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Immunoresponses to *Neisseria meningitidis* Epitopes: Suppression of Secondary Response to Phosphorylcholine Is Carrier Specific

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Results of our previous work have shown that *Neisseria meningitidis* serogroup B M986 can induce a phosphorylcholine (PC)-specific plaque-forming cell immunoresponse in mice. Also, a single injection of a relatively low dose of meningococci in NBF1 female mice induced a priming time-dependent suppression on subsequent meningococcus challenge. This suppression was not due to switching to another class of immunoglobulin nor to the presence of a capsule on *N. meningitidis*. In this study we show that suppression induced by meningococcus is carrier specific. Furthermore, we offer evidence suggesting that the structure(s) on meningococcus that trigger this suppression is heat labile and different from the antigenic structure(s) recognized by the suppressed B cells. In addition, we found that there is a gradual increase in antibody secretion rates of *N. meningitidis*-induced anti-PC plaque-forming cells that correlates with *N. meningitidis* priming time. Rather unexpected was the fact that pretreatment of mice with PC-keyhole limpet hemocyanin (thymus-dependent antigen) had a great influence on the subsequent PC-specific immunoresponses induced by *N. meningitidis* and PC-coupled heat-inactivated meningococcus [PC-(NMB)HI], as shown by (i) a striking decrease in T15 idiotype expression, (ii) concomitant direct anti-PC plaque-forming cells reduction, (iii) switching to immunoglobulin G (*N. meningitidis*-induced immunoresponse) or immunoglobulin G plus immunoglobulin A [PC-(NMB)HI-induced immunoresponse], and (iv) a significant increase in heterogeneity of plaque-forming cell secretion rates. The possibility that *N. meningitidis*, PC-(NMB)HI, and PC-KLH stimulate B lymphocytes pertaining to three different subpopulations embedded in distinct regulatory circuits is discussed, with emphasis on the interrelationships between T-dependent and T-independent lymphocyte compartments. We focus on the possibility of the existence of high-level regulatory circuits in which lymphocyte subpopulations or sets of lymphocyte subpopulations with different requirements of activation are connected.

Many infectious agents modulate to some extent the immune response (49). One of these is *Neisseria meningitidis*, about which little is known in this respect (10, 43, 47, 49, 52). Immunomodulatory effects of bacteria can be nonspecific or specific and can be due to factors released into the surrounding medium or to substances on the cell wall (13, 28, 34, 35, 49). Also, these effects can be quantitative, qualitative, or both (49).

The immunogenic behavior of *N. meningitidis* has been studied mainly with regard to the humoral *N. meningitidis*-induced immunoresponse against its capsular polysaccharide (29, 39, 58), lipopolysaccharide (44, 54, 58), or serotype antigens (14, 15, 38, 44). These studies have been hindered by the fact that the precise epitopes which are the target of such immunoresponses are unknown. Furthermore, it has been shown that lipopolysaccharide and serotype antigens vary greatly in their immunogenic and protective capacities against meningococcal disease, depending on culture conditions, the grade and method of purification of these antigens, and the retention of their natural membrane conformation (44, 48).

In recent years several reports have been published concerning certain aspects of cellular immunity to *N. meningitidis*. They describe macrophage- or polymorphonuclear leukocyte-mediated cytotoxicity (10), antibody-dependent antimeningococcal activity mediated by K cells and monocytes (30, 31), the in vitro mitogenic activity of heat-killed meningococcus B and several of their isolated surface antigens on murine B lymphocytes (36), and the immunomodulating activity of meningococcal antigens on the mitogen-induced proliferation of T lymphocytes and on the T-cell-dependent immune response (51, 52). Also, the presence of human immunoglobulin A1 (IgA1)-specific proteases on the outer membrane of the pathogenic *Neisseria* spp. recently has been described (42, 43), as has the fact that human IgA antimeningococcal antibodies and their Fab fragments block the lytic activity of bactericidal antimeningococcal IgG and IgM (19, 20). The earlier suggestion that bacteria or their products can influence qualitatively the immunoglobulin class of antibody (49) has now received indirect support with the recent description of T-cell-dependent class-regulatory mechanisms which can be influenced by the antigen (2).

One approach to the study of the immunomodulating properties of *N. meningitidis* is to define an epitope on the meningococcal surface and to use the whole cell as antigen. This permits an accurate study of the B-cell, T-cell, and antigen-presenting cell compartments in an epitope-specific immune response and the meningococcal influences on that response. We recently have described such a study system with *N. meningitidis* serogroup B M986 (12). We reported that in mice, *N. meningitidis* can elicit a phosphorylcholine (PC)-specific immunoresponse (suggesting that PC is present, at least functionally, on the bacteria) and that the PC-specific immunoresponse after meningococci challenge of meningococci-primed mice is suppressed, in a priming time-dependent fashion, without switching to other classes of immunoglobulins. These findings strongly support the

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view that *N. meningitidis* is a potent immunomodulator, but they also raise a number of questions: (i) is this suppression mediated by specific or nonspecific suppressor cells (T suppressor lymphocytes [17, 21, 25, 35], B lymphocytes [1, 26, 28], or antigen-presenting cells [41])?; (ii) is it due to the direct effect of suppressor meningococcal products on target B cells?; (iii) is the *N. meningitidis*-induced secondary immunoresponse diversified toward other public epitopes (32)?

In this report we extend our previous observations. We show, by means of the plaque inhibition assay, the influence of the carrier used for priming on the inhibitory profile of the *N. meningitidis*-induced anti-PC plaque-forming cells (PFC), and we demonstrate that the anti-PC PFC suppression observed in the secondary immune response to *N. meningitidis* is carrier specific, at least in the triggering step. Different explanatory mechanisms are discussed, with emphasis on the probable involvement of different lymphocyte, antigen-presenting cell subpopulations, or both.

