We report the selective inactivation of proteolytic antibodies (Abs) to an autoantigen, the neuropeptide vasoactive intestinal peptide (VIP), by a covalently reactive analog (CRA) of VIP containing an electrophilic phosphonate diester group at the Lys20 residue. The VIP-CRA was bound irreversibly by a monoclonal Ab that catalyzes the hydrolysis of VIP. The reaction with the VIP-CRA proceeded more rapidly than with a hapten CRA devoid of the VIP sequence. The covalent binding occurred preferentially at the light chain subunit of the Ab. Covalent VIP-CRA binding was inhibited by VIP devoid of the phosphonate diester group. These results indicate the importance of noncovalent VIP recognition in guiding Ab nucleophilic attack on the phosphonate group. Consistent with the covalent binding data, the VIP-CRA inhibited catalysis by the recombinant light chain of the variable region of the previously described vipase CRA. Catalytic hydrolysis of VIP by a polyclonal VIPase autoantibody preparation that cleaves multiple peptide bonds located between residues 7 and 22 essentially was inhibited completely by the VIP-CRA, suggesting that the electrophilic phosphonate at Lys20 enjoys sufficient conformational freedom to react covalently with Abs that cleave different peptide bonds in VIP. These results suggest a novel route to antigen-specific covalent targeting of pathogenic Abs.

Specific antigen recognition by the variable domains underlies the pathogenic effects of certain Abs1 produced as a result of autoimmune, allergic, and anti-transplant reactions. For instance, Abs found in myasthenia gravis (reviewed in Ref. 1) and hemophilia (reviewed in Ref. 2) bind important epitopes of the acetylcholine receptor and Factor VIII, respectively, that interfere with the biological activity of these proteins by a steric hindrance mechanism. Other Abs utilize their constant region to mediate pathogenic effects, but antigen recognition by Ab variable domains is the stimulus initiating these effects, e.g. Ab recognition of erythrocyte antigens stimulates complement activation by the constant region in autoimmune hemolytic anemia and incompatible blood transfusions. Similarly, allergen recognition by IgE bound to receptors for the constant region on the surface of mast cells stimulates their degranulation. In other diseases, the mechanism of Ab pathogenicity is less clear. For example, Abs to nucleic acids in lupus (reviewed in Ref. 3) and to thyroglobulin in Hashimoto's thyroiditis (reviewed in Ref. 4) are unambiguously disease-associated but additional immune abnormalities are also evident in these diseases and the precise functional effects of the Abs remain debatable. Recently, a novel variable domain mechanism underlying Ab pathogenicity has emerged, viz. the catalytic cleavage of antigens. Hydrolytic catalysts such as Abs to polypeptides (5–8) and nucleic acids (9) hold the potential of interfering with the biological activity of these proteins by a catalytic mechanism reminiscent of serine proteinases. This is suggested by studies in which replacement of the active site Ser residue resulted in the loss of catalytic activity (15) and by inhibition of catalysis by haptenic phosphonate diesters (10). These compounds form adducts with the activated nucleophiles of enzymes by virtue of the covalent reactivity of the electrophilic phosphorus atom (reviewed in Ref. 16) and have been developed recently as probes for the active site nucleophiles in Abs displaying serine protease and serine esterase activity (17, 18), designated covalently reactive antigen analogs (CRAs). As in the case of ordinary Abs, traditional noncovalent antigen recognition is hypothesized to underlie the specificity of the proteolytic Abs for VIP. Therefore, CRAs of the VIP sequence represent a potentially specific means to target the Abs by virtue of offering a reaction surface that combines covalent

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1 The abbreviations used are: Ab, antibody; AMC, 7-amino-4-methylcoumarin; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CRA, covalently reactive analog; DMF, N,N-dimethylformamide; MeSO, dimethyl sulfoxide; Vapp, apparent reaction velocity; VIP, vasoactive intestinal peptide; DFP, diisopropyl fluorophosphate.
binding to the Ab active site with noncovalent binding at neighbor-boring peptide epitope(s). Here we describe the antigen-specific covalent reaction of monoclonal and polyclonal Abs with a synthetic VIP-CRA compound. Despite positioning of the phosphoryl group at a single site, Lys20, the covalent reaction resulted in irreversible inhibition of polyclonal Abs that cleave VIP at several peptide bonds located between residues 7 and 22. The results suggest the feasibility of targeted inactivation of individual Ab populations based on their antigenic specificity.

