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Biological mimicry of antigenic stimulation: analysis of the *in vivo* antibody responses induced by monoclonal anti-idiotypic antibodies

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SUMMARY

In this study, the induction of protective antibodies against a bacterial pathogen in mice was used as a model for idiotype vaccine development. The antibody responses induced in different strains of mice by the hapten phosphorylcholine (PC) coupled to ovalbumin, PC-OVA, were compared with the responses induced by carrier conjugates of two different anti-idiotopic antibodies. One anti-idiotope, 4C11, exhibits the characteristics of an internal image of phosphorylcholine, and therefore is classified as an Ab2β; the other, F6, does not mimic antigen, and therefore is classified as an Ab2α. The analysis of the temporal kinetics of the IgM and IgG1 anti-PC responses induced by nominal and idiotope antigens revealed dynamic responses characterized by changes in the quality and quantity of the antibody populations during the course of the immune response. All three antigens could stimulate antibodies that were PC-specific and T15 idiotope-positive in BALB/c and A/St mice. The highest titre of T15$^+$ anti-PC antibodies was achieved with an immunization protocol which involved priming with Ab2α followed by challenge with PC-OVA. Antibodies specific for the extended hapten, diazophenylphosphorylcholine, and hapten–carrier bridge determinants were being stimulated late in the responses to PC-OVA. BALB-c, A/St and CBA/N (Xid) mice all produced, late in the response to Ab2α, high T15$^+$ antibody titres which do not bind PC. The induction of T15$^+$, non-PC binding, antibody suggests that T15 is a regulatory idiotope, expressed on antibodies having differing antigenic specificities. With regard to vaccine development, these results support the contention that effective induction of antibodies does not depend on stimulating a unique idiotope but can be achieved by anti-idiotypes reacting with different idiotopes. In addition, these results suggest that the combined use of idiotope and nominal antigens in an immunization protocol may provide the maximal protective immunity.

INTRODUCTION

The network hypothesis of interacting idiotypes (Jerne, 1974) has become the basis recently for developing novel kinds of vaccines (reviewed by Kieber-Emmons *et al.*, 1986; Bona & Moran, 1985). Unlike conventional vaccines, which contain intact pathogens or materials derived from pathogens, the so-called idiotype vaccines consist of anti-idiotypic antibodies. The reason why certain anti-idiotypic antibodies can be used as surrogate antigens lies in the molecular mimicry of antigenic structures by idiotypic determinants. Idiotype vaccines may have advantages over conventional vaccines where immaturity of the immune system or acquired unresponsiveness render conventional vaccines ineffective (Gray *et al.*, 1981; Sell *et al.*, 1981; Sharma & Gupta, 1985).

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The stereochemical similarity of haptenic structures and idiotypic loops on the surface of immunoglobulins provides the basis for molecular mimicry of antigens by anti-idiotypic antibodies (Kohler, Muller & Bona, 1985). By virtue of the large variability of the immunoglobulin surface (Kieber-Emmons & Kohler, 1986), it can be assumed that every potential antigenic structure could be mimicked and represented as an internal image in the idiotypic repertoire. In order to conceptualize internal image idiotopes, a formal distinction between different types of anti-idiotypic antibodies has been proposed (Jerne, Roland & Cazanave, 1982; Bona & Kohler, 1984). Anti-idiotypes which mimic antigens are termed Ab2β, and those which do not mimic antigens are Ab2α. In addition, a further distinction of anti-idiotypes by identifying a third category which recognizes idiotypic determinants located physically close to the binding site has been made. Binding to such idiotypes results in steric interference of antigen binding. This type of AB2 has been termed gamma (Bona & Kohler, 1984).

Although the distinction of α, β or γ anti-idiotypic antibodies has been deemed crucial in the development of idiotype

vaccines, recent experimental evidence suggests that the classification of anti-idiotypic antibodies into those which mimic antigen and others which are anti-idiotypic antibodies a la Oudin is irrelevant in the design of idiotope vaccines. For example, anti-idiotypes which do not meet the criteria of internal images have been shown to be effective in inducing antigen-specific antibody responses (Huang, Ward & Kohler, 1986). Since anti-idiotypic stimulation is not dependent on idiotypic–anti-idiotypic complexes involving unique idiotopes on Ig or T-cell receptors, the topography of idiotope targets is only important in the context of the linkage of target idiotope and binding site (paratope). Assuming a tight linkage of target idiotope and paratope in the antibody population, biologically significant stimulation can be achieved by an anti-idiotype which does not mimic antigen, but interacts with this idiotope.

