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Primary Murine Immunoglobulin M Responses to Certain Pneumococcal Capsular Polysaccharides Consist Primarily of Anti-Pneumococcal Cell Wall Carbohydrate Antibodies

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The antibody induced in mice immunized with a vaccine preparation of type 6 (Danish type 6A) pneumococcal capsular polysaccharide (S6) reacted with several chemically disparate pneumococcal capsular polysaccharides. Equivalent numbers of plaque-forming cells were observed when sheep erythrocytes coated with either S6, type 19 pneumococcal capsular polysaccharide (S19), or the pneumococcal cell wall carbohydrate (PnC) were used to detect the response to S6 or to S19. The addition of exogenous PnC to the plaquing mixtures of spleen cells from S6-, S19-, or PnC-immunized mice inhibited the appearance of most (≥85%) of the plaque-forming cells. Furthermore, the addition of monoclonal antibody specific for the dominant (TEPC 15) idiotype of anti-phosphorylcholine (a component of PnC) antibodies also inhibited the appearance of most of the plaque-forming cells. A suppressed S19 response was induced by priming mice with a low dose of S19 or PnC 3 days before immunization with an optimal dose of S19 (low-dose paralysis). These results demonstrated that most, if not all, of the antibody stimulated by these preparations of S6 and S19 was actually induced by and was specific for PnC.

A major emphasis of this laboratory has been the study of murine antibody responses to pneumococcal polysaccharides. Recently, we began to examine the response to S6, the capsular polysaccharide from type 6 pneumococci, which is among the least immunogenic of the polysaccharides in the multivalent pneumococcal vaccine. Immunization with S6 elicited a low, but measurable plaque-forming cell (PFC) response in several strains of mice (9). Like responses to other polysaccharide antigens, the ability to respond to S6 was not dependent upon the presence of thymus-derived (T) cells. Several observations, however, indicated that the magnitude of the S6 response in mice was regulated by T cells. For instance, priming with a low dose of S6 3 days before immunization with an optimal dose of S6 resulted in a suppressed PFC response (9). This phenomenon, termed low-dose paralysis, has been shown by other investigators to be antigen specific (3, 12) and to be mediated by T suppressor cells (Ts) (1, 3).

During a more extensive study of low-dose paralysis to S6, we observed that priming mice with several other pneumococcal polysaccharides in the multivalent vaccine also induced suppression of the response to an optimal dose of S6. These results suggested either that induction of low-dose paralysis to S6 was not antigen specific or that these polysaccharides shared a common determinant which was inducing the suppression. The purpose of the present investigation was to determine whether these polysaccharides shared a common determinant, perhaps due to a contaminant in the vaccine preparations, and to determine whether each of the polysaccharides could stimulate an antibody which reacted with this determinant.

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MATERIALS AND METHODS

Mice. BALB/c mice were obtained through Clarence Reeder at the National Institutes of Health, Bethesda, Md. CAF_1 mice were obtained from the Jackson Laboratory, Bar Harbor, Maine.

Antigens and immunization. Capsular polysaccharides from type 6 (S6) and type 19 (S19) pneumococci were supplied by Merck & Co., Inc., Rahway, N.J. Capsular polysaccharides from type 3 (S3), type 14 (S14), and type 23 (S23) pneumococci were supplied by Eli Lilly & Co., Indianapolis, Ind. The carbohydrate from the pneumococcal cell wall (PnC) was kindly provided by Michael J. Caulfield, Department of Molecular and Cellular Biology, Cleveland Clinic Foundation, Cleveland, Ohio. All polysaccharides were stored as stocks (5 mg/ml) in saline and were diluted in saline for use. Animals were immunized intraperitoneally with a previously determined optimal dose of polysaccharide (0.6 μg of S3 [4]; 1 to 5 μg of S6 [9], S14, S19 and S23; and 1 μg of PnC [6]) unless stated otherwise.