**MATERIALS AND METHODS**

**CRAs**—Diphenyl N-6-biotinamidoheaxanoylaminoglycosaminidophosphorylmethanephosphonate (compound 1) was prepared from diphenylamino-4-amidophenylmethanephosphonate (compound 1) prepared by solid-phase peptide synthesis with standard 9-fluorenlymethoxycarbonyl protocol (21) with the exception that 4-methyltrityl (22) was used for side-chain protection of Lys20. The peptide resin was treated with 1% trifluoroacetic acid in dichloromethane (5 min × 10) to remove the 4-methyltrityl group, and the deprotected amino group of Lys20 was acetylated with compound 2 in 1-methyl-2-pyrroldione containing 0.1 M N,N-diisopropylethylamine. The peptide resin was treated with trifluoroacetic acid-ethanedithiol-thioanisole-phenol (90:1:1:8) at room temperature for 2 h. After removing the resin by filtration, diethyl ether was added to the solution to afford a precipitate, which was collected by centrifugation and washed with diethyl ether.

**Monoclonal anti-VIP IgG clone c23.5 and control isotype-matched IgG clone UPC10 (IgG2a, k, Sigma) were purified from ascites by affinity chromatography on immobilized protein G-Sepharose (23). Polyclonal IgG from the serum of a human subject with chronic obstructive pulmonary disease (designated HS-2 in ref. 24) was also purified by protein G-Sepharose chromatography. The recombinant light chain of anti-VIP Ab clone c23.5 (GenBank accession number L34775) was expressed in bacterial periplasmic extracts and purified by the binding of the His tag to a nickel-affinity column (15). All of the Abs were electrophoretically homogeneous. Protein concentrations were determined with Micro BCA protein assay kit (Pierce).

**CRA Adducts**—Covalent binding assays were carried out as described previously (17, 20). IgG (1 μg) was incubated with compound 1 or 2 (10 μM) in 1 M sodium phosphate, 0.1 M NaCl, pH 7.4, containing 1 mM CHAPS and 0.1% Me2SO (compound 3 binding experiments) or 0.1% DMSO (dilution 1 binding experiments) at 37 °C. In some experiments, the reaction was carried out in the presence of human plasma collected in EDTA (pooled from eight healthy blood donors; 1% v/v). Aliquots of the reaction mixtures at 10, 20, 40, 60, 90, and 120 min were boiled in 2% SDS containing 3.3% 2-mercaptoethanol and 1% v/v. After removal of the resin by filtration, diethyl ether was added to the solution to afford a precipitate, which was collected by centrifugation and washed with diethyl ether.

**RESULTS**

VIP-CRA—Important features in design of the VIP-CRA (compound 3 in Fig. 1A) are as follows. (a) Inclusion of the electrophilic phosphonate diester group capable of selective reaction with activated nucleophiles, for example, is found in serine proteases (16). (b) The location of the positively charged amido group is in proximity to the phosphate to allow recognition by the model proteolytic IgG clone c23.5, which cleaves peptide bonds preferentially on the C-terminal side of basic amino acids (Arg/Lys) (23, 25). (c) The incorporation of these groups is on the side chain of Lys20 in the sequence of VIP. Hapten CRA 1 contains the phosphate diester and amido groups but is devoid of the VIP sequence. Location of the covalently reactive moiety at Lys20 is based on observations that the Lys20-Lys23 peptide bond is one of the bonds cleaved by monoclonal Ab clone c23.5 (23) and polyclonal human IgG preparations containing Abs to VIP (24). Peptide inhibitors of proteases customarily contain the covalently reactive group located within the peptide backbone or at the peptide termini (e.g. Refs. 26 and 27). In this study, our purpose was to maximize the opportunity for approach of the phosphate group within covalent binding distance of the nucleophile contained in diverse Ab active sites. For this reason, the phosphate group was placed at the side chain of Lys20 using a flexible linker, which allows rotation at several C-C bonds (as opposed to inclusion of the phosphate within the peptide backbone, which may impose a greater level of conformational constraints on accessibility of this group).

