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The effects of idiotype on the ability of IgG_1 anti-phosphorylcholine antibodies to protect mice from fatal infection with *Streptococcus pneumoniae**

Anti-phosphorylcholine (PC) antibodies of the mouse are found in three different idiotype families: T15, M603 and M511. These subgroups exhibit different specificities for PC analogs and utilize light chains of different V_L subgroups. In this study we have found that IgG_1 antibodies of the T15 idiotype are much more protective against pneumococcal infection than IgG_1 antibodies of the M511 or M603 idiotypes. This finding provides additional evidence that the T15 V_H and V_L genes may have evolved to protect mice from infection with PC-bearing pathogens.

1 Introduction

Definitive studies of the biological significance of idiotype have been difficult to perform, in part because appropriate anti-pathogen antibodies have not been available. The recent observations that anti-phosphorylcholine (PC) antibodies can bind to live pneumococci [1] and protect mice from infection with certain pneumococcal strains [2–4], coupled with the existence of a large number of highly characterized hybridoma and myeloma anti-PC antibodies [5–8], have provided an ideal system with which to investigate this question. The V_H region of virtually all mouse anti-PC antibodies is coded for by the same V_H and J_H gene segments [8]. Anti-PC antibodies, however, consist of three distinct families, distinguished by having light chains of different V_L subgroups. Each family is also characterized by having distinctive binding specificities for PC analogues and expresses different serological idiotypes *i.e.* T15, M603 or M511 [5, 6].

Because of the large panel of monoclonal anti-PC antibodies available it is possible to compare the protective effects of antibodies of the three different families by testing antibodies of the same isotype but different idiotypes. Previously we have demonstrated that IgM antibodies of the T15 idiotype family were more protective than IgM antibodies of either the M603

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Abbreviations: **CFU:** Colony-forming units **LD_{50}:** Median lethal dose, *i.e.* dose of bacteria lethal to 50% of the animals **PC:** Phosphorylcholine **PD_{50}:** Median protective dose, *i.e.,* dose of antibody at which 50% of the animals survive **V_H:** Heavy chain variable region **V_L:** Light chain variable region

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or M511 idiotype families [9]. In this report we have examined the relative protective capacity of IgG_1 anti-PC antibodies of each of the three anti-PC families. This new investigation was important since the relative protective effects of antibodies with these three binding-site structures might be either amplified or minimized by differences in valence of IgG and IgM antibodies, as well as the fact that IgG and IgM anti-PC antibodies may bring about opsoniphagocytosis by different mechanisms, that rely on different relative involvements of Fc and C3b receptor-mediated phagocytosis. This study was done with IgG_1 antibodies since IgG_1 was the only IgG isotype for which antibodies of all three idiotypes were available. Our findings indicate that the protective superiority of antibodies of the T15 idiotype over those of the M603 and M511 idiotypes was, if anything, even more pronounced among IgG_1 than among IgM anti-PC antibodies.

2 Materials and methods

2.1 Bacteria

S. pneumoniae type 3, strain WU2, was grown from frozen stock cultures in Todd-Hewitt broth (Difco Laboratories, Detroit, MI) supplemented with 0.5% yeast extract (Difco) and 2% heparinized human blood [2]. One subsequent passage was made in broth without blood, and early log phase pneumococci were harvested. Bacteria were enumerated by absorbance and plating, and were injected i.v. in 2% heat-inactivated fetal calf serum-Ringer's lactate (FCS-Ringer's) as described [2].

2.2 Hybridoma antibodies

The isolated hybridoma antibodies to PC that were used in this study have been described [5–7]. All hybridoma antibodies were made by fusion of BALB/c or C57L spleen cells (both Igh^a, $Igh\text{-}PC^b$) with the nonsecreting plasmacytoma fusion line P3X63-Ag8.653, except HP293, HP1613 and HP2857, which were generated with the nonsecreting line SP2/0-Ag14. Antibodies 159.4D5.2 and 1613 were generated from mice immunized with *S. pneumoniae* strain R36A; 167.4G5.5, 137.7C9, 293 and 2857 from mice immunized with PC-keyhole limpet hemocyanin (KLH); and 180.2G6.2 and 180.7B6.3 from mice immunized with *Morganella morganii*. The idiotypes of these anti-PC hybridoma antibodies have been characterized by a solid-phase radioimmunoassay using heterologous anti-idiotype antisera to T15, M603 and M511 [10].