Hemolytic plaque assay. Splenic PFC were enumerated by the slide modification of the Jerne plaque assay (13). Indicator sheep erythrocytes (SRBC) were coated with a particular polysaccharide by using a modification of the chromic chloride ($CrCl_3$) method (4). Briefly, 0.5 ml of washed packed SRBC was added to 1 mg of polysaccharide in 1.1 ml of saline. Then 1 ml of $CrCl_3$ (1.5 mg/ml in saline) was added and the mixture was gently shaken. After 5 min at room temperature, 9 ml of saline was added and the SRBC were pelleted by centrifugation. The cells were then washed three times and diluted to 5% for use. A rabbit anti-mouse immunoglobulin M (IgM) serum diluted 1:100 was used to facilitate plaque development as previously described (5). When plaque inhibition studies were performed, various amounts of a pneumococcal polysaccharide or of AB1-2 (a monoclo-

TABLE 1. Effect of low-dose priming with pneumococcal polysaccharides on the S6 PFC response in BALB/c mice

Priming on day −3 (μg)[a]	PFC response ± SE (%)[b] for the following expts:			
	1, S6-SRBC	2, S6-SRBC	3	
			S3-SRBC	S6-SRBC
None (control)	6,844 ± 792	4,113 ± 1,185	11,720 ± 828	1,967 ± 133
S6 (0.5)	1,475 ± 341 (21)	1,731 ± 337 (42)	11,300 ± 400 (96)	569 ± 268 (29)
S14 (0.5)	2,675 ± 744 (39)	1,956 ± 358 (48)	13,010 ± 3,075 (111)	785 ± 438 (40)
S19 (0.5)	770 ± 135 (11)	ND	16,650 ± 3,430 (142)	1,044 ± 243 (53)
S23 (0.5)	ND[c]	815 ± 211 (20)	14,740 ± 1,514 (126)	837 ± 538 (42)
S3 (0.06)	ND	6,563 ± 1,315 (160)	425 ± 59 (4)	2,925 ± 1,258 (149)

[a] Groups of mice were given the indicated polysaccharide 3 days before immunization with 5 μg of S6 (experiments 1 and 2) or with 0.6 μg of S3 and 5 μg of S6 (experiment 3).
[b] Mean number of S6-specific (experiments 1 and 2) or S3-specific and S6-specific (experiment 3) IgM PFC per spleen ± standard error and percent response compared with controls for groups of 4 to 5 mice 5 days after immunization.
[c] ND, Not determined.

nal antibody binding to the T15 idiotype [11] kindly provided by Michael Caulfield, Cleveland Clinic Foundation) were added to the plaquing mixture, which was then poured onto a slide, and the plaque assay was completed. Spleen cells were diluted so that approximately 90 to 150 PFC per slide would appear in the absence of exogenously added polysaccharide or antibody. In all experiments, results are expressed as PFC per spleen, but the conclusions would not differ if the results were expressed as PFC per 10^6 spleen cells.

RESULTS

Induction of low-dose paralysis to S6 with capsular polysaccharides from other types of pneumococci. When mice are primed with a low dose (0.006 μg) of S3, the response to a subsequent injection of an optimal dose (0.6 μg) of S3 is suppressed (2, 3). Low-dose paralysis to S3 has been shown to be antigen specific, since priming with a suboptimal dose of type 1 pneumococcal polysaccharide (S1) did not alter the response to S3 (3). In an earlier report, we demonstrated that low-dose paralysis to S6 was induced by giving mice a suboptimal dose (0.1 to 0.5 μg) of S6 before challenge with an optimal dose (1 μg) of the polysaccharide (9). It was of interest, therefore, to examine the specificity of induction of low-dose paralysis to S6. BALB/c mice were given a low dose of S3, S6, S14, S19, or S23. Three days later, mice were immunized with 5 μg of S6 or with 5 μg of S6 and 0.6 μg of S3, and the PFC response was determined 5 days later. The PFC response to S6 was substantially suppressed in mice primed with a low dose of either S6, S14, S19, or S23 (Table 1). Although low-dose priming with S3 resulted in suppression of the response to an optimal dose of S3, a low dose of S3 did not diminish the number of PFC induced in response to S6 (Table 1). Furthermore, suboptimal doses of S6, S14, S19, or S23 had no effect on the response to an optimal dose of S3 (Table 1). These results could suggest that the induction of low-dose paralysis to S6 is not antigen specific. Since low-dose priming with S3 did not suppress the S6 response, however, this did not seem a likely explanation for the results. An alternative possibility is that the preparations of S6, S14, S19, and S23 used in these experiments had a common determinant toward which the low-dose paralysis was directed.