VIP-CRA 3 was synthesized by the regioselective on-resin acylation as outlined in Fig. 1B. The VIP sequence was constructed by solid-phase peptide synthesis with standard 9-fluorenlymethoxycarbonyl chemistry with the exception that the 4-methyltrityl group was used for side-chain protection of Lys. The 4-methyltrityl group was used for side-chain protection of Lys. The peptide resin containing 0.1 M N,N-diisopropylethylamine. The peptide resin was treated with trifluoroacetic acid-ethanedithiol-thioanisole-phenol (90:1:1:8) at room temperature for 2 h. After removing the resin by filtration, diethyl ether was added to the solution to afford a precipitate, which was collected by centrifugation and washed with diethyl ether.

**Catalysis Assays**—Pro-Phe-Arg-AMC (0.2 mM, Peptides International, Louisville, KY) was incubated with Ab (0.8 μM) in 96-well plates in 50 mM Tris-HCl, 0.1 mM glucose, pH 8.0, containing 0.6% Me2SO and 0.025% Tween 20 at 37 °C, and the release of AMC was determined by fluorometry (λex 470 nm; λem 360 nm, Cary Eclipse spectrometer, Varian, Palo Alto, CA). Preparation and assay of cleavage of [35S]VIP (125I/VIP were described previously (24). To determine whether the CRAs inhibit Abs irreversibly, IgG (2 μg) was incubated (37 °C) with compound 1 or 3 for 16 h in 50 mM Tris-HCl, 0.1% glycerol, pH 8.0, containing 2.5% Me2SO and 0.025% Tween 20. After 16 h, the VIP sequence was characterized in the reaction mixture the reaction mixture (0.2 ml) on protein G columns as described previously (23) (50 μl of settled gel; washed with 0.8 ml of 50 mM Tris-HCl, pH 7.4; eluted with 0.2 ml of 0.1% glycerol, pH 2.7; neutralized with 1 M Tris-HCl, pH 9). 50-μl aliquots of the recovered IgG (and IgG-CRA complexes) were incubated with [35S]VIP (88,000 cpmp/μg) for 1 h, and peptide cleavage was determined by measuring the radioactivity soluble in trichloroacetic acid. Control IgG samples were incubated without CRA, chromatographed, and analyzed for VIP-cleaving activity in the same way.
recognition of VIP. The covalent reaction was visualized by boiling the reaction mixtures followed by denaturing SDS-electrophoresis and detection of biotin-containing adducts (Fig. 2A, inset). Accumulation of covalent VIP-CRA adducts with the anti-VIP Ab increased linearly as a function of time 2 with the light chain subunit accounting for the majority of the adducts (nominal mass 29 kDa determined by comparison with molecular mass standards). Adducts of VIP-CRA with the control Ab were formed at lower levels. Similarly, hapten CRA reacted with anti-VIP and control Abs slowly compared with the VIP-CRA and there was no preference for covalent binding of the hapten CRA at the light chain subunit. Apparent reaction velocities ($V_{app}$) were obtained from the slopes of linear regression curves fitted to the progress data by least square analysis ($[\text{Ab-CRA}] / [\text{H}] = \frac{V_{app}}{t}$, where $[\text{Ab-CRA}]$ represents the intensity of Ab-CRA adduct band in AAU and $t$ is the reaction time). $V_{app}$ values are compiled in Table I. For the anti-VIP Ab, $V_{app}$ of the VIP-CRA reaction with the light chain was 66-fold greater than the corresponding reaction with the control Ab light chain. These observations indicate the selective nucleophilic reactivity of the anti-VIP light chain. Inclusion of VIP-CRA and  