MATERIALS AND METHODS

Animals. BALB/c and CBA/N mice were originally purchased from OLAC Ltd. (Bicester, Oxon, England) and were kept in our animal facilities. Hybrids (BALB/c × CBA/N)F1 (BNF1) and (CBA/N × BALB/c)F1 (NBF1) were also bred. Unless otherwise stated, mice were 6 to 12 weeks of age when used for experimentation.

Antigens. Two meningococcal antigens were used. They were prepared as described previously (12). Briefly, nonencapsulated *N. meningitidis* serogroup B M986 protein serotypes 2 and 7 (lipopolysaccharide immunotypes 3 and 7) were obtained from C. Ferreirós (Departmento de Microbiologia, University of Santiago). They were grown at 37°C on Columbia blood agar plates for 36 h; they were then aseptically scraped from the plates, washed three times with sterile saline by centrifugation (10,000 × *g* for 10 min), and suspended in sterile saline. One portion was maintained for 48 h at 4°C and was termed NMB, and another portion was autoclaved (121°C for 10 min). PC was coupled to this portion, as described below for sheep erythrocytes, by using *p*-aminophenyl phosphorylcholine (Biosearch, San Rafael, Calif.) diazotized by the procedure of Chesebro and Metzger (4), and the resulting antigen was termed PC-(NMB)HI. All these antigens were used diluted to a transmittance of 86% at 530 nm in saline and frozen at −30°C until use. The inability to grow was verified for each batch before use.

PC was also coupled to keyhole limpet hemocyanin (KLH; Calbiochem-Behring, La Jolla, Calif.) by using diazo-*p*-aminophenyl phosphorylcholine as described by Chesebro and Metzger (4).

Antisera. Monospecific rabbit anti-mouse IgG subclasses and anti-mouse IgA antisera were obtained in our laboratory by the method previously described by Good (18), with minor modifications (55).

Immunization. Mice were inoculated intravenously at the retroorbital plexus with 0.1 ml of a meningococcal preparation or 200 μg of PC-KLH in saline 5 days before the primary immune response was quantified. Primed mice received intraperitoneally the same amount of antigen as challenged mice, appropriately emulsified (1:1) in incomplete Freund adjuvant (IFA; Behring Institute, Marburg, Federal Republic of Germany) at least 1 month before the second (challenge) injection.

PFC assay. PFC responses were assayed by using PC-sheep erythrocytes as target cells by the modification described by Cunningham and Szenberg (7) of the hemolytic plaque assay described by Jerne and Nordin (23). PC was coupled to sheep erythrocytes as described previously (12). All determinations were run in duplicate. The magnitude of the anti-PC response was expressed as the number of direct or direct plus indirect anti-PC PFCs per spleen. IgG and IgA PFCs were developed by incorporating a pool of rabbit anti-mouse IgG subclasses and anti-mouse IgA antisera, respectively, into the plaque mixture. With 1:900 as the final dilution for anti-IgG and 1:180 as the final dilution for anti-IgA, direct PFC inhibition was always less than 15%. The anti-PC T15 idiotype PFC was determined by using plaque inhibition with a monoclonal anti-idiotypic antibody (GB4) kindly donated by J. Quintáns (La Rabida-University of Chicago Institute, Chicago, Ill.).

PFC inhibition by PC-chloride. Serial twofold dilutions of PC-chloride were prepared, starting at a concentration of 10^{-3} M before addition to the plaque mixture, and each dilution was used at a final concentration of 1:17. PFCs in the presence of the corresponding dilution of free PC were developed and counted. The percentage of inhibition of anti-PC PFC increased as a sigmoid function of the log amount of inhibitor added.

Data analysis. Two methods were employed to calculate the amount of PC-chloride that supresses 50% of PFC (PC_{50}): (i) the parametric method, in which the linear regression from probit transformation of PFC percent inhibition on the log amount of PC-chloride added (11) was used; (ii) the nonparametric method in which the following function developed from the work of Collomb (5) and adapted to our particular case by W. González (Departamento Estadística, Facultad Matemáticas, University of Santiago) was used to obtain estimated values from the observed ones, for a continuous range of inhibitor concentrations: $\psi_n(x) = \Sigma \bar{y}_i K[(x - x_i)/h_N]/\Sigma K[(x - x_i)/h_N]$, where y_i is the mean of PFC inhibition percentages corresponding to the ith PC dilution; x_i is the −log of the molar concentration of the ith PC dilution; n is the total number of PC dilutions; m is the number of mice (replicates) used in the experiments; N is $n \times m$; h_N is $N^{-1/5}$; and $K[(x - x_i)/h_N] = k(z_i) = 0.75(1 - z_i)^2$ if $|z_i| \leq 1$ or 0 for other cases.

Heterogeneity index (Hi) is a parameter that quantifies the spread of the PFC inhibition curve over the dose range of free PC used and is expressed by the slope of the linear regression indicated in method (i) above.

When necessary, significant comparisons between means were done by Student's t test or an analysis of variance (ANOVA).

RESULTS

Induction of nonresponsiveness to the PC epitope in NBF1 female mice is not a nonspecific effect of priming with NMB. NMB-primed NBF1 female mice showed a drastic reduction in anti-PC PFCs induced after NMB challenge, but not after challenge with PC-KLH or PC-(NMB)HI, as compared with controls (unprimed) (Table 1). Furthermore, this drop of anti-PC PFCs was not due to immunoglobulin class switching to IgG or IgA. Moreover, when PC-KLH or PC-(NMB)HI was used for priming, slightly increased PC-specific immune responses were observed after PC-KLH or PC-(NMB)HI challenge, respectively, with concomitant switching to IgG in the PC-KLH secondary immunoresponse. The results in Table 1 also illustrate some interesting relationships among the different subpopulations involved in anti-PC responses. After PC-(NMB)HI challenge NMB-primed mice exhibited strikingly greater anti-PC PFC num-