2.3 Mouse protection with hybridoma antibodies

All mice used in these studies were 6–8-week-old CBA/N mice obtained from Dominion Lab., Dublin, VA.

To determine the median protective dose (PD_{50}) of each hybridoma antibody *vs.* 100 colony-forming units (CFU) of type 3 strain, WU2, mice were injected i.p. with either 100, 20, 2 or 0.2 μg of hybridoma antibody in FCS-Ringer's 1–2 h before i.v. injection of the *S. pneumoniae*.

We also determined the dose of WU2 required to kill 50% of unprotected mice (LD_{50}), and mice protected with 20 μg of each hybridoma antibody. After injection of diluent or antibody mice were divided into smaller groups and infected with doses of WU2 ranging, in 10-fold increments, from 10^1 to 10^8 CFU. For determining LD_{50} we always had from 5–10 mice infected with the dose just above and just below the LD_{50}.

Mice were observed for 10 days, and deaths were recorded at 24 h intervals. Other mice were infected at each of several other doses as well. The rank order statistical test was used to compare the death order of mice in the different groups. For groups in which over one-half of the mice died within the 10-day period, the median survival time was estimated by calculating the reciprocal mean survival time for the mice in each group. In groups where less than one-half of the mice died in 10 days, the median survival time was simply listed as >10 days. The median PD_{50} and LD_{50} were calculated by the method of Reed and Muench [11].

2.4 Blood clearance of *S. pneumoniae*

Mice were injected i.p. with either antibody or diluent 1 h prior to i.v. challenge with pneumococci. Blood samples were collected from the retroorbital plexus at 1, 60 and 240 min post-inoculation in calibrated heparinized capillary pipets, serially diluted in FCS-Ringer's, and plated on blood agar to determine the number of viable pneumococci in the blood of each mouse, assuming a blood volume of 3 ml [12].

3 Results

3.1 General remarks

In this study we have examined the ability of 5 different hybridoma IgG_1 anti-PC antibodies of the T15, M603 and M511 idiotype families [4, 5] to protect mice from infection with type 3 *S. pneumoniae*. CBA/N mice, which carry the *xid* defect [14], have been used for all the protection studies described below. These mice are ideal for this purpose [2, 9, 14] since they have little or no detectable anti-PC antibody in their normal serum [2, 15] and make only very low levels of anti-PC antibody when immunized [16].

3.2 Relative protective capacity of T15, M603 and M511 IgG_1 hybridoma anti-PC antibodies

We compared the abilities of two T15, two M603, and one M511 IgG_1 anti-PC hybridoma antibodies to protect mice from fatal infection with *S. pneumoniae*. We observed marked protection with the T15 antibody but very little protection with M603 and M511 antibody (Table 1). The 10-day PD_{50} for the T15 IgG_1 antibodies was found to be between 4 and 6 μg/mouse, whereas with the non-T15 antibodies the PD_{50} was not reached even at 100 μg/mouse (Table 2). The protective capacities of these antibodies were also analyzed by protecting all mice with 20 μg of either T15, M603 or M511 IgG_1 anti-PC antibodies and determining the change in LD_{50} of type 3 *S. pneumoniae* WU2. The results indicate that T15 provides 7 logs of protection compared to M603 and M511 antibodies which provided no more than 1 log of protection (Table 3).