In the present study we have explored the use of different anti-idiotypes to induce biologically important responses. Our work is based on the results of Briles *et al.* (1984), who showed that the anti-PC antibody response in mice provides effective protection against infection with *Streptococcus pneumoniae*. Furthermore, the anti-PC antibodies which provide the best protection express the T15 idiotype (Briles *et al.*, 1982). We have recently been able to induce such protective immunity in mice using a monoclonal anti-idiotypic antibody, 4C11 (McNamara, Ward & Kohler, 1984b). Immunization with KLH conjugate of 4C11 induced a significant titre of anti-PC, T15 idiotype-positive antibodies. If these mice were challenged with lethal doses of bacteria, significant improvement of survival over non-immunized controls was observed. From these studies, it is evident that a high titre of T15-positive anti-PC antibodies is needed to achieve protection. Thus, the aim of protective immunization in this system is to stimulate $T15^+$ anti-PC antibodies.

Here, we have studied the temporal kinetics of the T15-positive anti-PC response in mice induced by carrier-conjugates of 4C11 and another anti-idiotype, F6, which does not fall into Ab2β category. Furthermore, we compared these responses with the response induced by PC-carrier antigen, and asked whether a combination of antigen and anti-idiotype stimulation increases the T15-positive anti-PC response. Based on our results, we propose to introduce the term Biological Response Mimicry in addition to the existing concept of Internal Image or Stereochemical Mimicry. The concept of Biological Mimicry thus becomes the decisive factor in the development of idiotype-based vaccines.

MATERIALS AND METHODS

Mice

BALB/c and A/St mice were obtained from West Seneca Laboratories, West Seneca, NY. CBA/N females were obtained from Old Dominion Laboratories, Dublin, VA. All mice used were between 6 and 16 weeks of age.

Antigens

Limulus polyphemus haemocyanin (Hy), keyhole limpet haemocyanin (KLH), human gamma globulin (HGG) and chicken ovalbumin (OVA) were purchased from Worthington Diagnostic Systems Inc., Freehold, NJ. *P*-aminophenyl phosphorylcholine was obtained from Sigma Chemical Co., St Louis, MO, and converted to diazophenyl phosphorylcholine according to Chesebro & Metzger (1972). PC-conjugated proteins (PO_5–BSA, PC_5–OVA, and PC_{18}–HGG) were prepared by the reaction of diazophenyl phosphorylcholine with carrier proteins according to the procedure of Lee, Cosenza & Kohler (1974). 4C11–Hy, F6–Hy and 4D9–KLH conjugates were prepared according to the method of Avrameas (Avrameas & Ternynck, 1971), using glutaraldehyde as a coupling reagent.

Reagents

Bovine serum albumin (BSA) was obtained from the United States Biochemical Co. PC, nitrophenylphosphorylcholine (NPPC), alkaline phosphatase, enzyme substrate (*p*-nitrophenylphosphate), diethanolamine, polyoxyethylenesorbitan (Tween 20), and glutaraldehyde were obtained from Sigma Chemical Company. Sephacryl S-200 and Protein A-Sepharose CL-4B were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were purchased from Difco Laboratories, Detroit, MI. Alkaline phosphatase-conjugated polyclonal goat antisera to mouse μ, $\gamma 3$, $\gamma 1$, $\gamma 2b$, $\gamma 2a$, and α heavy chains were purchased from Southern Biotechnology Associates Inc., Birmingham, AL.

Monoclonal antibodies (Abs)

The BALB/c anti-PC hybridomas, HPCM2 [μ, κ, T15 idiotope-positive (Id^+)] were gifts of Dr P. Gearhart, Carnegie Institution of Washington, Baltimore, MD. S107 (α, κ, T15-Id^+) myeloma was a gift of Dr M. D. Scharff, Albert Einstein College of Medicine, Bronx, NY. MAbs from these hybridomas were affinity purified by passage of ascites fluids over a PC–Sepharose column by the method of Cuatrecases (1970). The PC-binding hybridoma protein 167.4G5.5 ($\gamma 1$, k, T15-Id^+) was the gift of Dr J. L. Claflin, University of Michigan Medical School, Ann Arbor, MI. The anti-T15 idiotypic hybridomas F6 and 4C11 (both $\gamma 1$, κ) were generated in our laboratory as previously described (Wittner, Bach & Kohler, 1982). F6 and 4C11 Abs were affinity purified by passage of ascites fluids over a T15–Sepharose CL-4B column according to Cuatrecasas (1970). MAb 3A6 ($\gamma 2b$, κ) and Ab 11C1 ($\gamma 1$, κ), which bind to the murine mammary tumour L1210/GZL, and MAb 4D9 ($\gamma 1$, κ), which binds to idiotopic determinants on 11C1, were gifts of Dr S. Raychaudhuri in our laboratory. The BALB/c MPC11 ($\gamma 2b$, κ) myeloma protein was purified from ascites fluid by standard methods of ammonium sulphate precipitation followed by fractionation on a Protein A column.

Preparation of PC-specific, T15-Id^+ polyclonal antibody standard

Thirty BALB/c mice were immunized with 100 μg PC_{18}HGG in CFA i.p. Sera were collected on Day 30, and antibodies were purified by subsequent PC–BSA and F6 affinity chromatography. Sixty percent of the purified PC-specific T15-Id^+ polyclonal antibodies were found to be IgG1 according to both total IgG1 and PC-specific IgG1 ELISA, when HPCG12 ($\gamma 1$, κ, T15-Id^-) and 167·4G5.5 ($\gamma 1$, κ, T15-Id^+) Abs were used as standards. We used this affinity-purified polyclonal antibody as the PC-specific T15-Id^+ IgG1 standard for the direct binding assay.