TABLE 1. Suppression of anti-PC immune response induced by NMB in NBF1 female mice is carrier specific[a]

Antigen	Priming	No. of direct anti-PC PFCs/spleen[b]	% Direct anti-PC T15 idiotype PFCs	% IgG[c]	% IgA[d]
PC-KLH	None	59,735 (4.78 ± 0.09)	51 ± 1	130 ± 13	125 ± 9
	NMB-IFA	168,317 (5.23 ± 0.03)	37 ± 7	127 ± 8	108 ± 8
	PC-KLH-IFA	104,137 (5.02 ± 0.13)	28 ± 11	228 ± 18[e]	103 ± 2
	PC-(NMB)HI-IFA	160,917 (5.21 ± 0.12)	47 ± 16	149 ± 10	105 ± 11
NMB	None[f]	145,748 (5.16 ± 0.14)	39 ± 3	108 ± 9	93 ± 10
	NMB-IFA[f]	13,373 (4.13 ± 0.14)[e]	61 ± 6	112 ± 13	107 ± 6
	PC-KLH-IFA[f]	110,341 (5.04 ± 0.02)	6 ± 4[e]	132 ± 8	91 ± 5
	PC-(NMB)HI-IFA[f]	184,503 (5.27 ± 0.08)	29 ± 7	109 ± 11	102 ± 9
PC-(NMB)HI	None	69,700 (4.84 ± 0.22)	55 ± 13	101 ± 10	97 ± 4
	NMB-IFA	235,598 (5.37 ± 0.15)	47 ± 14	120 ± 6	107 ± 7
	PC-KLH-IFA	19,004 (4.28 ± 0.18)	17 ± 15	293 ± 52[e]	133 ± 54
	PC-(NMB)HI-IFA	95,350 (4.98 ± 0.15)	44 ± 8	121 ± 20	90 ± 5

[a] Groups of three to five mice were used, and direct and direct plus indirect PFCs were counted 5 days after challenge. Mice were challenged 1 to 3 months after priming.
[b] Given as the geometric mean; the numbers in parentheses represent the log of the geometric mean ± the standard error.
[c] Percent IgG was calculated by the following formula: [direct (IgM) + indirect (IgG) anti-PC PFCs] × 100/(IgM anti-PC PFCs).
[d] Percent IgA was calculated by the following formula: [direct (IgM) + indirect (IgA) anti-PC PFCs] × 100/(IgM anti-PC PFCs).
[e] $P < 0.01$ versus the control (not primed), as determined by Student's t test.
[f] Mice were 16 to 24 weeks of age when challenged after priming.

bers than did control (unprimed) or PC-(NMB)HI-primed mice. Priming with NMB or PC-(NMB)HI also appeared to increase slightly the anti-PC response induced by PC-KLH and unlike NMB priming, PC-(NMB)HI priming had some influence on the switching to IgG in mice challenged with PC-KLH. The strongest support for the concept of a relationship among subsets responding to thymus-dependent (TD) and thymus-independent (TI) PC antigens, however, comes from the results with PC-KLH-primed mice (Table 1); when these animals were challenged with NMB, the anti-PC showed a partial switch to IgG, and even more intriguing, almost all direct anti-PC PFCs had a non-T15 idiotype, unlike the control group (not primed). Similarly, when PC-KLH-primed mice were challenged with PC-(NMB)HI, a striking decrease in the direct anti-PC PFC T15 idiotype was observed, but this effect was accompanied by intense switching to IgG and less switching to IgA (193 and 33%, respectively, over the direct anti-PC PFCs); these changes were qualitative only, without net changes in total anti-PC PFCs with respect to controls (not primed).

Similar experiments carried out at different times gave essentially the same results, mainly those related to the decrease in the T15 idiotype in PC-KLH-primed mice challenged with NMB versus nonprimed NMB-challenged mice (5 ± 6 and 36 ± 3% anti-PC T15 idiotype as a percentage of PFC responses, respectively), and in PC-KLH-primed mice challenged with PC-(NMB)HI versus nonprimed PC-(NMB)HI-challenged mice (24 ± 12 and 64 ± 9% anti-PC T15 idiotype as a percentage of PFC responses, respectively), and the strong switch to IgG of anti-PC PFC immunoresponse in PC-KLH-primed versus nonprimed mice challenged with PC-(NMB)HI, which responded with practically the same total number of anti-PC PFCs (524 ± 295 and 20 ± 3% anti-PC IgG as a percentage of the total PFC responses, 65,096 [log of geometric mean ± the standard error, 4.81 ± 0.01] total anti-PC PFCs per spleen, and 94,897 [4.98 ± 0.03] total anti-PC PFCs per spleen, respectively). Also, in other experiments carried out under the same conditions as those described in Table 1, the results obtained for anti-PC PFCs in NMB-primed and nonprimed mice challenged with PC-KLH and PC-(NMB)HI were very close to the ones reported here (data not shown).

To further determine the relationships among subsets responding to NMB, PC-(NMB)HI, and PC-KLH, the consequences of priming NBF1 female mice were examined by determining PFC secretion rate profiles (8, 11) (rather than avidity profiles [22]) by inhibition of plaque formation with free antigen. Results of some of these studies involving TD and TI anti-PC PFC responses are presented in Fig. 1. It can be seen that the more diluted the free hapten, the higher the number of PFCs; in fact, in most curves the maximum overshot the 100% point (which corresponds to the number of PFCs without free hapten), and then decreases until it reaches approximately the 100% level (Figures 1A, B, and C). This overshooting is seen most clearly in the curve representing NMB-primed NMB-challenged mice (Fig. 1B). This inhibition pattern is probably due to the so-called augmentable PFCs (9). Plaque inhibition curves from the group of mice primed with PC-(NMB)HI and challenged with PC-KLH exhibited significantly lower PC_{50}s than curves from PC-KLH-challenged, unprimed and NMB-primed mice ($P < 0.05$); however, no significant differences were noted when compared with results from PC-KLH-primed, PC-KLH-challenged mice (Fig. 1D). Although there were no significant differences among the Hi corresponding to the curves described above ($P > 0.1$), it should be noted that the secretion rate of anti-PC PFCs corresponding to the secondary response to PC-KLH appeared to be less heterogeneous than that of the other groups (low values of Hi indicate high heterogeneity). Among NMB-challenged mice, significant differences were noted only between the Hi from PC-KLH-primed mice and the Hi from unprimed and NMB-primed mice ($P < 0.005$) (Fig. 1E), with the former being lower (more heterogeneous secretion rates) than the latter. Although not significant, a larger PC_{50} value for NMB-primed mice, compared with unprimed mice, can be observed (Fig. 1). Also, among PC-(NMB)HI-challenged mice, the Hi from PC-KLH-primed mice was significantly lower than the Hi from unprimed and NMB-primed mice ($P < 0.025$) (Fig. 1F). However, the pattern of the plaque inhibi-

