodies directed to either polysaccharide or protein antigens of a single strain can be protective against infection with streptococci containing these antigens.

Received for publication 17 March 1975.

References

1. Lancefield, R. C. 1933. A serological differentiation of human and other groups of hemolytic streptococci. *J. Exp. Med.* **57**:571.
2. Lancefield, R. C. Specific relationship of cell composition to biological activity of hemolytic streptococci. *In* The Harvey Lectures, 1940–41, Series XXXVI, pp. 251–290.
3. Lancefield, R. C. 1934. A serological differentiation of specific types of bovine hemolytic streptococci (Group B). *J. Exp. Med.* **59**:441.
4. Lancefield, R. C. 1938. Two serological types of Group B hemolytic streptococci with related, but not identical, type-specific substances. *J. Exp. Med.* **67**:25.
5. Stableforth, A. W. 1946. Serological classification of the mastitis streptococci. *In* Bovine Mastitis. R. B. Little and W. N. Plastridge, editors. McGraw-Hill Book Company, New York. 203–245.
6. Pattison, I. H., P. R. J. Matthews, and W. F. Maxted. 1955. Type classification by Lancefield's precipitin method of human and bovine Group B streptococci isolated in Britain. *J. Pathol. Bacteriol.* **69**:43.
7. Maxted, W. R. 1949. Occurrence of the M substance of type 28 Group A in streptococci of Lancefield Groups B, C, and G. *J. Gen. Microbiol.* **3**:1.
8. Butter, M. N. W., and C. E. de Moor. 1967. Streptococcus agalactiae as a cause of meningitis in the newborn, and of bacteraemia in adults. Differentiation of human and animal varieties. *J. Microbiol. Serol.* **33**:439.
9. Haug, R. H. 1972. Type classification of the Group B streptococci by means of Lancefield's precipitin method. *Nord. Vet. Med.* **24**:631.
10. Lancefield, R. C. 1954. Cellular constituents of Group A streptococci concerned in antigenicity and virulence. *In* Streptococcal Infections. M. McCarty, editor. Columbia University Press, New York. Chap. 1.

11. Jelinkova, J. 1963. Serological typing of group B hemolytic streptococci. *Cesk. Epidemiol. Mikrobiol. Imunol.* **12**:74.
12. Wilkinson, H. W., R. R. Facklam, and E. C. Wortham. 1973. Distribution by serological type of Group B streptococci isolated from a variety of clinical material over a five-year period (with special reference to neonatal sepsis and meningitis). *Infect. Immun.* **8**:228.
13. Wilkinson, H. W., and R. G. Eagon. 1971. Type-specific antigens of group B, type Ic streptococci. *Infect. Immun.* **4**:596.
14. Lancefield, R. C., and E. H. Freimer. 1966. Type-specific polysaccharide antigens of group B streptococci. *J. Hyg.* **64**:191.
15. Freimer, E. H. 1967. Type-specific polyyaccharide antigens of Group B streptococci. II. The chemical basis for serological specificity of the Type II HCL antigen. *J. Exp. Med.* **125**:381.
16. Russell, H., and N. L. Norcross. 1972. The isolation of some physiochemical and biologic properties of the Type III antigen of Group B streptococcus. *J. Immunol.* **109**:90.
17. Rotta, J., R. M. Krause, R. C. Lancefield, W. Everly, and H. Lackland. 1971. New approaches for the laboratory recognition of M types of Group A streptococci. *J. Exp. Med.* **134**:1298.
18. Lancefield, R. C. 1972. Cellular antigens of Group B streptococci. *In* Streptococci and Streptococcal Diseases, Recognition, Understanding, and Management. L. W. Wannamaker and J. M. Matsen, editors. Academic Press, Inc., New York. 57–65.
19. Wilkinson, H. W. 1975. Immunochemistry of purified polysaccharide type antigens of Group B streptococcal types Ia, Ib, and Ic. *Infect. Immun.* **11**:845.