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Structural and Stereochemical Specificity of Mouse Monoclonal Antibodies to the Organophosphorus Cholinesterase Inhibitor Soman

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SUMMARY

To test the usefulness of immunotherapy in organophosphate poisoning, two mouse monoclonal antibodies were prepared to the chemical warfare agent soman. The antibodies bound reversibly to soman and afforded considerable protection to acetylcholinesterase *in vitro*. However, they were only marginally effective in preventing the consequences of soman poisoning in mice (these data have been published elsewhere). Since potential for immunotherapeutic usefulness resides in antibody affinity and specificity, we conducted experiments to define these parameters to enable us to maximize them in the production of later antibodies. Interaction of the antibodies (CC1 and BE2) in affinity-purified form with a series of soman analogs in a competitive inhibition enzyme immunoassay was used to assess the contribution to binding affinity of each functional group on the soman molecule. Neither antibody interacted with the —P═S analog of soman or methylphosphonic acid. A decrease in the number of methyl groups on the pinacolyl side chain reduced or eliminated binding with both antibodies while increasing the size of this group had a mixed result. The major metabolite of soman, its basic hydrolysis product, interacted weakly with BE2 and failed to interact with CC1. Alkyl ester group substitution at the fluorine position increased antibody binding up to the symmetrical dipinacolyl analog. Stereochemical specificity was determined by measuring the apparent decrease in the rate of inhibition of cholinesterases (acetylcholine acetylhydrolase, EC 3.1.1.7, or acylcholine acylhydrolase, EC 3.1.1.8) by pure soman stereoisomers in the presence of increasing concentrations of each antibody. CC1 demonstrated specificity that varied as C(+)P(+) < C(−)P(+) < C(−)P(−) < C(+)P(−). Although affinities were much lower, BE2 also showed a preference for the more toxic P(−) isomers.

INTRODUCTION

Methylphosphonofluoridic acid, 1,2,2-trimethylpropyl ester (soman, I in Table 1), is a potent acetylcholinesterase inhibitor with potential for use in chemical warfare (1, 2). Prior research has shown that immunologic means can be used to alter the pharmacologic and toxic effects of chemicals (3). We recently reported the preparation of mouse subclass IgG1 monoclonal antibodies that bind soman (4), the ability of these antibodies to compete with acetylcholinesterase for soman *in vitro*, and their modest ability to protect animals against the effects of soman when they were administered in a passive immunization (5).

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Theoretically, this lack of *in vivo* effectiveness arises out of the inability of a reversible reaction, the soman-antibody interaction, to compete with an irreversible reaction, the phosphonylation of acetylcholinesterase. Presumably, the structural elements on a small organic molecule such as soman offer antibody interactions too infrequent or too weak to enable an antibody to reduce the concentration of this exceedingly neurotoxic substance at its site of action. Yet, there are numerous reports of polyclonal sera to small molecules with affinities in the 10^7–10^{11} liters/mol range (6–9). This was an indication to us that an understanding of the chemical nature and frequency of the sites on soman contributing to the strength of the antibody-soman interaction may be helpful in the design of conjugates for the production of antibody species with higher affinities.

In the present work, we have examined both structural and stereochemical aspects of the specificity of our two

TABLE 1

Fine specificity of antisoman monoclonal antibodies

Data were generated by CIEIA using six inhibitor concentrations in log increments from 10^{-3} to 10^{-8} M, and an antibody concentration of 2 μg/ml. Four replicates were run at each concentration of each analog. An uninhibited control, also in quadruplicate, was included with each inhibitor and served as an indicator of maximum possible optical density for the series.