Preparation of F(ab')$_2$ fragment of F6 Ab

F(ab')$_2$ fragments of the F6 Ab were prepared by pepsin digestion according to Lamoyi & Nisonoff (1983). After

digestion, various fractions were separated by passing through a Sephacryl S-200 column and further purified by passage through Protein A–Sepharose CL-4B columns, which absorbed out contaminated undigested F6 molecules. The purity and T15-binding activity of these $F(ab')_2$ fragments were tested by enzyme-linked immunosorbent assay (ELISA).

Immunizations

For the primary serum response, four to six mice per group were immunized with 100 μg PC–OVA, 4C11–Hy or F6–Hy emulsified in CFA i.p. Sera were collected on Day 10 and Day 30 after immunization. For the secondary response, mice were boosted 2 months later with 100 μg of these antigens emulsified in IFA. Sera were collected on Days 10 and 30 post-immunization.

ELISA

ELISAs were used to quantify PC-specific antibodies. Four different ELISAs (total anti-PC ELISA, T15-Id$^+$ ELISA, hapten inhibition ELISA, and idiotype inhibition ELISA) were set up to analyse the serum antibody response induced by the three vaccine antigens. Both IgM and IgG1 isotypes were measured for each sample.

A modified ELISA procedure of Engvall & Perlman (1972) was employed. Briefly, 0·1 ml antigen solution [PC–BSA for the anti-PC assay or F $(ab')_2$ fraction of F6 Ab for the T15 assay] diluted in phosphate-buffered saline (PBS), pH 7·6, with 0·02 NaN_3 was coated to each well of 96-well microtitre trays (Becton-Dickinson, Oxnard, CA) by incubating at 37° for 4 hr. After washing the plates eight times with PBS, 0·2 ml of 1% BSA–PBS was added to each well at room temperature for 30 min to block uncovered sites on the microtitre plate. After washing, duplicate samples of sera serially diluted in 1% BSA–PBS containing 0·05% Tween 20, in a volume of 0·1 ml per well, were added and incubated overnight at 4°. After washing the plates, 0·1 ml of the enzyme-labelled antibodies diluted at 1:1000 in 0·05% Tween 20–1% BSA–PBS was then added and incubated at room temperature for 3 hr. The plates were then washed and developed with 0·1 ml of phosphatase substrate, dissolved in diethanolamine buffer mixture (1 mg substrate/1 ml buffer). The absorbence at 405 nm was read on an Artek ELISA Reader (Artek System Corp., Farmingdale, NY).

For PC-specific antibody quantification, 0·1 ml of a 10-μg/ml solution of PC–BSA was coated to each well; alkaline phosphatase-conjugated goat anti-mouse μ and γ1 heavy chain-specific antisera were used to develop PC-specific IgM and IgG1, respectively.

For T15-Id$^+$ antibody quantification, 0·1 ml of a 5-μg/ml solution of a F1 $(ab')_2$ fragment of F6 Ab was coated to each well; alkaline phosphatase-conjugated goat anti-mouse μ and γ1 heavy chain antisera was added to develop the T15-Id$^+$ IgM and IgG1. HPCM2 Ab and the affinity-purified PC-binding T15-Id$^+$ polyclonal IgG1-containing antibody standards were used as standards to quantify serum PC-specific and T15-Id$^+$ IgM and IgG1 concentrations, respectively.

For hapten inhibition assays, 0·1 ml of a 2 μg/ml solution of PC–BSA was coated to each well. MAb of HPCM2 and affinity-purified PC-binding T15-Id$^+$ polyclonal IgG1-containing antibodies were used to set up standard curves (in the absence of hapten inhibitors) to quantify serum hapten-inhibitable IgM and IgG1 antibodies, respectively. Haptens (PC or NPPC) were added, prior to the addition of standard proteins or sera samples, to inhibit the binding of IgM and IgG1 antibodies. We found that 10^{-3} M and 10^{-4} M hapten (PC or NPPC) could inhibit more than 90% HPCM2 IgM and polyclonal PC-specific IgG1-containing antibody standard, respectively. Therefore, these inhibitor concentrations were chosen to determine the fine antigen specificity (PC or NPPC) of the serum antibody response to the three antigens. The antibody concentration (μg/ml) of the immune serum was measured in the presence and absence of hapten inhibitors and the percentage inhibition was calculated according to the following formula:

$$\frac{(1 - \text{Ab concentration with inhibitor})}{\text{Ab concentration without inhibitor}} \times 100.$$

For the T15-Id$^+$ IgG1 inhibition assays, 0·1 ml of a 1-μg/ml solution of $F(ab')_2$ fragment of F6 was coated to a 96-well plate, and serial dilutions of T15 (α, κ) (6·25–400 ng in a volume of 50 μl) were added to inhibit diluted immune sera (which contained about 10 ng per well PC-binding T15-Id$^+$ IgG1 antibodies) binding to the F6 $(ab')_2$-coated plates. F6 $F(ab')_2$ MPC11 (γ2b, κ) were used as controls in the T15-Id inhibition assay. Alkaline phosphatase-conjugated goat anti-mouse γ1 heavy chain antiserum was added to develop the F6 $(ab')_2$-binding IgG1.