3.3 Relative blood clearance of pneumococci by IgG_1 anti-PC antibodies of the T15, M603 and M511 idiotypes

The T15 anti-PC IgG_1 antibodies efficiently cause blood clearance of *S. pneumoniae* such that during the first hour the

Table 1. Protection of *xid* mice from type 3 *S. pneumoniae* with hybridoma IgG_1 anti-PC antibody[a]

Idiotype	Hybridoma	Dose (μg/ mouse)	No. of mice alive at indicated day 0	1	2	3	4	5	6	>10	Median days alive[b]
T15	167.4G5.5	20	8	8	7	6	6	6	6	6	>10**[c]
		2	10	10	3	3	3	3	3	3	2.6
		0.2	6	6	0	0	0	0	0	0	2
	159.4D5.2	20	13	13	12	12	12	12	12	12	>10***
		2	14	14	7	4	4	4	4	4	2.9*
		0.2	4	4	0	0	0	0	0	0	2
M603	180.2G6.2	100	5	5	2	1	1	1	1	1	2.6
		20	10	10	3	3	3	3	3	3	3.0
		2	5	5	2	2	2	2	2	2	2.9
	180.7B6.3	100	5	5	2	1	1	1	1	1	2.6
		20	10	10	6	6	5	5	5	5	3.6*
		2	5	5	1	1	1	1	1	1	2.4
M511	137.7C9	100	3	3	2	0	0	0	0	0	2.6
		20	3	3	0	0	0	0	0	0	2
Diluent			11	11	0	0	0	0	0	0	2

a) Mice were injected i.p. with antibody or diluent 1 h prior to i.v. inoculation with 100 CFU of type 3 *S. pneumoniae*, strain WU2.

b) For values < 10, the median days alive is the reciprocal mean days alive for each mouse in the group. That is, median days alive $= n\left(\sum_{i=1}^{n} \frac{1}{x_i}\right)$, where x is the number of days that mouse i lived and n is the number of mice in the group.

c) Statistically different from diluent control at $P < 0.03$ (*); $P < 0.01$ (**); $P < 0.001$ (***); by two-sample rank test.

Table 2. Comparison of the protective ability of T15, M603 and M511 IgG_1 antibodies in *xid* mice

Idiotype	Hybridoma	PD_{50} on day[a] 2	10	P *vs.* T15 IgM[b]
T15	167.4G5.5	4.6	5.5	-
	159.4D5.2	2.3	4.4	-
M603	180.2G6.2	>100	>100	<0.01
	180.7B6.3	32	>100	<0.01
M511	137.7C9	67	>100	<0.01
All non-T15		>100	>100	<0.002

a) PD_{50} doses were calculated at 2 and 10 days post-infection.

b) Calculated by rank order test on days alive data for mice protected with 2 μg.

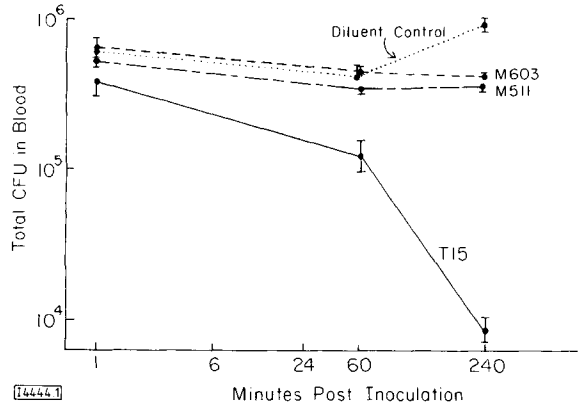

Figure 1. IgG_1 antibody-dependent blood clearance of *S. pneumoniae*. Mice were injected i.p. with 20 μg of T15, 159.4D5.2; M603, 180.2G6.2; or M511, 137.7C9 IgG_1 anti-PC antibody. Control mice received diluent only. One hour later all mice received 10^6 WU2, *S. pneumoniae* i.v.

blood level of circulating bacteria is reduced by 67% and by 4 h it is reduced by almost 98% (Fig. 1). In contrast, the non-T15 IgG_1 antibodies reduced the circulating level of pneumococci by less than 40% during the same 4-h period, while in diluent-injected mice the number of circulating bacteria actually increased by 62% (Fig. 1).

Table 3. LD_{50} of *S. pneumoniae* in mice protected with 20 μg IgG_1 antibody

Hybridoma	Idiotype	LD_{50} WU2[a]	P *vs.* T15[b]
159.4D5.2	T15	10^4	-
180.2G6.2	M603	$<10^2$	<0.002
137.7C9	M511	$<10^2$	<0.002
None	-	$<10^1$	<0.002

a) Calculated values were the same at 2 and 10 days post-infection.

b) Calculated by rank order test on days alive for the 10^6 dose.