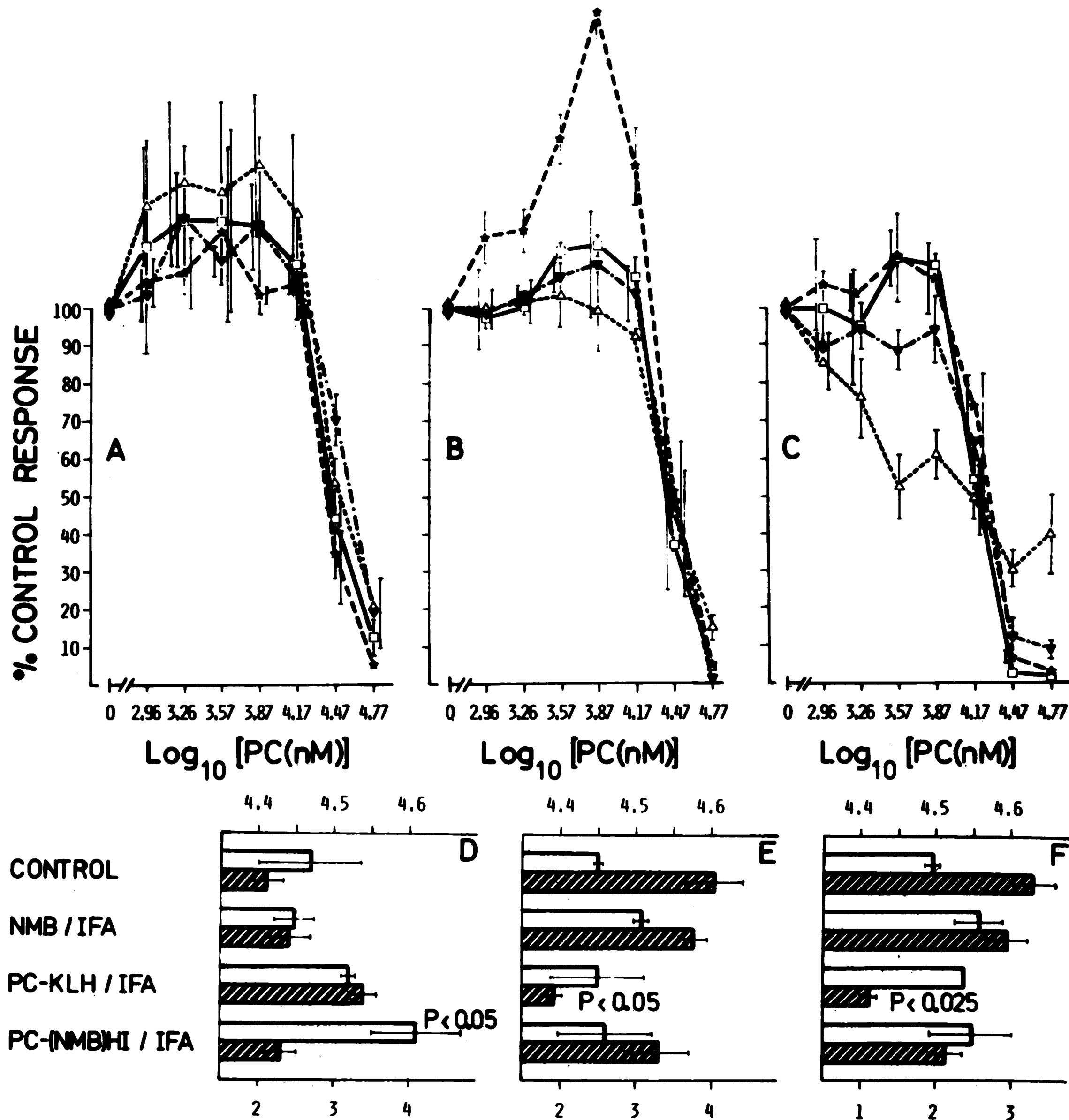

FIG. 1. Inhibition of direct hemolytic plaque formation with different concentrations of free PC hapten in NBF1 female mice. The source of cells for these plaque assays was the spleens used in experiments reported in Table 1. Primed mice were inoculated intraperitoneally with 0.2 ml of NMB diluted in saline (86% transmittance at 530 nm), 0.2 ml of PC-KLH (200 μg), or 0.2 ml of PC-(NMB)HI; and each was emulsified 1:1 in IFA. At least 1 month after priming, mice were challenged with appropriate antigens. Five days later direct anti-PC PFC counts without inhibitor were made (100% of response). Rate secretion profiles were obtained by determining the extent of inhibition after the incorporation of twofold serial dilutions of hapten in the plaque mixture, with a maximum of 58.82 μM final concentration of free PC. (A and D) Mice challenged with PC-KLH; (B and E) mice challenged with NMB; (C and F) mice challenged with PC-(NMB)HI. Panels A, B, and C show the pattern of inhibition curves. Each point represents the percent response (mean ± standard error) of the total number of PFCs at each hapten concentration. Symbols: □, control mice (not primed); ★, NMB-primed mice; △, PC-KLH-primed mice; and ▼, PC-(NMB)HI-primed mice. The histograms represent the log of the PC_{50} (open bars; expressed as nM) and Hi (hatched bars; see text). Statistical analysis was performed by ANOVA. P, Probabilty of significance when compared with the control.

tion curve from PC-KLH-primed PC-(NMB)HI-challenged mice is anomalous (Fic. 1C), in the sense that with our standard dilutions of free PC neither 0 nor 100% inhibition was achieved; moreover, the decline of PFCs with increased amounts of inhibitor was not monotonic.