These assays were specific in distinguishing various isotypes, with less than 1% cross-reactivity between the anti-μ and anti-γ1 antisera. The sensitivities were greater than 0·5 ng and 0·1 ng specific antibodies per well for IgM and IgG1, respectively, in the direct binding assay.

RESULTS

Primary antibody response of BALB/c mice to PC–OVA and anti-idiotypes conjugated to Hy

For the purpose of obtaining protective immunity against infection with *S. pneumoniae*, antibodies which are PC-specific and express the T15 idiotype are desired (Briles *et al.*, 1982). Thus, we compared the antibody responses induced in three groups of BALB/c mice immunized with either PC-OVA, 4C11–Hy, or F6–Hy. 4C11 and F6 are anti-idiotypic hybridomas of the IgG1 class, as described previously (Wittner *et al.*, 1982). While the binding of 4C11 to T15 can be completely inhibited by free PC, F6 binding is not inhibited by PC, and only partially inhibited by PC carrier. Thus, 4C11 can be classified as an Ab2β or Ab2γ, while F6 is Ab2α.

Normal BALB/c mice were immunized with PC–OVA, 4C11–Hy, or F6-Hy in CFA i.p., and bled on Days 10 and 30 after immunization. The sera were analysed for antibodies binding to PC–BSA-coated plates, and to the $F(ab')_2$ fragment of F6. The data were calculated to show the amount of total anti-PC–BSA-binding antibodies (anti-PC), and the amount of T15-positive antibodies (T15$^+$). IgM and IgG1 classes of antibodies were assayed separately. In order to determine the hapten specificity of the antibodies, inhibition assays were performed. Antibodies binding to PC–BSA which were inhibited by both PC (PC$^+$) and NPPC (NPPC$^+$) are PC-specific. Antibodies specific for the diazophenyl derivative of PC are inhibited only by NPPC, not by PC.

As shown in Table 1, all three antigens could stimulate antibodies which are PC specific and T15 positive both early and late in the response. Thus, even though F6 is not binding-site specific, this anti-idiotope antibody, coupled to a carrier, can

Table 1. Primary response of BALB/c mice*

Specificity‡	Day 10 IgM(μg/ml)†	Day 10 IgG1(μg/ml)	Day 30 IgM(μg/ml)	Day 30 IgG1(μg/ml)
BALB/c primary response to PC–OVA				
Anti-PC	140±10§	108±11	93±19	676±195
T15$^+$	136±1	93±8	73±5	318±16
PC$^+$	119±7	75±13	64±12	155±81
NPPC$^+$	91±21	85±10	69±12	433±88
T15$^+$PC$^-$	17±3	18±7	9±6	163±37
BALB/c primary response to 4C11–Hy				
Anti-PC	66±10	211±15	13±2	112±16
T15$^+$	63±3	213±8	12±1	101±11
PC$^+$	48±4	200±9	10±1	105±4
NPPC$^+$	52±5	200±9	10±1	104±4
T15$^+$PC$^-$	15±2	13±5	2±0	0±0
BALB/c primary response to F6–Hy				
Anti-PC	39±7	183±28	23±7	157±27
T15$^+$	38±1	278±54	23±1	436±93
PC$^+$	34±3	172±5	20±0	152±3
NPPC$^+$	31±4	170±4	16±2	146±3
T15$^+$PC$^-$	0±0	106±24	0±0	284±41

*Mice were immunized once with 100 μg of either PC–OVA, 4C11–Hy or F6–Hy, and bled 10 and 30 days after immunization.

†Antibody concentrations were quantified by ELISA as described in the Materials and Methods.

‡PC-BSA was coated to the plates in order to measure PC-binding antibodies. F6 F(ab′)$_2$ was coated to the plate to measure T15-Id$^+$. PC or NPPC antibodies were measured using an inhibition ELISA.

§Numbers represent the mean±SEM of assays from four to six individual mice.