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FIG. 1. Structure of hapten CRA 1, VIP-CRA 3, and synthetic intermediate 2 (panel A), and scheme for synthesis of VIP-CRA 3 (panel B). Reagents and conditions for steps i–vi in panel B are as follows. Step i, solid-phase peptide synthesis by 9-fluorenylmethoxy carbonyl (Fmoc) chemistry (deprotection, 20% piperidine in DMF (3 min × 2; 20 min × 1); coupling, Fmoc-amino acid (2.5 eq), PyBOP (2.5 eq), 1-hydroxybenzotriazole (2.5 eq), and $N,N$-disopropyl ethylamine (7.5 eq) in DMF (60 min); step ii, 20% piperidine in DMF (3 min × 2; 20 min × 1); step iii, d-biotin (2.5 eq), PyBOP (2.5 eq), 1-hydroxybenzotriazole (2.5 eq), and $N,N$-disopropyl ethylamine (7.5 eq) in DMF (60 min); step iv, 1% trifluoroacetic acid in CH$_2$Cl$_2$ (5 min × 10); step v, compound 2 (3 eq), 0.1 mM $N,N$-disopropyl ethylamine in DMF (overnight); and step vi, trifluoroacetic acid-ethanedithiol-thioanisole-phenol (90:1:8:2 h). All of the steps were done at room temperature. Protecting groups: Boc, t-butoxycarbonyl; tBu, tert-butyl; Pmc, 2,2,5,7,8-pentamethylchrome-n-6-sulfonyl; Trt, trityl; Mtt, 4-methyltrityl.

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2 The CRA-Ab reactions were predicted to follow the second-order rate law, but linear adduct accumulation occurred in the initial stage of the reaction.
CRA adduct appeared at the position of the light chain subunit of the VIPase Ab (Fig. 2C). Little or no reaction of the VIP-CRA with plasma proteins and the control IgG subunits was observed. Similarly, the reaction mixtures of hapten CRA 1 yielded little or no adduct formation with plasma proteins or the exogenously added monoclonal Abs. Faint biotin bands were observed upon prolonged exposure in each of the lanes shown in Fig. 2C at a mass of 67–70 kDa. These bands presumably reflect a low level adduct formation of the hapten-CRA and VIPase with albumin, the major protein present in plasma (see silver-stained electrophoresis lane in Fig. 2C). Covalent reactions of albumin with organophosphorus compounds have been reported previously (28, 29).

Disopropyl fluorophosphatase (DFP), a well-established serine hydrolase inhibitor, was previously reported to inhibit catalysis by anti-VIP light chain c23.5 (15). In this study, DFP inhibited the covalent VIP-CRA binding to the light chain (Fig. 2D), consistent the presence of a serine protease-like binding site(s).

**Inhibition of Catalytic Activity**—The cleavage of the model peptide substrate Pro-Phe-Arg-AMC by the recombinant light chain of anti-VIP Ab c23.5 has been reported previously (15). Site-directed mutagenesis studies have suggested that the light chain contains a catalytic triad similar to the active site of serine proteases (15). Here, the progress of Pro-Phe-Arg-AMC cleavage by the light chain was measured fluorometrically by determining AMC generated due to cleavage at the Arg-AMC amide bond. As expected, a linear increase of AMC fluorescence was evident (Fig. 3A). Inclusion of VIP-CRA 3 in the reaction mixture inhibited the reaction in a time-dependent manner. The deviation of the progress curve from linearity in the presence of VIP-CRA suggests an irreversible inhibition mode (30).