Change of hemolytic plaque inhibition curve pattern of NMB-challenged mice with NMB priming time. The differences in pattern and PC_{50} between curves obtained from NMB-primed and unprimed mice challenged with NMB (Fig. 1B and E) could be due to chance variations; however, there could also be real differences between PC_{50}s that were not detected because the number of mice used in the experiments was insufficient for this statistical analysis. We tried to overcome this difficulty by studying the evolution of the inhibition curve with NMB priming time (Fig. 2). Once the changes of PC_{50} and Hi with priming time were analyzed by ANOVA of linear regression, the PC_{50} was, in fact, seen to increase significantly in NMB-challenged mice which

were NMB primed for four weeks ($P < 0.01$), but Hi showed no significant decreases ($P \geq 0.1$).

Suppression induced in secondary immunoresponse to NMB has an effect on PC-(NMB)HI-induced immunoresponse but not on that induced by PC-KLH. Results of the experiments reported in Table 1 show that suppression occurs only when mice are both primed and challenged with NMB, thus ruling out the possibility of nonspecific suppression. Moreover, NMB induces suppression not only in NBF1 female mice primed for 1 month but also in BNF1 male and female and NBF1 female mice challenged with NMB at different times (from slightly more than 1 month to at least 6 months) after priming with NMB, always giving less than 20,000 anti-PC PFCs per spleen (data not shown). However, it remains to be determined whether this suppression is triggered by priming or by challenge after priming, and furthermore, if the latter is the case, once the suppressive mechanism is triggered, would it affect anti-PC responses induced by PC antigens other than NMB? To answer these questions, we simultaneously injected NMB plus PC-(NMB)HI or NMB plus PC-KLH into NMB-primed mice and compared the results with those obtained by injecting only NMB, PC-(NMB)HI, or PC-KLH into NMB-primed mice. The results (Table 2), indicate that the subpopulations involved in the immune responses to PC-KLH and NMB are independent. However, the PC-(NMB)HI-induced anti-PC immune response decreased when this antigen was injected simultaneously with NMB into NMB-primed mice; in some experiments the same level of unresponsiveness as that seen with NMB alone was noted (Table 2, experiment 3).

These experiments were carried out simultaneously with the examination of PFC secretion rate profiles by inhibition of plaque formation with free antigen. Patterns of inhibition displayed by curves corresponding to experiments 1 and 2 in Table 2 are represented in Fig. 3A and B, respectively. The three curves from experiment 1 (Table 2) are very similar, with no differences in their PC_{50} and Hi values ($P \geq 0.1$) and with no clear overshooting of the 100% level. In contrast, there were significant differences between the PC_{50} corresponding to the curve from PC-(NMB)HI- plus NMB-challenged mice and the PC_{50} corresponding to the curve from NMB-challenged mice ($P < 0.05$) (Fig. 3D); moreover, in Fig. 3B a slight overshooting can be seen in the NMB and PC-(NMB)HI plus NMB curves in the region of highest dilutions of inhibitor.