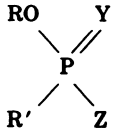
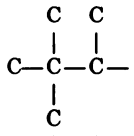
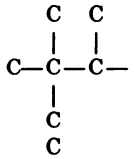
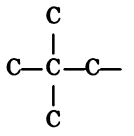
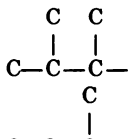
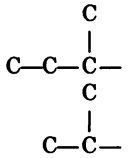
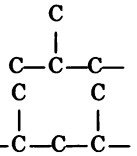
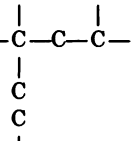
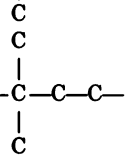
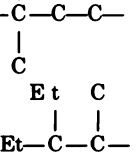
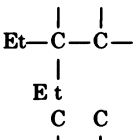
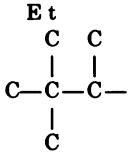
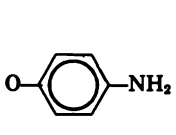
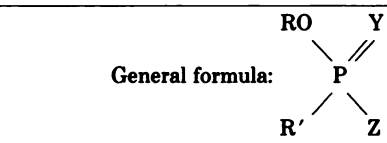
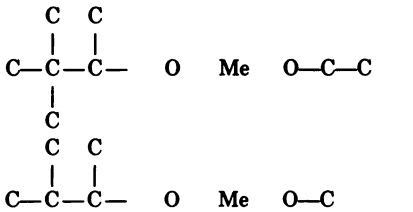
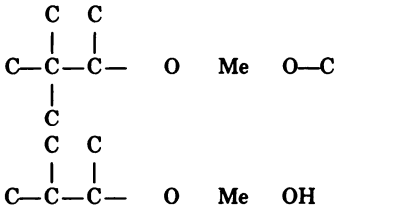
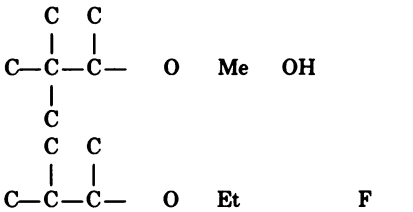
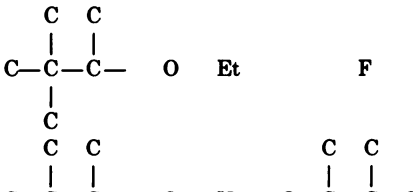
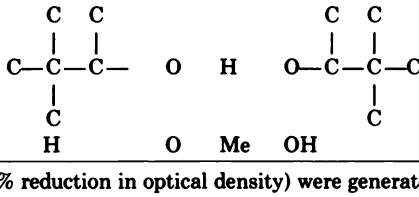
	Compound name	General formula: 				IC_{50}[a]		IC_{50} Ratio with soman[b]	
		R	*Y*	*R′*	*Z*	Be2	CC1	BE2	CC1
						μM			
I	Methylphosphonofluoridic acid, 1,2,2-trimethylpropyl ester (soman)		O	Me	F	84.0	6.5	1.0	1.0
II	Methylphosphonofluoridothioic acid, 1,2,2-trimethylpropyl ester		S	Me	F	NI[c]	NI		
III	Methylphosphonofluoridic acid, 2,2-dimethylpropyl ester		O	Me	F	790.0	780.0	0.1	<0.1
IV	Methylphosphonofluoridic acid, 1,2-dimethylpropyl ester		O	Me	F	>1000.0	NI		
V	Methylphosphonofluoridic acid, 1-methylpropyl ester		O	Me	F	NI	NI		
VI	Methylphosphonofluoridic acid, 1-methylethyl ester (sarin)		O	Me	F	NI	NI		
VII	Methylphosphonofluoridic acid, 2-methylpropyl ester		O	Me	F	NI	NI		
VIII	Methylphosphonofluoridic acid, 1,3,3-trimethylbutyl ester		O	Me	F	43.0	70.0	2.0	0.1
IX	Methylphosphonofluoridic acid, 3,3-dimethylbutyl ester		O	Me	F	600.0	420.0	0.1	<0.1
X	Methylphosphonofluoridic acid, 1-methyl-2,2-diethylbutyl ester		O	Me	F	60.0	40.0	1.4	0.2
XI	Methylphosphonic acid, *p*-amino(−) phenyl-1,2,2-trimethylpropyl diester		O	Me		4.9	4.5	17.1	1.4
XII	Methylphosphonic acid, bis-1,2,2-trimethylpropyl ester		O	Me		3.8	1.8	22.1	3.6