Table 4. Relative protective capacity of IgM anti-PC antibodies of T15, M511 and M603 idiotypes

Idiotype	Hybridoma	PD_{50} on day[a] 2	10
T15	22.1A4	47	85
	HP293	26	33
	All T15	38	51
M603	HP1613	120	>300
	180.6B6.5	100	>300
	180.7C9.2	150	>300
	All M603	120	>300
M511	HP2857	100	190

a) See footnote a) of Table 2.

3.4 Relative protective capacity of T15, M603 and M511 IgM hybridoma antibodies

To provide a comparison with the above IgG results, we have determined the protective capacity of IgM T15 and non-T15 anti-PC antibodies. In this study we have included 5 IgM hybridoma anti-PC antibodies whose PD_{50} values we had not previously determined (Table 4). Our findings indicate, as expected, that IgM T15 anti-PC antibodies are in general more protective than IgM non-T15 anti-PC antibodies and that IgM anti-PC antibodies are less protective than IgG anti-PC antibody [9, 14].

4 Discussion

The data presented here demonstrate that the protective superiority of T15, *vs.* non-T15, anti-PC antibodies can be seen with IgG_1 as well as with IgM anti-PC antibodies. This finding indicates that the superior protective ability of anti-PC antibodies of the T15 idiotype is independent of both the valence of the antibody molecule and any isotype-mediated differences (IgG_1 *vs.* IgM) in the bacteriocidal mechanisms used for protection. Since the heavy chain of the anti-PC antibodies of all 3 idiotypes is made from the same germ-line gene, and since somatic mutations, D region segments, or J_H segments specific for particular anti-PC public idiotypes have not been detected [7, 8], it seems likely that the differences in protective capacity of the antibodies in the three idiotype groups are related to the fact that they use light chains from different V_L families. The fact that antibodies of the 3 anti-PC idiotype groups differ in their specificity has previously been established in studies showing that the relative reactivities of antibodies within each of the 3 idiotype families for a panel of PC analog molecules is very similar, yet distinct from the reactivities of the antibodies of the other two families ([5, 6] Claflin, Hudak and Maddalena, manuscript submitted). In this context it is important to note that the mouse protection data in this report indicate that the ability of the different anti-PC antibodies to bind the complex PC-containing antigen on the pneumococcal surface is not related to their ability to bind free PC, since the binding constant of anti-PC antibodies for this antigen is virtually the same for anti-PC antibodies of each of the 3 idiotype families [7, 8].

Similarities in anti-PC specificity of antibodies within a family, particularly T15 and M603, are especially striking [5, 6] and help to explain why all IgM and IgG_1 hybridoma antibodies within an idiotype family protect equally well (or poorly). In fact, immunization with the T-dependent (PC-KLH) or T-independent (killed pneumococci) form of PC has had no demonstrable effect on the fine specificity of M603 or T15 antibodies for PC antigens from microorganisms and parasites (Claflin, Hudak and Maddalena, manuscript in preparation), or a series of PC-containing soluble molecules [5, 6].

As we have argued previously [9], the fact that direct translation of the T15 germ line V_L and V_H genes [7, 8, 17] leads to antibody protective against pneumococcal infection, may be an indication that these genes have evolved to produce antibody to the phosphorylcholine present in pneumococcal C polysaccharide. The effects of somatic mutations on the protective capacity of anti-PC antibodies of the T15, M603 or M511 idiotypes is presently a matter of speculation, since amino acid sequences are not available for most of the hybridoma antibodies we have used. However, even if somatic mutation could result in even more protective anti-PC antibody, it is doubtful that they would commonly play an important biological role in immunity to pneumococci, since anti-carbohydrate responses are relatively T cell independent [13, 18], develop slowly [18–20], and thus show little if any affinity maturation [19–21]. Therefore, in the cases of antibodies that bind to surface carbohydrates of common pathogens, it may be important to have functional V_H and V_L genes, as well as an idiotype regulatory system that can insure their expression in antibody responses.

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