**Table 1**

| Ab                  | Subunit | $V_{app}$ ± S.D. (AAU min$^{-1}$) |
|---------------------|---------|----------------------------------|
| Anti-VIP IgG, c23.5 | Light   | 19.8 ± 0.4                       |
| Anti-VIP IgG, c23.5 | Heavy   | 3.0 ± 0.3                        |
| Control IgG, UPC10  | Light   | 0.3 ± 0.1                        |
| Control IgG, UPC10  | Heavy   | 2.0 ± 0.3                        |

* ND, not determined.

Next, we turned to a human polyclonal IgG preparation isolated from a subject with airway disease (designated HS-2 in Ref. 24). Cleavage of VIP by this preparation has been attributed to IgG autoantibodies based on retention of the activity in Fab fragments, adsorption of the activity by IgG binding agents, and absence of VIP cleavage by control identically isolated sera. The stoichiometry of the inhibition was determined by titration with limiting amounts of VIP-CRA 3 (IC$_{50}$ = 1.5 and 27 μM, respectively; Fig. 3B). The superior potency of VIP-CRA 3 is consistent with the covalent adduct data reported in the preceding section and may be attributed to improved noncovalent recognition of the peptide component of VIP-CRA 3.

**Fig. 2.** Specific covalent VIP-CRA binding by monoclonal anti-VIP IgG (clone c23.5). Panel A, accumulation of VIP-CRA 3 or hapten-CRA 1 adducts shown in arbitrary area units (AAU) of the adduct bands determined by electrophoresis and densitometry. Reaction conditions: 1 μM IgG, 10 μM CRA, at 37°C. Data are means of closely agreeing duplicates. Correlation coefficients for curves fitted to progress curves by linear regression were 0.9 or greater. All of the reactions were analyzed at 6 time points as shown for anti-VIP L chain. For clarity, only the final data points at 120 min are shown for anti-VIP H chain and control Ab H and L chains (UPC10 IgG). Inset, streptavidin-peroxidase-stained blots of SDS gels showing 3-adducts of the c23.5 light (29 kDa) and heavy (58 kDa) chains. Lanes 1–6 correspond to the reaction time shown in the graph (10, 20, 40, 60, 80, and 120 min). Panel B, representative plot showing inhibition by VIP (10 μM) of formation of anti-VIP light chain adducts with VIP-CRA 3. Percent inhibition was determined as follows: 100 – 100($V_{app}$($V_{app}$)*), where +VIP and –VIP refer to the presence and absence of VIP, respectively. Inset, streptavidin-peroxidase-stained electrophoresis cutouts showing light chain adducts formed in the absence and presence of VIP. Headers 1–6 correspond to the progressively increasing reaction time shown in the graph. Panel C, streptavidin-peroxidase-stained blots of SDS-electrophoresis gels showing CRA binding to anti-VIP Ab in the presence of human plasma (1% v/v, 1 h; 10 μM each of CRAs; 10 μM exogenously added Abs). Exogenous Abs and CRAs used are as follows: anti-VIP c23.5 IgG + VIP-CRA 3 (lane 1); Control UPC10 IgG + VIP-CRA 3 (lane 2); VIP-CRA 3 alone (lane 3); anti-VIP c23.5 + hapten 1 (lane 4), UPC10 IgG + hapten 1 (lane 5); and hapten 1 alone (lane 6). Biotin-containing bands in lanes 1–6 were detected as in panel A. Lanes 7 and 8 are silver-stained bands of human plasma (1% v/v) and molecular weight standards, respectively. Panel D, streptavidin-peroxidase-stained blots of reducing SDS-electrophoresis gels showing inhibition of VIP-CRA binding to anti-VIP c23.5 light chain by DFP. Anti-VIP IgG c23.5 (1 μM) was incubated with or without DFP (5 mM) for 5 min and then was allowed to react with VIP-CRA 3 (2 μM) for 60 min.