TABLE 1—Continued

	Compound name	General formula: 				IC_{50}[a]		IC_{50} Ratio with soman[b]	
		R	*Y*	*R′*	*Z*	BE2	CC1	BE2	CC1
						μM			
XIII	Methylphosphonic acid, propyl 1,2,2-trimethylpropyl diester		O	Me	O—C—C—C	6.2	4.4	13.6	1.5
XIV	Methylphosphonic acid, ethyl 1,2,2-trimethylpropyl diester		O	Me	O—C—C	5.6	6.2	15.0	1.1
XV	Methylphosphonic acid, methyl-1,2,2-trimethylpropyl diester		O	Me	O—C	5.5	7.8	15.3	0.8
XVI	Methylphosphonic acid, 1,2,2-trimethylpropyl ester		O	Me	OH	720.0	NI	0.1	
XVII	Ethylphosphonofluoridic acid, 1,2,2-trimethylpropyl ester		O	Et	F	18.0	3.5	4.7	1.9
XVIII	Phosphonic acid, bis-1,2,2-trimethylpropyl ester		O	H		130.0	100.0	0.7	0.1
XIX	Methylphosphonic acid	H	O	Me	OH	NI	NI		

[a] IC_{50} values (molar inhibitor concentrations causing 50% reduction in optical density) were generated by converting average optical densities to per cent reduction of maximum optical density (y) and converting this value to a logit according to the expression logit = ln $y/(100 - y)$. Logits were plotted against molar inhibitor concentrations on semilog paper and the IC_{50} values were determined graphically by dropping a vertical line to the x axis from the point where the curve crossed logit = 0. With the exception of the stereoisomers of soman, the actual antibody affinities for these compounds remain undetermined. Therefore, these values can only be compared with values generated in the same system, preferably at the same time.

[b] This ratio generates a value indicative of the relative affinity of the antisoman antibody for each analog tested: better inhibitor than soman > 1.00 (soman) > poorer inhibitor than soman.

[c] NI, no interaction at the highest inhibitor concentration tested (1.0×10^{-3} M), as indicated by no reduction in absorbance.

antibodies. Using analogs that deviate systematically in structure from soman, we determined specificity by testing each analog's relative inhibitory power in a CIEIA.[1] Access to unique preparations of the stereoisomers of soman in complete resolution made it possible to establish antibody specificity at the configurational level (10). The presence of two centers of optical activity in the soman molecule gives rise to four distinct spacial arrangements (10, 11). Since the acute toxicity of soman resides primarily in the epimeric pair with the (−) configuration at phosphorus (10), it was of particular importance to determine whether one or both of these antibodies interacted preferentially with the more more potent stereoisomers.

[1] The abbreviations used are: CIEIA, competitive inhibition enzyme immunoassay; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; EIA, enzyme immunoassay; GLC, gas-liquid chromatography.

MATERIALS AND METHODS

Preparation of immunogenic soman-protein conjugates. The low molecular weight of soman (I) required that it be convalently bound to protein to render it immunogenic. This was accomplished by diazotization and reaction with hemocyanin from giant keyhole limpets (Schwarz-Mann, Orangeburg, NY) or crystallized bovine serum albumin (12) (Miles Laboratories, Kankakee, IL) using the *p*-aminophenoxy analog of soman supplied by the U. S. Army Medical Research Institute of Chemical Defense (USAMRICD), Aberdeen Proving Ground, Aberdeen, MD. Epitope densities (moles of soman analog/mole of protein), determined by the method of Fenton and Singer (13), were found to be 700 and 15 for KLH and BSA, respectively.

Preparation of monoclonal antibodies. Monoclonal antibodies were prepared as described by Hunter *et al.* (4). Briefly, this involved

immunization of adult female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) with 100 μg of soman-BSA or soman-KLH administered subcutaneously in Freund's complete adjuvant. The initial immunization was followed at 2-week intervals by intraperitoneal injections of conjugate in saline at 50, 10, and 1 μg/mouse. Cell fusion was done on day 4 after the 1-μg booster using 10^8 spleen cells and 10^7 cells of the nonsecreting mouse plasmacytoma line P3-X63-Ag8.653 (14). Hybridomas were selected in hypoxanthine-aminopterin-thymidine medium, and at 14 days the cultures were tested by EIA for the presence of antibody that bound to a heterologous soman-protein conjugate (15). The immunizing conjugate was avoided at this stage to prevent the detection of antibodies specific for the carrier protein.