Table 2. Primary response of A/St*

Specificity‡	Day 10 IgM(μg/ml)†	Day 10 IgG1(μg/ml)	Day 30 IgM(μg/ml)	Day 30 IgG1(μg/ml)
A/St primary response to PC–OVA				
Anti-PC	139±35§	55±5	25±8	822±116
T15$^+$	104±14	30±5	20±1	8±3
PC$^+$	126±42	9±7	21±0	0±0
NPPC$^+$	121±6	36±8	21±1	82±49
T15$^+$PC$^-$	0±0	21±4	0±0	8±2
A/St primary response to 4C11–Hy				
Anti-PC	79±16	146±61	12±2	35±2
T15$^+$	53±4	93±8	7±2	19±4
PC$^+$	57±8	139±5	9±0	33±0
NPPC$^+$	62±8	139±5	9±1	33±1
T15$^+$PC$^-$	0±0	0±4	0±0	0±0
A/St primary response to F6–Hy				
Anti-PC	35±6	92±14	30±13	113±37
T15$^+$	33±2	111±23	29±2	420±114
PC$^+$	32±2	87±3	22±1	105±5
NPPC$^+$	30±4	76±4	22±1	105±5
T15$^+$PC$^-$	1±1	24±10	7±1	315±51

*Mice were immunized once with 100 μg of either PC–OVA, 4C11–Hy or F6–Hy, and bled 10 and 30 days after immunization.

†Antibody concentrations were quantified by ELISA as described in the Materials and Methods.

‡PC-BSA was coated to the plates in order to measure PC-binding antibodies. F6 F(ab′)$_2$ was coated to the plate to measure T15-Id$^+$. PC or NPPC antibodies were measured using an inhibition ELISA.

§Numbers represent the mean±SEM of assays from four to six individual mice.

stimulate T15-specific antibodies. This result supports the contention that B-cell activation by idiotope-carrier antigens is not dependent on a unique paratope-related idiotope.

There were, however, quantitative differences in the antibody isotypes associated with these antibodies. PC–OVA stimulated more T15$^+$, PC-specified IgM than IgG1 antibodies; on the contrary, anti-idiotope antigens stimulated more T15$^+$, PC-specific IgG1 than IgM antibodies.

Significant differences were seen in the titres of T15-positive, non-PC binding antibodies, which are high in the PC–OVA and F6–Hy groups and absent in the 4C11–Hy group. Furthermore, antibodies binding to PC–BSA which are not inhibited by either PC or NPPC were present in the PC–OVA group, but absent in the two other groups.

One problem associated with our interpretation of these results stems from using the same monoclonal anti-idiotype (F6) both as an immunogen and as the antibody used to measure T15 idiotype antibodies. The exact quantification of T15$^+$ idiotype antibodies is difficult, due to the simultaneous presence of both T15$^+$ (Abl-like) antibodies together with anti-F6 idiotype (Ab3) antibodies. This problem is addressed further below.

These results can be summarized by stating (i) all three antigens induce T15$^+$ PC$^+$ antibodies; (ii) the two idiotope antigens stimulate antibodies having a different isotype make-up than those stimulated by PC–OVA; and (iii) only 4C11–Hy does not induce a T15$^+$ PC$^-$ response (Ab3β type of antibodies).

Primary antibody response of A/St mice to PC–OVA and anti-idiotypes conjugated to Hy

Next, we wanted to analyse the primary responses to the same three antigens in A strain mice, because these mice, unlike BALB/c mice, are not T15 dominant. In these experiments, the anti-idiotope induced responses are syngeneic, since 4C11 and F6 were raised in A strain mice.

The data shown in Table 2 again indicate that all three antigens induced significant titres of T15$^+$PC$^+$ antibodies, but only early in the response. Only F6-Hy induced significant titres of T15$^+$ PC$^+$ antibodies late in the response. In addition, antibodies which are T15$^+$ PC$^-$ dominated the late IgG response to F6–Hy. When these data are compared to the results obtained in BALB/c mice, significant differences are noted. The T15$^+$ PC$^+$ titres induced by PC–OVA and 4C11–Hy are lower in A/St mice, as compared to BALB/c, while the titres induced by F6–Hy are similar in both mouse strains. Based on the T15$^+$ PC$^+$ titres, F6-Hy would therefore be the best immunogen of the three to stimulate protective immunity against *S. pneumoniae* infection.

Primary response of CBA/N mice to PC–OVA, 4C11–Hy and F6–Hy

Mice with the Xid defect (Scher, 1982; Amsbaugh *et al.*, 1972) cannot produce anti-PC T15$^+$ antibodies when immunized with

Table 3. Primary response of CBA/N mice*

	Day 10		Day 30	
Specificity‡	IgM(μg/ml)†	IgG1(μg/ml)	IgM(μg/ml)	IgG1(μg/ml)
Primary response of CBA/N to PC–OVA				
Anti-PC	<10§	0	270±52	<10
T15$^+$	0	0	0	0
Primary response of CBA/N to 4C11–Hy				
Anti-PC	<10	<5	<10	<5
T15$^+$	0	0	0	0
Primary response of CBA/N to F6–Hy				
Anti-PC	<10	<5	<10	<5
T15$^+$	25	320	20	600

*CBA/N mice were immunized once with 100 μg of either PC–OVA, 4C11–Hy or F6–Hy and bled 10 and 30 days after immunization.

†Antibody concentrations were quantified by ELISA as described in the Materials and Methods.