Cross-reactivity of the antibody response induced by S6 with S14, S19, and S23. Since low-dose paralysis to S6 could be induced by priming with other polysaccharides, it was important to determine whether the antibody induced by immunization with S6 could also react with these polysaccharides. To evaluate the specificity of PFC produced by BALB/c mice immunized with S6, spleen cells from S6-immunized mice were assayed by using SRBC coated with either S3, S6, S14, S19, or S23 (Table 2). Few PFC were detected by using S3-SRBC, whereas nearly equivalent numbers of PFC were detected by using SRBC coated with either S6, S14, S19, or S23. The apparent cross-reactivity of antibody produced in response to S6 was further examined by comparing the reactivity of the antibody induced by immunization with S14, S19, and S23 with the antibody induced by S6. Groups of mice were injected with 1 μg of S6, S14, S19, or S23. Five days later, the PFC responses were determined by using SRBC coated with each of the four polysaccharides (Table 2). Although there were differences in the magnitude of the PFC response induced by each of the four pneumococcal polysaccharides, the number of detectable PFC was essentially equivalent when any one of the four polysaccharide-coated SRBC preparations was used to determine the PFC response. These results suggested that

TABLE 2. Reactivity of PFC responses to pneumococcal polysaccahrides

Immunogen[a]	Mean PFC ± SE detected with[b]:				
	S3-SRBC	S6-SRBC	S14-SRBC	S19-SRBC	S23-SRBC
S6	200 ± 102	6,583 ± 2,362	6,017 ± 2,292	6,450 ± 2,430	6,417 ± 2,412
S14	ND	6,700 ± 4,255	6,950 ± 4,767	7,133 ± 4,344	7,750 ± 4,840
S19	312 ± 75	11,633 ± 1,153	11,650 ± 231	12,717 ± 707	13,125 ± 429
S23	ND	1,650 ± 25	1,088 ± 87	1,638 ± 37	1,700 ± 50

[a] BALB/c mice received 1 μg of the indicated pneumococcal polysaccharide intraperitoneally.
[b] Mean number of IgM PFC per spleen ± standard error for groups of 4 mice 5 days after immunization. The polysaccharide-coated SRBC used to determine the PFC response is indicated.

TABLE 3. Antibody induced by S6 and by S19 reacted with PnC

Immunogen[a]	Mean PFC ± SE detected with[b]:			
	S3-SRBC	S6-SRBC	S19-SRBC	PnC-SRBC
S6	210 ± 56	4,190 ± 612	ND	4,005 ± 633
S19	490 ± 102	ND	12,012 ± 2,082	13,160 ± 1,294
PnC	260 ± 110	ND	6,765 ± 2,002	8,860 ± 137
PnC	ND	11,413 ± 2,978	ND	10,040 ± 2,180

[a] CAF_1 mice received 1 μg of PnC or 5 μg of S6 or S19 intraperitoneally.
[b] Mean number of IgM PFC per spleen ± standard error for groups of 4 mice 5 days after immunization. The polysaccharide-coated SRBC used to determine the PFC response is indicated.

immunization with either S6, S14, S19, or S23 induced antibody that was apparently completely cross-reactive with each of the other three polysaccharides. Furthermore, the addition of large amounts (5 μg or more) of S6, S14, S19, or S23 to mixtures of S6-SRBC and S6 immune spleen cells inhibited the appearance of all detectable S6 PFC (data not shown). This indicated that most, if not all, of the IgM antibody produced by BALB/c mice in response to immunization with S6 was also reactive with the S14, S19, and S23 preparations used in these experiments and in the multivalent pneumococcal polysaccharide vaccine.