Cultures positive for antibodies against soman conjugates were further tested by CIEIA to establish specificity for the soman hapten using either soman or a nontoxic soman analog (XII) as inhibitor. Cultures positive by both EIA and CIEIA were cloned by limiting dilution (0.5–1 cell/well) on irradiated mouse tumor macrophage (P388D1, Tumor Immunology Bank, American Type Culture Collection, Rockville, MD) feeder layers. The clones chosen for additional study (BSA-GD-BE2-IA10 and KLH-GD-CC1-IIA4, referred to subsequently as BE2 and CC1) were found to belong to the IgG1 subclass by Ouchterlony double diffusion against mouse immunoglobulin class and subclass specific reagents (Miles Laboratories, Elkhart, IN). The cloned hybridomas were cryopreserved in 15% dimethyl sulfoxide, 85% RPMI 1640 medium (Flow Laboratories, Vienna, VA) containing 20% fetal bovine serum in a rate-controlled freezer (Cryo-Med, Mt. Clemens, MI). Ascites cultures (16) were established and antibodies were harvested from the ascites fluid by 50% ammonium sulfate precipitation and affinity column chromatography on soman-protein conjugate bound to cyanogen bromide-activated Sepharose. Pharmacia Fine Chemicals (Piscataway, NJ) was the source for the matrix and the procedure. Antibody was eluted with 0.02 M glycine buffer, pH 2. The acidity was rapidly adjusted to pH 7–8 with base and dialysis was begun immediately.

CIEIA for molecular specificity. Affinity-purified antibodies and soman analogs were employed in a CIEIA as outlined in Ref. 4. The relative affinity of the antibodies for each analog was expressed as the molar concentration of analog that inhibited 50% of the binding of antisoman antibody to immobilized soman-protein conjugate (IC_{50}). Dry isopropyl alcohol (final concentration, 5%) was used to solubilize the more hydrophobic analogs, and was incorporated into all samples tested to allow compound to compound comparisons. Preliminary work (results not included) showed that the CIEIA could be performed in 10% isopropyl alcohol. Antibody plus inhibitor diluant and antibody plus buffer containing no alcohol were run as controls.

Preparation of structural analogs. Soman (I), sarin (VI), XI, XII, and methylphosphonic acid (XIX) were obtained in greater than 98% purity from USAMRICD. Other organophosphates were prepared at the Prins Maurits Laboratorium TNO. The sulfur analog of soman (II) was prepared by the procedure of Boter and Ooms (17) and purified by spinning band distillation to 99% as determined by GLC. The product contained 0.4% soman.

XVII was obtained from pinacolyl alcohol (1,2,2-trimethylpropan-1-ol), ethylphosphonodichloridic acid (18), and ethylphosphonofluoridic acid by the general procedure of Monard and Quinchon (19) modified by adding an equimolar amount of triethylamine to the alcohol (20). The purities were 97% by acidimetric titration and GLC. Compounds III–V and VII–X were also produced by this procedure with purities of 98% by acidimetric titration and GLC.

XIII–XV were obtained from methylphosphonochloridic acid, 1,2,2-trimethylpropyl ester (21), by reaction with the appropriate alcohol in the presence of an equimolar amount of triethylamine. Purities were 99% or greater by GLC. XVI was prepared by hydrolysis of methylphosphonochloridic acid, 1,2,2-trimethylpropyl ester (21), in aqueous alkali (22). The product was purified by molecular distillation (bath temperature, 110°) to 99% purity as measured by acidimetric titration. XVIII was prepared according to Goldwhite and Saunders (23), b.p. 80°, and was purified by preparative GLC to greater than 99% purity.

Preparation of individual stereoisomers. The four stereoisomers of soman designated C(+)P(+), C(+)P(−), C(−)P(+), and C(−)P(−), were obtained from C(+) and C(−) soman (11, 24) by stereospecific enzymatic reactions (10). The optical purity of the isomers, as 0.7–2.0 mM solutions in anhydrous ethyl acetate, was 99% or greater.