‡PC-BSA was coated to the plates in order to measure PC-binding antibodies. F6 F(ab')$_2$ was coated to the plate to measure T15-Id$^+$ antibodies.

§Numbers represent the mean±SEM of assays from four to six individual mice.

a variety of different PC antigens (Kenny *et al.*, 1981; Kohler, Smyk & Fung, 1981). Thus, these mice are excellent candidates for testing alternative immunization protocols using anti-idiotypes for inducing protective antibodies. Therefore, CBA/N mice were immunized with either PC–OVA, 4C11–Hy, or F6-Hy. The sera were bled on Day 10 or Day 30 after the last immunization and analysed in a similar manner as the BALB/c and A/St sera.

The results in Table 3 show that none of these antigens could induce anti-PC antibodies which are T15 positive. However, immunization with F6–Hy does induce significant titres of T15-positive antibodies which do not bind to PC. This is an interesting observation because it indicates that CBA/N mice have the capacity to induce T15$^+$ PC$^-$ antibodies, which may function in the immune network as so-called regulatory idiotypes (Victor-Kobrin *et al.*, 1985; Paul & Bona, 1982).

Secondary antibody responses of BALB/c mice to nominal and anti-idiotope antigens

We next compared the effectiveness of PC–OVA, 4C11–Hy and F6–Hy antigens in inducing secondary responses in BALB/c mice. Groups of BALB/c mice were primed with either PC–OVA, 4C11–Hy or F6–Hy. Each group of mice was boosted with 100 μg of either the same antigen or a different antigen in IFA 2 months after the first injection. Sera were collected and analysed on Days 10 and 30.

As shown in Table 4, the immunization protocols which produced the highest titres of T15$^+$ anti-PC antibodies were the ones which involved the stimulation of cells which had been primed with F6–Hy. In contrast, the protocols which involved the stimulation of PC-OVA-primed cells produced very low titres of T15$^+$ anti-PC antibodies. The protocols involving priming with 4C11–Hy produced higher titres of T15$^+$ anti-PC antibodies than the PC–OVA-priming protocol, but not as high as those produced by the F6–Hy-priming protocols. Based on these data, we conclude that the most protective immunity to *S. pneumoniae* may be stimulated by protocols involving priming with anti-idiotope antigen, followed by immunization with either nominal or idiotope antigen.

Characterization of the T15$^+$ PC$^-$ response

When anti-idiotypic antibody is used for immunization, two types of responses can be expected. The first type is due to stimulation of antibodies complementary to the paratope of the immunizing Ab2. In our experimental system, the paratope of Ab2 is a determinant of T15, i.e. the Ab2 is anti-T15. Thus, the resulting Ab3 repertoire will express T15-associated idiotopes, and some of these will bind PC. The second type of response possibly induced by immunization with Ab2 can be directed to any of the idiotopes which are expressed on Ab2. This kind of Ab3 is simply an anti-idiotypic response of the alpha type. To distinguish these two types of Ab3 responses, it is helpful to extend the alpha and beta nomenclature to the Ab3 level. Accordingly, Ab3α is the anti-idiotypic response to Ab2 immunization, producing antibodies unrelated to any determinant on Ab1; the Ab3β response, however, consists of antibodies which recognize determinants on the paratope of Ab2. Thus, the Ab3β antibodies express idiotopes present on the Ab1 (the T15 idiotype in our system).

To distinguish experimentally between the Ab3α and β antibodies, induced by Ab2 immunization, we used an inhibition assay whereby the binding of Ab3 sera to F6 is inhibited by different concentrations of T15. If complete inhibition is observed, this indicates the presence of Ab3 antibodies of the β type, i.e. induced by the paratope of Ab2. If there is partial or no inhibition by T15, this indicates the presence of antibodies directed against F6 idiotopes which are unrelated to T15 idiotopes, i.e. they are Ab3 of the α type. Although it is possible that AB3s exist which are T15 negative but still recognize the F6 paratope, previous results have shown that the F6-defined T15 idiotope is always associated with the expression of the canononical T15 idiotype, defined by polyclonal anti-idiotype sera.

The results from the antibody response induced in BALB/c by F6-Hy are summarized in Table 5. Ab3 sera from F6–Hy-immunized BALB/c contained T15-inhibitable antibodies only in the early primary response. This shows, therefore, that immunization with F6-Hy induces a mixture of Ab3α and Ab3β antibodies early in the response, and mostly Ab3α type antibodies late in the response.

The T15 inhibition assay was also used on Ab3 sera from A/St and CBA/N mice. The early response to F6–Hy in A/St mice contained antibodies which can be completely inhibited by T15, indicating β type Ab3 antibodies. However, in the late response, only partial inhibition was observed, demonstrating a mixture of α and β Ab3 antibodies. The inhibition experiments with Ab3 sera from CBA/N mice show that the Ab3 in CBA/N induced by F6–Hy are completely inhibited by T15, demonstrating the presence of β type Ab3.

These results provide evidence that, while both Ab3α and Ab3β antibodies can be stimulated by F6-Hy, Ab3β antibodies are usually stimulated earlier in the course of the response.