Antibody induced in response to S6 and to S19 reacted with PnC. The antibody induced by S6 reacted with several chemically disparate pneumococcal polysaccharides (i.e., S14, S19, and S23) (Table 2). It was important to define the determinant which was stimulating this antibody. A likely candidate was the PnC, a polysaccharide common to all pneumococci (18). To determine whether the antibody induced by S6 and by S19 was actually stimulated by PnC which might be present in these preparations, groups of CAF_1 mice were immunized with S6, S19, or PnC. Five days later, the spleen cells were assayed for PFC by using S3-, S6-, S19-, or PnC-coated SRBC (Table 3). Spleen cells from mice immunized with S6 had essentially equivalent numbers of PFC when either S6-SRBC or PnC-SRBC were used to determine the response. Equivalent numbers of PFC were also observed when S19-SRBC or PnC-SRBC were used to determine the response to S19. Moreover, equivalent numbers of PFC were observed when either S6-, S19-, or PnC-coated SRBC were used to assay the response to PnC. The use of S3-SRBC to assay the response to either S6, S19, or PnC resulted only in background numbers of PFC. These results suggested that the antibody induced by immunization with S6 or S19 was actually stimulated by PnC contaminating the polysaccharide preparations and that this antibody was, in turn, reacting with the PnC in these preparations.

This was examined in greater detail by determining the ability of exogenously added PnC to inhibit the appearance of PFC induced by other polysaccharides. Groups of CAF_1 mice were immunized with an optimal dose of S3, S6, S19, or PnC, and 5 days later the PFC were detected by using either S3-SRBC or S19-SRBC. Exogenously added PnC, S6, or S19 (1-μg amounts) was able to inhibit most of the PFC induced by either PnC, S6, or S19 (Table 4). Moreover, exogenously added amounts of AB1-2, a monoclonal antibody specific for the TEPC 15 idiotype of anti-phosphorylcholine (PC) antibodies (11), also inhibited the appearance of most of the splenic PFC from mice immunized with either PnC, S6, or S19. These results clearly demonstrate that most, if not all, of the antibody induced in the primary response to vaccine preparations of S6 and S19 is apparently directed to a PnC contaminant within these preparations. Furthermore, since TEPC 15^+ antibodies comprise 30 to 90% of the antibody response to the PC determinant of PnC (7, 8), these results indicate that the majority (70% or greater) of antibodies produced in the primary response to these preparations of S6 and S19 were of the dominant anti-PC idiotype (T15).

In contrast to the antibody response induced by S6 and S19, PnC and AB1-2 had little inhibitory effect on the appearance of PFC induced by S3 (Table 4). This indicated that most of the antibody stimulated by S3 was directed toward the specific S3 polysaccharide and suggests that this preparation of S3 contained a minimal amount of PnC contamination. Furthermore, this accounts for the observation that the antibody induced by these preparations of S6 and S19 did not react with S3 (Tables 2 and 3).

Low-dose priming with PnC induces low-dose paralysis to S19. Low-dose paralysis to S6 (Table 1) and to S19 (R. Fairchild, data not shown) is induced by priming with low doses of several chemically disparate pneumococcal polysaccharides (i.e., S6, S14, S19, or S23). Since the antibody responses to vaccine preparations of S6 and S19 appeared to be directed toward PnC contaminating these preparations, it was important to determine whether the ability of this wide range of polysaccharides to induce low-dose paralysis to S6 and S19 was also due to the PnC contaminant. Groups of BALB/c mice were given a subop-

TABLE 4. Inhibition of S6-, S19-, and PnC-induced PFC by exogenous polysaccharide or anti-TEPC 15 antibody

Immunogen (μg)[a]	Inhibitor[b]	Mean % inhibition of PFC appearance
S3 (0.6)	S3	92
	PnC	20
	AB1-2	9
S6 (5)	S3	21
	S6	85
	S19	96
	PnC	87
	AB1-2	83
S19 (5)	S3	15
	S19	91
	PnC	90
	AB1-2	74
PnC (1)	S3	0
	S6	90
	S19	92
	PnC	95
	AB1-2	81

[a] Groups of four CAF_1 mice were immunized with the indicated polysaccharide.
[b] Five days after immunization, 1 μg of the indicated polysaccharide or a 1:100 final dilution of AB1-2 ascites was added to the plaquing mixtures and the plaque assay was completed. S3-SRBC was used to detect the S3 response. S9-SRBC was used to detect the responses to S6, S19, and PnC.