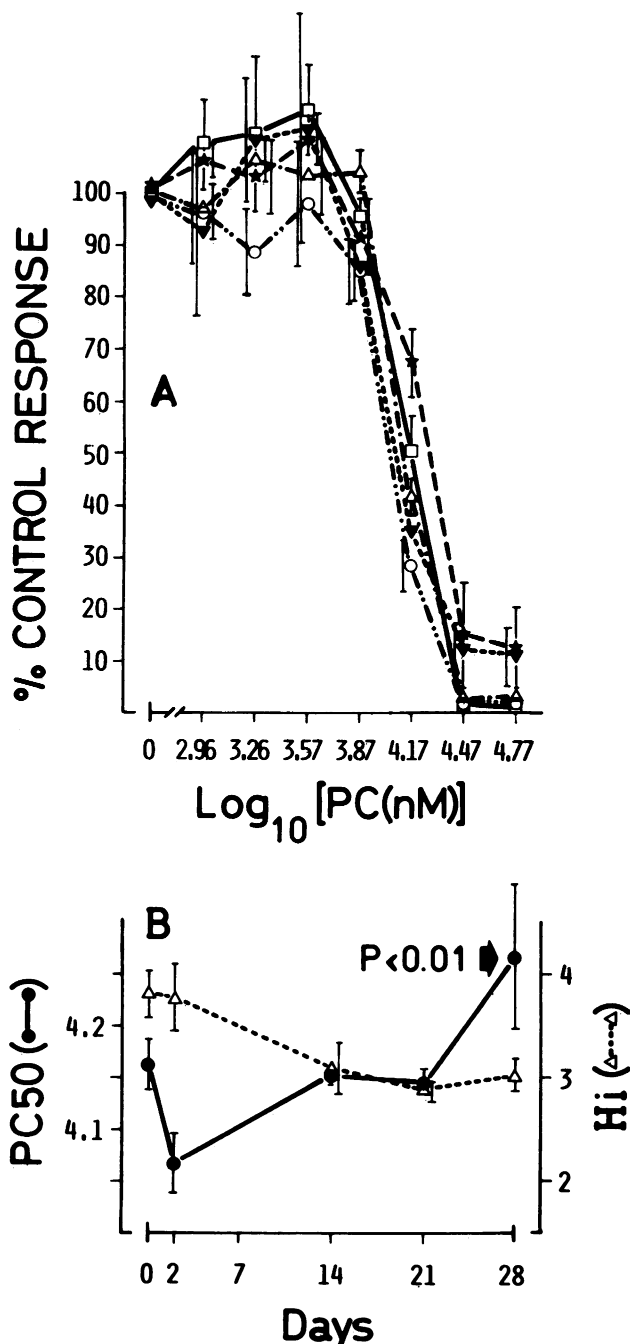

FIG. 2. Evolution of direct hemolytic plaque inhibition curve pattern of NBM-challenged mice with NMB priming time. NBF1 female mice were primed by intraperitoneal inoculation of 0.2 ml of NMB emulsified 1:1 in IFA (as described in the legend to Fig. 1). Five groups of animals were used. Control mice (not primed) and mice primed 2, 14, 21, and 28 days before challenge were challenged on the same day with 0.1 ml of NMB. Five days later anti-PC PFC counts without inhibitor were made (100% of response). Rate secretion profiles were obtained as described in the legend to Fig. 1. (A) Pattern of inhibition curves. Each point represents the percent response (mean ± standard error) of the total number of PFCs at each hapten concentration. Symbols: □, control mice (not primed); ○, mice primed for 2 days; △, mice primed for 14 days; ▼, mice primed for 21 days; ★, mice primed for 28 days. (B) PC_{50} and Hi are as described in the legend to Fig. 1. They were calculated from curves shown in panel A. Symbols: ●, PC_{50}; △, Hi. Statistical analysis was performed by ANOVA. P, Probability of significance when compared with the control.

DISCUSSION

A single injection of relatively low doses of NMB given to NBF1 female mice greatly impaired their ability to mount NMB-induced anti-PC responses after 1 month of priming. This remarkable and dramatic impairment was manifested by the alteration of the following parameters of the anti-PC PFC response (i) a permanent decrease in the capacity to give IgM PFC responses to PC without a detectable increase in the ability to give IgG or IgA anti-PC PFCs (12); (ii) the early loss and subsequent recovery of high T15 expression (suggesting that an idiotype–anti-idiotype circuit is initially involved in the anti-PC suppressive mechanism induced by

TABLE 2. Interference between NMB- and PC-(NMB)HI-induced anti-PC responses, but not between NMB- and PC-KLH-induced anti-PC responses, in NBF1 female mice primed with NMB[a]

Expt	Antigen	No. of direct anti-PC PFCs/spleen[b]	% Direct anti-PC T15 idiotype PFCs	% IgG[c]	% IgA[d]
1[e]	PC-KLH	92,292 (4.97 ± 0.05)	48 ± 8	91 ± 4	105 ± 8
	PC-KLH + NMB	108,099 (5.03 ± 0.03)	44 ± 6	90 ± 7	107 ± 9
	NMB	20,826 (4.32 ± 0.17)	58 ± 14	96 ± 12	93 ± 5
2[f]	NMB	12,236 (4.09 ± 0.17)	60 ± 3	111 ± 12	106 ± 5
	PC-(NMB)HI + NMB	103,331 (5.01 ± 0.12)	69 ± 11	83 ± 9	91 ± 5
	PC-(NMB)HI	226,222 (5.35 ± 0.16)	71 ± 10	91 ± 5	106 ± 3
3[g]	NMB	6,125 (3.79 ± 0.10)	55 ± 14	ND[h]	ND
	PC-(NMB)HI + NMB	16,116 (4.21 ± 0.09[i])	54 ± 5	ND	ND
	PC-(NMB)HI	203,348 (5.31 ± 0.17)	72 ± 6	ND	ND

[a] Groups of three to four mice were used, and direct and indirect PFCs were counted 5 days after challenge. In experiments 1 and 2, NMB-challenged mice were primed 6 months before challenge, and the other four groups of mice were primed 1 month before challenge. In experiment 3 all mice were primed 5 months before challenge.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*.
[d] See Table 1, footnote *d*.
[e] Mice were 36 weeks of age when challenged after priming.
[f] Mice were 28 to 32 weeks of age when challenged after priming.
[g] Mice were 32 to 34 weeks of age when challenged after priming.
[h] ND, Not done.
[i] $P < 0.001$ versus the PC-(NMB)HI-challenged group, as determined by Student's *t* test.

NMB) (12); (iii) a gradual decrease in antibody secretion rates of NMB-induced anti-PC PFCs (this study).

The results presented in Tables 1 and 2 rule out the possibilities that suppression would be nonspecific or strictly epitope (PC) specific and that priming with NMB is sufficient to give the signal necessary to activate suppression. Moreover, pretreatment with NMB appears to potentiate the immunoresponse particularly to PC-(NMB)HI (Table 1). Priming with PC-KLH induced a typcially secondary immunoresponse to PC-KLH, with a switch to IgG. A rather unexpected result, the meaning of which is unclear, is that the challenge with PC-(NMB)HI after pretreatment with PC-KLH causes a strong switch to IgG and a less pronounced one to IgA in the anti-PC PFC response (62% IgG, 34% IgM). However, unlike the secondary immunoresponse to PC-KLH, there was no concomitant increase in the number of total anti-PC PFCs (direct plus indirect); rather, the number of total anti-PC PFCs remained unchanged with respect to the PC-(NMB)HI-induced anti-PC response in normal (unprimed) mice. Moreover, it appears that the direct T15 idiotype anti-PC PFC tends to decrease in PC-KLH-primed mice injected with PC-(NMB)HI versus normal mice injected with PC-(NMB)HI. Similar results were observed in normal versus PC-KLH-primed mice when the same protocol was used with NMB instead of PC-(NMB)HI; there was a switch, although less marked, to IgG (24% IgG, 76% IgM) and a drastic reduction in the direct T15 idiotype anti-PC PFCs (38% T15 idiotype in normal mice versus 6% T15 idiotype in PC-KLH-primed mice). Also, as in the preceding case, the total number of anti-PC PFCs remained unchanged. As mentioned above, very similar results were observed in other experiments in which conditions were parallel to those described here (data not shown), suggesting a high reproducibility of phenomena. A similar phenomenon has been described in other idiotypic systems, such as that of the anti-ABA CRI$^+$ (azobenzene arsonate cross-reactive idiotype) antibodies (6). According to the model proposed below, our results can be explained by assuming that practically all IgG anti-PC PFCs in the PC-KLH-primed mice came from T15 idiotype B cells, an explanation supported by numerical counts. Furthermore, this diminution in T15 idiotype PFCs is paralleled by a diminution of the Hi value (more secretion rate heterogeneity) (Fig. 1B and C) but not of the PC_{50} value. Thus, it would be interesting to study the time dependence of these parameters (PC_{50}, Hi) in the NMB- and PC-(NMB)HI-induced anti-PC responses in PC-KLH-primed mice.