Optical purities were determined by GLC on sequential capillary columns coated with Carbowax 20M and Chirasil-Val (24). It was shown in separate experiments that only slight racemization or hydrolysis of the isomers occurred over the duration of the incubation in aqueous systems (10).

Measurement of affinity and stereochemical preference. Affinity values were generated by comparing the rate of inhibition of cholinesterase by each soman stereoisomer and by each isomer plus several concentrations of antibody. Antibody concentration was maintained in large excess relative to fixed soman stereoisomer concentration which, in turn, was in large excess relative to a constant concentration of enzyme (see Table 2 for details). Rate constants for inhibition were determined by sampling test mixtures at 10-, 15-, or 30-sec intervals and determining the residual enzyme activity spectrophotometrically (5, 10).

The scheme adopted for the interaction of antibody, inhibitor, and enzyme was

$$\begin{array}{l} I + E \xrightarrow{k} EI \\ + \\ Ab \\ \Updownarrow \quad K_A \\ AbI \end{array}$$

where Ab = antibody, E = enzyme, and I = individual stereoisomer. The relationship of the rate constant of inhibition in the presence of antibody (k_{obs}) to the association constant of the antibody and its hapten (K_A) was described by $k_{obs} = k[I_{tot}]/(1 + [Ab]K_A)$ where $k[I_{tot}]$ equals the inhibitor rate constant in the absence of antibody. As would be predicted, $k_{obs} = k[I_{tot}]$ when antibody concentration goes to zero. Values of K_A were calculated by fitting this equation to a set of k_{obs} and antibody concentrations using nonlinear regression.

Acetylcholinesterase from electric eel (Sigma Chemical Co., St. Louis, MO) was used to determine antibody affinity for the C(+)P(−) and C(−)P(−) isomers. The affinities for the weak anticholinesterases C(−)P(+) and C(+)P(+) were measured with butyrylcholinesterase from horse serum (Diosynth, Oss, The Netherlands).

RESULTS AND DISCUSSION

The generation of a molar IC_{50} for each test compound allowed quantitative comparisons between antibodies and among inhibitory ligands on the basis of relative affinity. Knowledge of the relative affinities of the antisoman monoclonal antibodies for soman and the soman analogs provided the basis for the determination of their fine specificity. Fine specificity is a function of the chemical structure on haptenic molecules in general and supplies insight into the physiochemical basis for the noncovalent interaction of specific antibody-ligand pairs.

In addition to their structural analogy to soman, all of the compounds in Table 1 except XIX were also racemic mixtures. We have largely ignored the issue of configurational effects in this discussion because preparations of the soman analogs in stereoisometric resolution were unavailable. It remained a possibility that some of the effects seen were the result of differing antibody affinities for each enantiomer of a racemic inhibitor mixture as was the case with soman (Table 2). When we compared the IC_{50} values for a series of haptens configurationally homologous to soman (compounds XI and XIII–XV), we

TABLE 2

Specificity and affinity of the antibodies for the stereoisomers of soman

Configuration at the chiral centers of soman	Affinity constant[a]	
	BE2	CC1
	liters/mol	
C(+) P(−)	$2.5–5.0 \times 10^3$	$5.9 \pm 0.4 \times 10^5$
C(−) P(−)	$1.0–2.5 \times 10^3$	$3.8 \pm 0.3 \times 10^5$
C(−) P(+)	$\leq 10^3$	$1.2 \pm 0.1 \times 10^5$
C(+) P(+)	$\leq 10^3$	$5.2 \pm 0.4 \times 10^4$

[a] The association constant of an antibody-stereoisomer complex was determined from the retarding effect of increasing antibody concentrations on the inhibition of cholinesterase by a fixed concentration of the stereoisomer. Alcoholic solutions of the stereoisomer were diluted to experimental concentration with phosphate-buffered saline and were added (10 μl) to a mixture of 10 μl of enzyme plus 180 μl of an appropriate concentration of antibody in phosphate-buffered saline. Nine samples were removed at intervals during the 25° incubation, and inhibition was halted by diluting the samples 1:250 with a solution of 0.05 M phosphate buffer, pH 7.0, made 0.001 M with respect to acetylthiocholine iodide and 5,5′-dithiobis(2-nitrobenzoic acid). Residual enzyme activity was determined by incubation of this mixture at 25° for 20 min (7 min with C(+) P(+) soman); then the absorbance at 412 nm was determined after the addition of sodium dodecyl sulfate (final concentration, 0.5%, w/v) to halt the enzymatic hydrolysis. Final stereoisomer and enzyme concentrations and the range of antibody concentrations used were as follows.