TABLE 5. Induction of low-dose paralysis to S19 by priming with a low dose of PnC

Priming dose on day −3 (μg)[a]	Mean PFC response ± SE (%)[b]	
	S3-SRBC	S19-SRBC
None (control)	6,825 ± 703	12,725 ± 2,493
S3 (0.06)	983 ± 97 (14)	12,617 ± 3,186
S19 (0.25)	10,800 ± 2,917	3,322 ± 1,208 (26)
PnC (0.25)	11,367 ± 1,851	5,680 ± 803 (44)

[a] Groups of BALB/c mice were primed with a suboptimal dose of the indicated polysaccharide 3 days before immunization with 0.6 μg of S3 and 5 μg of S19.

[b] Mean number of PFC per spleen ± standard error and percent response compared with control for groups of 4 mice 5 days after immunization.

timal dose of either S3, S19, or PnC. Three days later, all mice and an unprimed control group were challenged with an optimal dose of both S3 and S19. Five days after challenge, the number of splenic PFC was detected by using S3- and S19-SRBC. Low-dose priming with either S19 or PnC suppressed the PFC response to S19 but did not suppress the response to S3 (Table 5). Priming with a suboptimal dose of S3 had no effect on the response to S19 but did induce suppression of the response to an optimal dose of S3. In other experiments, low-dose priming with PnC but not with S3 was observed to suppress the PFC response to immunization with S6 (K. Sterner, data not shown). These results indicate that low-dose paralysis to these preparations of S6 and S19 is both induced by and directed to the PnC in the preparations.

DISCUSSION

A previous report from this laboratory showed that immunization of mice with a vaccine preparation of S6 resulted in a small PFC response composed entirely of IgM antibody-producing cells (9). More recent results indicated that priming with low doses of several chemically disparate pneumococcal capsular polysaccharides induced suppression of the response to an optimal dose of S6. Since the induction of low- dose paralysis was shown by other investigators to be antigen specific (3, 12), we began to question the specificity of the antibody induced in mice by S6. The purpose of the present investigation was to determine the specificity of antibodies induced by primary immunization with S6 and other pneumococcal polysaccharides.

The results demonstrated that most, if not all, of the IgM antibody induced in mice by several vaccine-grade pneumococcal capsular polysaccharides was actually stimulated by and was specific for PnC present in these polysaccharide preparations. Mice immunized with optimal doses of S6, S19, or PnC had equivalent numbers of PFC when S6-, S19-, or PnC-coated SRBC were used to detect the responses (Table 3). Moreover, the addition of exogenous PnC or a monoclonal antibody specific for the T15 idiotype to the plaquing mixtures of spleen cells from S6-, S19-, or PnC-immunized mice and S19-SRBC inhibited the appearance of most (75% or greater) of the PFC (Table 4). These results demonstrated that most of the antibody stimulated by these preparations of S6 and S19 was actually induced by PnC. More specifically, these antibodies appeared to be directed toward the PC component of PnC and were of the dominant anti-PC idiotype expressed in these strains of mice. It is reasonable to conclude that the broad range of reactivity of antibodies induced by these preparations of S6, S14, S19, and S23 (Table 2) can also be attributed to PnC contamination in each of the preparations.

These results also provide evidence that the PnC in these polysaccharide preparations accounts for the ability of low doses of either S6, S14, S19, or S23 to induce suppression of the PFC response to S6 (Table 1). Low-dose paralysis has been shown in other laboratories to be antigen specific (3, 12) and to be mediated by Ts activated by the low dose of antigen (1, 3). Since low-dose priming with PnC induced suppression of the responses to S19 (Table 5) and to S6 (data not shown), it is likely that the PnC within these polysaccharide preparations activated the Ts that mediated this suppression. To prove that PnC-induced Ts are mediating low-dose paralysis to these preparations of S6 and S19, polysaccharides free of PnC contamination, if they can be obtained, will have to be used to repeat these experiments.