The explanation given above of the effects of pretreatment with PC-KLH on the subsequent immune response to NMB and PC-(NMB)HI raises a number of questions about the role played by these antigens in the anti-PC immunoresponse that they trigger. Is pretreatment with PC-KLH sufficient to determine class, intensity of, and idiotype(s) involved in the switch (37, 56)? Or do the new antigenic stimuli also participate effectively? In the first case, PC-KLH-primed mice injected with PC-KLH, NMB, or PC-(NMB)HI should give similar results. In fact, however, our results are quite different from this prediction (Table 1). The other possibility, while it appears more likely, demands a more detailed and complex interpretation.

We have shown that (i) both antigens [NMB and PC-(NMB)HI] induce practically the same total anti-PC PFC numbers in PC-KLH-primed and normal mice, (ii) a similar T15 idiotype reduction is detected in both immune responses compared with that of the respective controls, and (iii) an important fraction of IgG anti-PC PFCs is also observed in both immune responses. Therefore, explicit differences between both immunoresponses appear to be quantitative rather than qualitative.

Current experimental evidence supports the idea of B-lymphocyte subpopulations with different activation requirements (53). One set is T cell dependent and responsible only to TD antigens (B_{TD} cells; could these be B cells Lyb 5^-?) (24, 27, 45, 50, 56, 57); another set is T cell independent and responsible only to TI antigens (B_{TI} cells; could these be B cells Lyb 5^+?) (24, 27, 45, 50, 56, 57); and a third B cell subpopulation or set of B cell subpopulations has activation requirements intermediate between those of B_{TD} and B_{TI}