Table 4. Secondary response of BALB/c mice

Primed with	Challenged with*	Day 10 Anti-PC†	Day 10 $T15^+$	Day 10 $T15^+PC^-$	Day 10 $T15^+PC^+$	Day 30 Anti-PC	Day 30 $T15^+$	Day 30 $T15^+PC^-$	Day 30 $T15^+PC^+$
PC–OVA	PC–OVA	456±184‡	5±4	0±0	23±18	1520±533	2±1	0±0	30±15
	4C11–Hy	47±10	54±12	0±0	25±6	38±6	82±14	0±0	31±5
	F6–Hy	62±14	47±6	0±0	29±4	38±7	118±10	7±4	38±4
4C11–Hy	4C11–Hy	11±4	128±20	4±2	11±2	16±3	139±39	4±3	16±3
	PC–OVA	163±27	101±6	2±10	163±10	201±20	85±8	0±0	171±16
F6–Hy	F6–Hy	58±22	763±235	385±61	58±61	61±21	643±200	331±54	61±54
	PC–OVA	205±29	171±28	146±29	205±29	292±34	94±28	0±0	274±81

*Mice were boosted with 100 μg of antigen in IFA 2 months after first immunization. Sera were collected at Day 10 or Day 30 post-secondary boost.

†Antibody concentrations were quantified by ELISA.

‡Numbers represent the mean μg/ml±SEM of assays from four to six individual mice.

Table 5. Inhibition by T15 of F6-induced Ab3 binding

Mice*	Day 10 Inhibition†	Day 10 I^{50}(ng)	Day 30 Inhibition	Day 30 I^{50}(ng)‡
BALB/c	Partial	223	None	ND
A/St	Complete	52	None	ND
CBA/N	Complete	61	Complete	124

*Mice were immunized once with 100 μg of F6-Hy and bled 10 and 30 days after immunization.

†Different amounts of T15 protein were used to inhibit the binding of Ab3 containing sera to F6-coated plates.

‡I^{50} is defined as the amount of inhibitor needed to achieve 50% inhibition.

ND, not determined.

DISCUSSION

In this study, we tested the effectiveness of two different anti-idiotypic antibodies in inducing antibody responses important in protection against infection with *S. pneumoniae*. As we have demonstrated previously (McNamara *et al.*, 1984b), protective anti-PC antibodies can be induced by an anti-idiotypic vaccine. Our results supported earlier studies showing that the most effective protective antibody is an anti-PC antibody expressing the T15 idiotype (Briles *et al.*, 1984, 1982).

Both monoclonal anti-idiotypic antibodies used in this study have the same isotype, but have different idiotypic fine specificities (Wittner *et al.*, 1982) and different primary structures (unpublished data). According to hapten inhibition experiments (Wittner *et al.*, 1982), one anti-idiotype, 4C11, falls into the class of either Ab2β or Ab2γ, and the other, which is not inhibited by PC, is an Ab2α type.

While the distinction of α, β or γ anti-idiotypic antibodies may be an important concept of the idiotype network, an important practical issue is the efficacy of protection such anti-idiotype vaccines can eventually achieve. The protection against an experimental streptococcal infection is a model to rationally design idiotype vaccines.

Using F6–Hy and 4C11-Hy as idiotype vaccines in different strains of mice, and comparing the antibody responses to the response induced by immunization with PC-OVA, we detected important differences at the level of hapten specificities and idiotype expression.

The dynamic nature of the response observed in the time period between Day 10 and Day 30 after immunization is evident with all three antigens. Early in the response to the three antigens, antibody which is PC specific and expressing the T15 idiotope dominates. The antibodies induced by PC–OVA shifted by Day 30 post-immunization, becoming mostly non-PC specific and T15 negative. This type of antibody response has been analysed previously by Chang, Brown & Rittenberg (1982a, b) and termed Group II anti-PC antibodies. The hallmark of Group II antibodies is that they are inhibited not by PC, but by the extended hapten NPPC. A third kind of PC antibody induced by PC-carrier antigens which has been observed is not inhibited by either PC or NPPC; these have been termed Group III antibodies. We observed Group III antibodies in the late primary and secondary response to PC–OVA in BALB/c and A/St mice. Group II and Group III antibodies do not bind PC and do not provide protection against a *S. pneumoniae* infection. Thus, using the anti-PC response as a model, PC-carrier antigens may not represent the best immunogen to use for a vaccine.

The antibody response induced by 4C11–Hy in BALB/c mice is more stable throughout the observed response time. Although the early IgG $T15^+$ PC^+ antibody titre is higher than in the PC–OVA group, there is a faster decline. There is no $T15^+$ PC^- antibody detected. This contrasts to the F6–Hy-induced antibody response, which contains, in the early and late primary response, substantial amounts of $T15^+$ PC^- antibodies. The titre of protective $T15^+$ PC^+ antibodies is roughly identical in both anti-idiotype-induced responses.