Stereoisomer	Enzyme active site	Antibody concentration range	
		CC1	BE2
C(−) P(+) 0.66 μM	0.9 mg/ml (~45 nmol)	5–35 μM	50–180 μM
C(+) P(+) 0.21 μM	0.3 mg/ml (~15 nmol)	4–25 μM	75–225 μM
C(+) P(−) 6 nM	1.9 μg/ml (~0.6 nmol)	1.4–13 μM	50–175 μM
C(−) P(−) 8 nM	1.9 μg/ml (~0.6 nmol)	1.4–13 μM	60–180 μM

Because of its lower affinity, only ranges or threshold values could be determined in this system for BE2. The high antibody protein concentrations required for the determination of more accurate values would have compromised the enzyme and yielded questionable values at best. Values for CC1 represent the mean ± the standard deviation of three determinations.

found them to be invariant within the limits of experimental technique. That being so, it was valid to compare the IC_{50} values obtained without concern for relative antibody affinities for a particular stereoisomer. Note, however, that conclusions based on configurational effects have been drawn where it seemed reasonable.

Fine specificity based on structure. The soman analogs used as inhibitors fell into four groups. Within each group, one of the four substituents around the central phosphorus atom was varied systematically while the others were maintained in their usual relationship. The IC_{50} values for these analogs were then compared to that of soman for each of the monoclonal antisoman antibodies.

Soman itself yielded the indicated IC_{50} values with BE2 and CC1 (I, Table 1). To examine the relative contribution of the phosphoryl oxygen (*Y*, see general formula, Table 1) as a molecular immunodeterminant, the P═S analog (II) was tested. Neither antibody showed an interaction with II. Similarly, early work in our laboratory with monoclonal antibodies to paraoxon, a phosphate, showed no cross-reactivity with its phosphorothionate analog parathion.[2] The P═O moiety, therefore, seems to be a very strong molecular immunodeterminant tending to make antiphosphate and antiphosphonate antibodies nonreactive toward the analogous sulfur-containing compounds.

Our initial inclination was to ascribe this behavior to the strong dipolar nature of the phosphoryl group, leading to a coulombic interaction with a charged site in the antigen-combining region. However, the P═S group has dipolar character much like that of the P═O group (25), making it difficult to explain the difference in affinity on the basis of differing polarity. The well characterized interaction between phosphorylcholine and phosphorylcholine-binding immunoglobulins is largely dependent on hydrogen bond formation between phosphate oxygens and amino acids in the antigen-binding site (26). This, coupled with the knowledge that the hydrogen-bonding capacity of phosphonates is reduced by almost 2 orders of magnitude in the corresponding phosphonothioates (27), makes it more likely that the reduction in affinity when P═S is substituted for P═O is a reflection of strongly reduced capacity for hydrogen bond formation between the organophosphate and a constituent amino acid in the antibody-combining site.

Compounds III–X were used to examine the effect of alkyl (pinacolyl, *R*, see general formula, Table 1) side chain structure and configuration on the strength of antibody-inhibitor interaction. Both antibodies showed reduced affinity with decreasing numbers of side chain methyl groups (III–VII, Table 1) with CC1 more sensitive to this effect than BE2. Removal of the pinacolyl α-methyl (III), and concomitant elimination of one site of optical activity, produced an inhibitor less effective than soman by a factor of 10 with BE2 and by a factor of 100 with CC1. Removal of a single pinacolyl β-methyl and restoration of α-carbon chirality (IV) produced a compound that did not inhibit the binding of CC1 to soman-protein conjugate at 10^{-3} M or less but interacted weakly with BE2. Sequential removal of additional pinacolyl β-methyl groups (V and VI) or removal of a β-methyl and the α-methyl (VII) produced species that caused no measurable inhibition of either antibody. Evidently, the hydrophobic tertiary butyl group of the pinacolyl moiety was a major determinant for binding to the antibody, although the data indicate both stereoisomeric and hydrophobic components to binding at this site. Pinacolyl alcohol alone is not inhibitory toward either BE2 or CC1.[2]