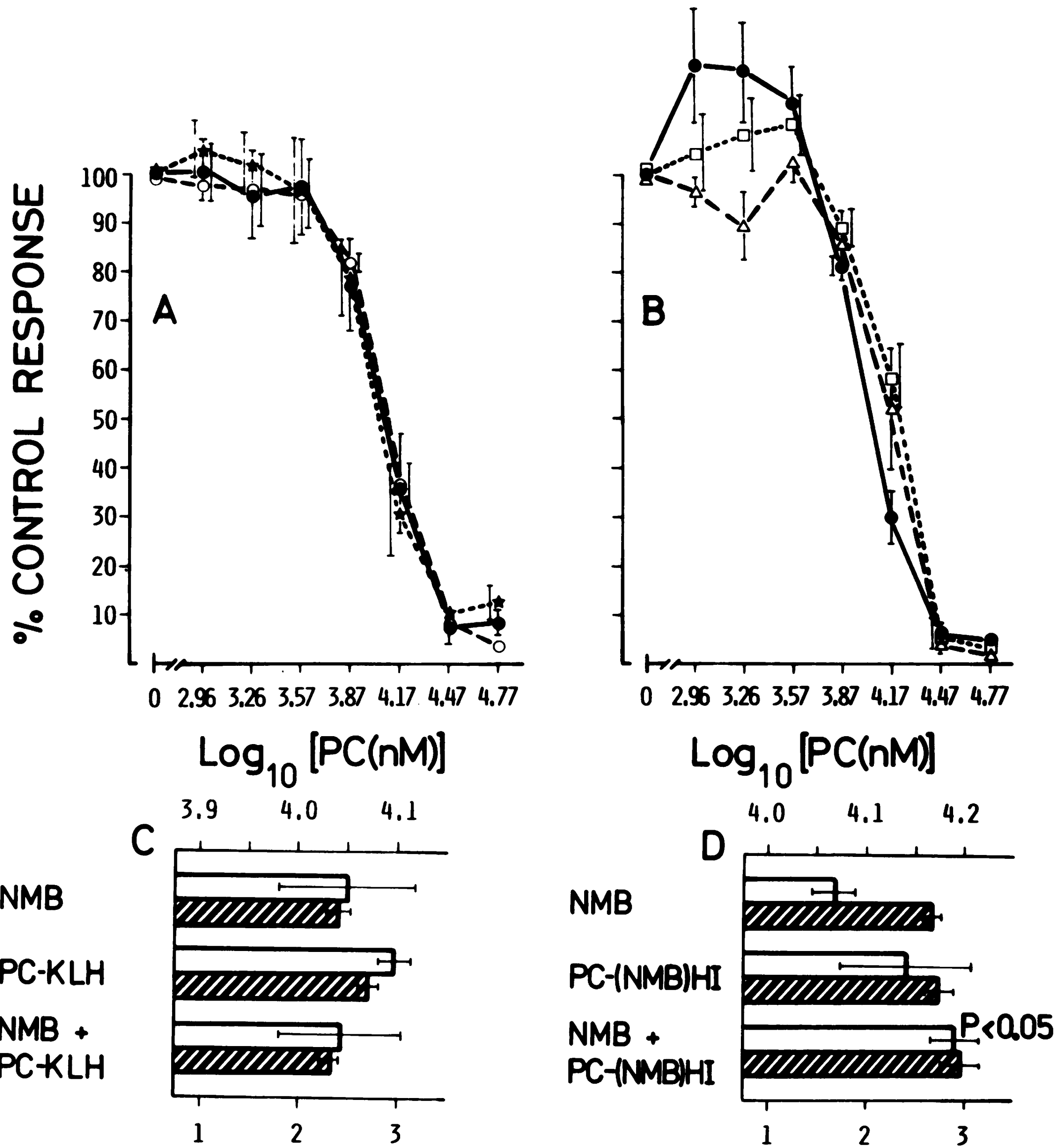

FIG. 3. Inhibition of direct hemolytic plaque formation with different concentrations of free PC in NMB-primed NBF1 female mice. Cells used were from spleens processed in experiments reported in Table 2. Primed mice were inoculated intraperitoneally with 0.2 ml of NMB emulsified 1:1 in IFA (as described in the legend to Fig. 1). At least 1 month after priming, mice were challenged with appropriate antigens. Five days later direct anti-PC PFC counts without inhibitor were made (100% of response). Rate secretion profiles were obtained as described in the legend to Fig. 1. Panels A and C correspond to experiment 1, Table 2; panels B and D correspond to experiment 2, Table 2. (A) and (B) Pattern of inhibition curves. Each point represents the percent response (mean ± standard error) of the total number of PFC at each hapten concentration. Symbols: ●, challenged with NMB; ○, challenged with PC-KLH; ★, challenged with PC-KLH plus NMB; △, challenged with PC-(NMB)HI; □, challenged with PC-(NMB)HI plus NMB. The histograms represent the log of the PC_{50} (expressed as nM; open bars) and the Hi (hatched bars). The statistical analysis was performed by ANOVA. P, Probability of significance when compared with the control.

cells (16). We have called these B_{TD+TI}. Based on the evidence given above and being aware that our results, although quite reproducible, do not offer incontrovertible data to support our model, we present the following interpretation as a working hypothesis. (i) PC-specific B_{TD} and a fraction of B_{TD+TI} cells are induced to switch after PC-KLH pretreatment; (ii) almost all PC-specific T15 idiotype B lymphocytes are B_{TD+TI} cells; (iii) NMB and PC-(NMB)HI stimulate different sets of B cell subpopulations, with NMB stimulating mainly B_{TI} cells and a fraction of B_{TD+TI} cells and PC-(NMB)HI stimulating the B_{TD+TI} cells, a fraction of B_{TI} cells, and another fraction of B_{TD} cells.

Results presented in Table 1 are in agreement with those reported by Wicker et al. (56), in that pretreatment with different PC antigens determines the type of anti-PC response (i.e., idiotypic and class expression of antibodies). However, data given in this report suggest that the opposite is also true. PC-KLH-primed mice vary in their anti-PC immunoresponses, depending on the antigen subsequently used to induce them.

Finally, results in Table 2 offer evidence that (i) activation of the suppressive mechanism in NMB-primed NMB-challenged mice is not altered or affected by simultaneous injection of a TD or TI PC antigen with NMB, and (ii) once the suppressive mechanism is triggered, it at least partially affects the anti-PC response induced by PC-(NMB)HI but does not affect that induced by PC-KLH. Although this result seems to argue against carrier specificity of suppression, it must be remembered that autoclaving of NMB does not ensure the destruction or alteration of all meningococcal epitopes or antigenic structures, and it is possible that some of these structures remain intact (44). Rather, the observed suppression of the PC-(NMB)HI-induced immunoresponse, when this antigen is injected simultaneously with NMB in NMB-primed mice, is due to structures on it recognized by the previously triggered suppressive mechanism. These structures are the same as those on NMB and are not heat labile. Such structures are probably different from those involved in the triggering of the suppressive mechanism; these triggering structures are present in an active form only in NMB and are probably heat labile. Thus, PC-(NMB)HI cannot trigger suppression, but once suppression is activated, the cells normally stimulated by PC-(NMB)HI become a target for suppression. Therefore, suppression must be considered carrier-specific.

In this report, data are offered that suggest clearly that NMB, PC-(NMB)HI, and PC-KLH stimulate different PC-specific B cell subpopulations or sets of B cell subpopulations. These data concern fundamental parameters of these immune responses, such as the number of anti-PC PFCs, T15 idiotype PFCs, antibody class, PC_{50} and Hi. We suggest that the suppressor mechanism activated by NMB belongs to an NMB-specific immune circuit or reticulum, i.e., to an immune reticulum in which interactions revolve mainly around the NMB-specific B cells. Also, PC-KLH, the immune reticulum of which seems to be independent of that of NMB, is able to interact directly or indirectly with B cells from PC-(NMB)HI or NMB immune reticuli. Our data suggest, although not unambiguously, that this interaction is indirect and probably acts through an idiotype–anti-idiotype mechanism (3, 46) based on the T15 idiotype. Finally, PC-(NMB)HI seems to stimulate an immune reticulum that partially overlaps with that of NMB [pretreatment with NMB potentiates the immune response to PC-(NMB)HI (Table 1), and suppresses the immune response to PC-(NMB)HI plus NMB (Table 2)].

At present, we can only speculate about the mechanism of such suppression and its molecular antigenic origin. Several meningococcal structures might be responsible for triggering this suppression: endotoxin or lipopolysaccharide (28, 34, 54); cell wall proteins, lipoproteins, or peptidoglycans (13, 49). Also, the effector phase of suppression can be mediated by carrier-specific suppressor T cells (17, 21, 25, 35); suppressor B lymphocytes (26, 28), or plasma cells (1); macrophages (41); or even, though this is less likely, to an excess of C-reactive protein induced by NMB (33, 40). We are currently analyzing the effector phase of such meningococci-induced suppression.

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