Devoid of the phosphate group in the reaction mixture inhibited the formation of VIP-CRA 3 adducts with the anti-VIP light chain (Fig. 2B; inhibition in three repeat experiments, 41.0 ± 7%). It may be concluded that selective covalent binding of VIP-CRA 3 by the anti-VIP Ab is made possible by noncovalent interactions due to the presence of the VIP sequence.

Pooled plasma from healthy humans was included in the reaction along with VIPase c23.5 to investigate further the selectivity of the VIP-CRA. As expected, the predominant VIP-CRA 3 adduct appeared at the position of the light chain subunit of the VIPase Ab (Fig. 2C). Little or no reaction of the VIP-CRA with plasma proteins and the control IgG subunits was observed. Similarly, the reaction mixtures of hapten CRA 1 yielded little or no adduct formation with plasma proteins or the exogenously added monoclonal Abs. Faint biotin bands were observed upon prolonged exposure in each of the lanes shown in Fig. 2C at a mass of 67–70 kDa. These bands presumably reflect a low level adduct formation of the hapten-CRA and VIPase with albumin, the major protein present in plasma (see silver-stained electrophoresis lane in Fig. 2C). Covalent reactions of albumin with organophosphorus compounds have been reported previously (28, 29).
initially confirmed the ability of the polyclonal IgG preparation to cleave multiple peptide bonds in VIP. Three new radioactive peaks were generated from [Tyr10-125I]VIP by treatment with the IgG (Fig. 4A). The observed radioactive product peaks in Fig. 4A probably represent mixtures of peptide fragments, as the VIP fragments generated by cleavage at the afrostatic peptide bonds have previously been noted to elute from the HPLC with similar retention times (24).

To determine whether VIP-CRA 3 is an irreversible inhibitor, aliquots of the IgG treated with varying concentrations of this compound (10, 20, 40, and 80 µM) were subjected to affinity chromatography on protein G to remove the unreacted inhibitor followed by assay of the cleavage of [Tyr10-125I]VIP (Fig. 4B). Control IgG was subjected to an identical incubation without VIP-CRA followed by the chromatographic procedure. Dose-dependent inhibition of catalytic activity was evident, and near-complete inhibition of catalysis was observed at VIP-CRA concentrations >20 µM. The observed irreversible inhibition suggests that VIP-CRA forms covalent adducts with the polyclonal Abs, similar to its behavior with the monoclonal Ab examined in the preceding section. Selectivity of the VIP-CRA inhibitory effect was confirmed by comparison with hapten CRA 1. As expected, the VIP-CRA inhibited the cleavage of VIP more potently than the hapten CRA (IC50 = 7 and 36 µM, respectively).

**DISCUSSION**

The following conclusions may be drawn from these data. (a) Functionally coordinated noncovalent and covalent interactions allowed nucleophilic anti-VIP Abs to form specific and covalent adducts with the VIP-CRAs. (b) The VIP-CRA inhibits each of the reactions involving cleavage of VIP at several peptide bonds, indicating its potential as a universal inhibitor of diverse anti-VIP catalytic Abs. The importance of noncovalent Ab paratope-antigen epitope binding in directing the VIP-CRA to the Ab nucleophile is evident from the following observations: lower reactivity of the anti-VIP monoclonal Ab with the hapten CRA devoid of the VIP sequence; limited reactivity of the irrelevant isotype-matched Ab and plasma proteins with the VIP-CRA; and inhibition of the anti-VIP Ab covalent reaction with the VIP-CRA by VIP devoid of the CRA moiety.