The effect of increasing the size of the group at the *R* position was examined with compounds VIII–X. The interposition of a methylene spacer between α- and β-pinacolyl carbons (VIII) produced an inhibitor equal to or slightly better than soman with respect to BE2 but 10-fold less inhibitory than soman for CC1. Removal of the α-methyl from VIII to give IX reduced binding to both antibodies but did not eliminate it, as with compound III above. In this case as well, the configuration at the pinacolyl α-carbon seemed to be subordinate to hydrophobicity. Unfortunately, the analog with a meth-

[2] Unpublished data.

ylene spacer added between the pinacolyl α-carbon and the ester oxygen was not available. With such an analog, it would have been possible to determine whether a critical factor for antibody recognition was the distance from tertiary butyl to α-methyl or from phosphoryl group to α-methyl.

Substituting β-ethyl groups (X) for pinacolyl β-methyl groups produced an effect equivalent to that seen with VIII where only chain length was increased; binding by BE2 remained the same or was slightly enhanced while that of CC1 was reduced. Thus, there is a strong likelihood that BE2 favors a bulky hydrophobic group at *R* with a methyl group on the carbon α to the ester oxygen. CC1 is more fastidious in this respect, showing reduced binding with increasing chain length and a requirement for an α-methyl and terminal tertiary butyl character. As we reported earlier (4, 5), both antibodies failed to recognize sarin (VI) in which all terminal methyl groups of the pinacolyl moiety are absent.

In compounds XI and XIII–XVI, fluorine (*Z*, see general formula, Table 1) was replaced with aryloxy or alkoxy groups while molecular architecture (optical activity) was maintained. Compound XI was the soman analog conjugated to protein to produce the immunogen used for both antibodies. Compound XII, in which configuration was not conserved, was employed as the competitive inhibitor in the selection of BE2; soman (I) itself was used in the selection of CC1. Testing of the compounds in this group, therefore, was an investigation of our antibody selection procedure as well as a fine specificity determination.

In most cases, these structures proved to be better inhibitors than soman. This tendency was markedly greater for BE2 than for CC1, a fact which probably reflects the use of soman as a selection reagent for CC1. The inhibitory potential of the compounds in this series was striking. They were effectively equal in strength to the *p*-aminophenyl analog (XI) which was closest in stucture to the *p*-azophenyl soman used in the immunogen. Immunologic dogma (28) predicts primary specificity toward azophenyl soman and by extension toward XI. Additionally, previous work with rabbits had shown the *p*-azophenyl group to be a strong locus of immunogenicity (5). Therefore, we had expected the *p*-aminophenyl analog to be the superior inhibitor. The equal effectiveness of the dipinacolyl analog (XII) may be partly due to the statistical advantage inherent in a symmetrical molecule able to present itself in two orientations leading to successful antibody hapten interaction. The inhibitory potential of compound XII assumes increased significance in light of the fact that the chiral center at phosphorus had been eliminated. The results in Table 2 (discussed more completely below) indicate that configuration about the phosphorus was an important consideration in antibody hapten interaction. The inhibitory potential of XI, XIII, XIV, and XV must be a reflection of strong hydrophobic interaction, engendered during immunization, between the antibody-combining site in this region and the group at *Z* on the inhibitor, since the chiral nature of these analogs remained the same as that of soman and the immunogen.

The uniformly greater relative affinity of BE2 for this entire series of analogs, compared to CC1, may reflect the use of compound XII as a competitive inhibitor in the selection of BE2. Soman, which lacks a bulky nonpolar group in the *Z* position, was used as a competitive inhibitor in the selection of CC1. Since the *p*-azophenyl soman derivative was the hapten used in immunization in both cases, the dichtomy in antibody preference for this group of analogs may reflect an initial immune response to the relatively nonpolar phenyl group selected for by the use of compound XII (BE2) and selected against by the use of soman (CC1).