In contrast to the preparations of S6 and S19, immunization with S3 induced few PFC that reacted with S6-, S19-, or PnC-coated SRBC (data not shown). Exogenous amounts of PnC or the anti-TEPC 15 antibody were able to inhibit the appearance of only a small percentage of the S3 PFC (Table 4). These results indicate that the antibody response induced by S3 is primarily directed toward the cellobiuronic acid units that compose S3 (16) rather than toward any putative PnC contamination of the preparation. Furthermore, spleen cells from mice immunized with S6, S19, or PnC did not produce PFC when assayed with S3-SRBC (Table 1) and priming with a dose of S3 was unable to induce suppression of the response to S19 (Table 5). These results strongly suggest that the S3 preparation used in this study had little or no PnC contamination. Moreover, the data presented in this report address questions recently raised concerning the specificity of the antibody response to S3. It has been suggested that certain preparations of S3 are contaminated with PC, resulting in nonspecific enhancement of the antibody response to S3 (10). As shown in these studies, the S3 preparation used in this and other studies from this laboratory appears to have little or no PnC contamination. This indicates that the anti-S3 response induced by immunization with this preparation of S3 is stimulated entirely by S3 and not by PnC.

PnC is a natural component of the pneumococcal cell wall and is common to pneumococci of all serotypes (17). Several analytical studies have demonstrated the presence of PnC in vaccine preparations of pneumococcal polysaccharides (14, 15, 17, 18). Sørensen, Henrichsen, and co-workers (17, 18) recently reported that 15% of the 14-valent pneumococcal vaccine was PnC. Furthermore, vaccination of human subjects with this vaccine has been shown to result in significant increases in anti-PnC antibody titers (15).

A major question of interest to this laboratory remains unanswered. Are mice able to mount a primary antibody response which is specific for the type 6 or type 19 pneumococcal capsular polysaccharides? The results of this report have demonstrated that most, if not all, of the antibody induced by S6 and by S19 was specific for PnC, even though PnC is likely to be a minor component of these preparations. The induction of anti-PnC antibodies by these preparations is not unique to BALB/c and CAF_1 mice. Responses of several other strains of mice to S6 and S19 have also been examined (e.g., CB.20, C57BL/6, BALB.K and BALB.B). All of these mice also produced antibody which was $T15^+$ and reacted with several chemically disparate pneumococcal capsular polysaccharides and with PnC (R. Fairchild and G. Milligan, unpublished results). More recently, we have immunized mice with lower doses of the S6 or S19 preparations to attempt to dilute out the PnC contaminant. Although not striking, this has generally resulted in a slightly lower

percentage of PnC-inhibitable PFC (K. Sterner, unpublished results). Sørensen and co-workers (17) attempted to examine murine anti-S6 antibodies by generating hybridomas from mice immunized with a pneumococcal type 6 whole-cell vaccine. All of the hybridomas generated, however, produced antibody that was specific for the PC determinant of PnC.

Although these results provide evidence that mice may be incapable of producing primary IgM anti-S6 or anti-S19 antibodies, murine antibodies specific for the type 6 and type 19 capsular polysaccharide can be stimulated by using certain experimental protocols. When spleen cells from mice primed with S6-coated chicken erythrocytes (S6-CRBC) are transferred to irradiated mice and the recipients are challenged with S6-CRBC, IgM- and IgG-producing PFC are stimulated (R. Fairchild, G. Milligan, K. Sterner, and H. Braley-Mullen, manuscript in preparation). The IgM antibody stimulated in the primary and secondary responses to S6-CRBC, using this protocol, is reactive with several chemically disparate pneumococcal polysaccharides, including PnC. By contrast, most of the IgG antibody induced in the memory response is specific for S6 and does not react with these other polysaccharides. Studies to determine the specificity of the precursors of these S6-specific memory B cells are currently in progress.

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