In A/St mice, the response pattern is different. PC–OVA is very ineffective in inducing protective $T15^+$ PC^+ titres in the IgG1 class. The best IgG $T15^+$ PC^+ antibody titres is induced by immunization with F6–Hy. However, F6–Hy also induced the

highest T15$^+$ PC$^-$ titres. 4C11–Hy induced more IgG T15$^+$ PC$^+$ antibodies than PC–OVA, but less than F6–Hy.

The results in the Xid CBA/N mice are surprising. Xid mice are known not to produce anti-PC antibodies which express the T15 idiotype (Kenny *et al.*, 1981; Kohler *et al.*, 1981). Although the presence of T15 expressing precursors induced by LPS has been reported (Primi *et al.*, 1986), no data are available to show induction of T15-positive antibodies by immunization *in vivo*. Our finding of large amounts of T15-positive antibodies, which do not bind PC, in CBA/N mice after immunization with F6–Hy demonstrates that T15-positive antibodies may play an important role in the immune repertoire of Xid mice. It would be interesting to analyse further the 4C11–Hy antisera for antibodies binding to 4C11. Our results show that 4C11–Hy immunization of CBA/N mice produced no PC-binding or F6-binding antibodies. It is possible that, as in the case of F6 immunization, there may be 4C11-binding non-PC binding-antibodies present.

Secondary response protocols were tested with combinations of priming and challenge using PC–OVA, 4C11–Hy and F6–Hy. The results shown in Table 3 clearly indicate that priming with F6–Hy followed by challenge with PC–OVA induces the highest titres of T15$^+$ PC$^+$ antibodies in the Day 10 and Day 30 responses. Priming with 4C11–Hy is somewhat less effective, while in PC–OVA-primed mice, the titre of T15$^+$ PC$^+$ antibodies to all challenges is very low. With respect to vaccine design, the use of a combination of anti-idiotype and nominal antigen would be expected to be more effective than the use of the bacterial carbohydrate antigen, since this T-independent antigen induces mainly IgM and IgG3 antibodies, and is ineffective in inducing a memory response (Perlmutter *et al.*, 1978).

Although the use of anti-idiotypes as vaccines has been demonstrated for viral, bacterial, and parasite pathogens, very little is known about the duration of protective immunity and possible shifts in the response during the course of the response. In addition, a problem in using hapten-carrier antigens could arise from carrier-induced hapten-specific inhibition observed with some haptens *in vivo* (Herzenberg & Tokuhisa, 1982). This would limit the use of hapten-carriers in vaccine development. Our data reported on the response to different anti-idiotypes by themselves and in combination with hapten-carrier antigens is the basis for ongoing *in vivo* protection experiments using nominal and idiotype vaccination combination protocols to extend our earlier observation on anti-idiotype induced protection (McNamara *et al.*, 1984b).

The final point which arises from this study addresses the role of regulatory idiotypes in vaccine development. Bona and colleagues (Victor-Kobrin *et al.*, 1985; Paul & Bona, 1982) have described the existence of a special class of idiotopes which are cross-reactive and associated with different antigen specificities. In the T15 system, we have evidence that a T-helper cell population recognizes a cross-reactive idiotope on T15 (McNamara, Gleason & Kohler, 1984a). Such T15-recognizing helper cells might be involved in the dominance of T15 in the anti-PC response (McNamara, Kang & Kohler, 1985). The finding here of high T15$^+$ PC$^-$ antibody titres induced by anti-idiotype stimulation relates to the role of T15 as a regulatory idiotope in the anti-PC response. If this model is correct, the induction of T15 might be important for stimulating idiotype-specific T-helper cells. Such T helpers could become involved in the maintenance of long-term B-cell memory. We are presently addressing the potential role of a regulatory T15 idiotype response induced by idiotype vaccines in B-cell memory.

The concept of antigenic mimicry in the development of idiotype vaccines has stimulated much interest recently. While the mimicry concept has been conceived as strictly stereochemical molecular mimicry (Kohler *et al.*, 1985; Kieber-Emmons & Kohler, 1986), more recent experience with anti-idiotypes as antigen substitutes, as shown in this report, clearly indicate another level of antigenic mimicry which is not based on three-dimensional stimularities between idiotopes and epitopes. Our data shown here, and recent data by Schick, Dreeseman & Kennedy (1987) demonstrate that anti-idiotypes which do not meet the criteria of internal images are effective in inducing specific antibodies. Thus, we propose the concept of biological mimicry to account for the response induced by non-internal image anti-idiotypes. Such anti-idiotypes are typically not or only partially inhibited by hapten in their binding to idiotype, and thus would not qualify as Ab2β. This idiotope may be a regulatory idiotope shared by other antibodies of different specificities but linked in a biological context. Further dissection of the idiotype connections is needed to define the concept of biological mimicry more precisely; however, even at the present state of knowledge, the concept of a biologically relevant topographical linkage of idiotopes and paratopes is very attractive and should be taken into the consideration designing idiotype vaccines.

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