Compound XVI, the alkaline hydrolysis product of soman, technically belongs in this group with substitution at the *Z* position. However, substituting hydroxy for fluorine yields a phosphonic acid monoester that is completely dissociated at the pH of our assay system (29). As a negatively charged species, XVI differs from the rest of the compounds in this group and this difference seems to be reflected in its interaction with the antibodies. In contrast to the phosphonate diesters (XI–XV), in which antibody-analog affinity was approximately equal to that for soman, BE2 reacted only poorly with the anionic species and CC1 did not react at all. This was probably due to strongly reduced hydrogen-bonding capacity of the $[>PO_2]^-$ moiety in correspondence with the highly acidic nature of the undissociated >P(O)OH moiety. This result supports our previous assumption (see above) that ionic interactions are not a major factor in binding to these antibodies. Taken together, the results for compounds XI–XVI seem to indicate that the structure at *Z* (see Table 1) is not as critical for affinity as is the unperturbed P═O group (hydrogen bond acceptor).

Compounds XVII and XVIII were included to assess the contribution of the substituents at the *R′* (see general formula, Table 1) position to antibody specificity. In XVII, an ethyl group was substituted for the phosphonate methyl group. This compound was a slightly better inhibitor than soman for both antibodies. However, the antibody to antibody comparison continued to reflect greater specificity on the part of CC1; BE2 exhibited 5-fold greater affinity for XVII than for soman while CC1 reacted with only 2-fold greater affinity. In compound XVIII, which was included to evaluate the loss of phosphonate alkyl substitution, the phosponate methyl of XII was replaced with hydrogen. In comparison to XII, this substitution reduced interaction with BE2 by a factor of 30 and interaction with CC1 by a factor of 50. These results indicate that the phosphonate alkyl group, like the α-methyl of the pinacolyl group, participates weakly in the interaction with the antigen-combining site.

Compound XIX (methylphosphonic acid) was included to examine antibody recognition of the CH_3—P═O moiety. This material had no inhibitory effect on either antibody at the highest concentration tested. However, the electron redistribution seen with hydroxysoman (XVI) undoubtedly applies here as well.

Fine specificity based on soman configuration. Soman, with two asymmetric centers, is a racemic mixture of isomers as were all of the analogs in Table 1 with the

exception of XIX. Of the four stereoisomers of soman, the potential for acetylcholinesterase inhibition is invested primarily in the epimeric pair that is (−) about the phosphorus (10, 11). Since we had unique preparations of the enantiomers of soman available in resolution and antisera can be raised with configurational specificity (30, 31), it was of obvious interest to us to evaluate the ability of these antibodies to make configurational distinctions.

The results of these determinations are presented in Table 2. As expected from the IC_{50} measurements, CC1 had a higher affinity for each of the single stereoisomers, respectively, than BE2. Although the data for BE2 are somewhat incomplete, the two antibodies seem to have had the same stereoisomeric preference. The data indicate that primary antibody specificity was directed towards the configuration around the phosphorus since highest affinity was measured for the P(−)-isomers. The binding contribution of pinacolyl group chirality was subordinate to that of hydrophobicity as we indicated in the discussion above. This subordination was reiterated in the data from Table 2 where C(+) provided the best interaction in combination with P(−) while C(−) bound best in concert with P(+).

The rather modest stereospecificity of the monoclonal antibodies may be related to our results showing that only two loci on the soman molecule, i.e., the phosphonyl oxygen and the *t*-butyl group of the pinacolyl moiety, provide the major source of interaction with the antibody. In general, three major interactions would be needed for expression of a more outspoken configurational preference, a situation that undoubtedly existed in the presence of the nonpolar *p*-aminophenoxy group on the immunogenic conjugate but no longer exists in soman because of the relatively polar nature of the fluorine.

Work is proceeding on this problem in two directions. New soman-protein conjugates have been synthesized with linkage through the pinacolyl and phosphonate methyl groups and lacking the structurally irrelevant *p*-azophenyl moiety. Mice are now being treated with these compounds in preparation for additional fusions and the production of new monoclonal antibodies. Also, the interaction of BE2 and CC1 with soman is being computer modeled to define the precise stereoelectronic parameters involved in the recognition of soman by the antibodies.

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