**Background fluorescence in the absence of catalyst corresponded to 0.05 ± 0.03 µM AMC. Panel B, comparison of VIP-CRA 3 (●) and hapten CRA 1 (○) inhibition of light chain-catalyzed Pro-Phe-Arg-AMC hydrolysis. Curves are fitted to the equation, percent inhibition = 100(1 + e−1/(EC50,CRA) × log[CRA]), where EC50, is the concentration yielding 50% inhibition (r2 0.98). Reaction conditions are as in panel A with the exception that varying CRA concentrations were employed (1, 3, 10, and 30 µM). Percent inhibition was computed as, 100(Vi − Ve)/Vi, where Vi represents the residual activity after incubation for 13 h (tangents of the least-square fit progress curves obtained as in panel A). Values are means of three replicates ± S.D. In the absence of CRAs, the reaction rate was 22 nm AMC h−1. Panel C, stoichiometry of antibody light chain (c23.5) reaction with VIP-CRA 3. Shown is the plot of residual catalytic activity (Pro-Phe-Arg-AMC hydrolysis) of the light chain in the presence of varying VIP-CRA 3 concentrations (reaction conditions as in panel B with the exception that the VIP-CRA concentrations were 0.03, 0.1, 0.3, 1.0, and 3.0 µM and reaction time was 36 h). Residual activity was determined as 100VV/V, where V is the velocity in the absence of inhibitor and Vi is a computed value of the velocity under conditions of complete inhibitor consumption. Vi values were obtained from least-square fits to the equation [AMC] = Vi × t + A1 − e−kobs × t, where A1 and kobs represent, respectively, the computed AMC release in the stage when inhibitor consumption is ongoing and the observed first-order rate constant, respectively (r2 for individual progress curves, >0.97). The equation is valid for reactions with an initial first-order phase and a subsequent zero-order phase. The x-intercept shown in the plot was determined from the least-square fit for data points at [VIP-CRA 3]/[light chain] ratio <1. Inset, example progress curve from which Vi values were computed. VIP-CRA 3, 0.03 µM.**

**Fig. 3. Inhibition of anti-VIP light chain c23.5 catalyzed Pro-Phe-Arg-AMC hydrolysis by VIP-CRA 3. Panel A, progress curves of Pro-Phe-Arg-AMC (0.2 mM) cleavage by the light chain (0.8 µg) in the absence (○) and presence (●) of VIP-CRA 3 (3 µM). Curves are least-square fits to the equation [AMC] = V × t (r2 0.99) (○) or [AMC]/[AMC]max = 1 − e−ktobs × t (r2 0.89) (●), where V is the velocity of AMC release, [AMC]max is the extrapolated maximum value of AMC release, and kobs is the observed first-order rate constant. Data are means of three replicates ± S.D. Fluorescence values expressed as released AMC by comparison with a standard curve constructed using authentic AMC.**
Recently, CRA derivatives of other polypeptide antigens (human immunodeficiency virus glycoprotein 120 and epidermal growth factor receptor) have also been reported to form covalent adducts with specific Abs directed to irrelevant Abs (31, 32). Taken together, these considerations open the route toward permanent inhibition of individual Ab subpopulations based on their antigenic specificity.

The light chain subunit accounted for most of the covalent reactivity of the anti-VIP monoclonal Ab with the VIP-CRA. Reactivity with the hapten CRA serves as an index of Ab nucleophilicity independent of traditional noncovalent forces responsible for Ab-antigen complexation. Hapten CRA reactivities of the anti-VIP heavy and light chain subunits were comparable, suggesting that differences in intrinsic nucleophilic reactivity do not account for rapid formation of adducts of the light chain with the VIP-CRA. It may be concluded that the light chain nucleophile is in the immediate vicinity of the Ab noncovalent binding site and that the noncovalent binding interactions facilitate covalent binding. This statement is consistent with observations that the purified light chain of this Ab is capable of specifically catalyzing the cleavage of VIP (25).

Previously, the purified light and heavy chain subunits of the Ab were reported to bind VIP independently determined by a conventional assay for noncovalent Ab-antigen complexes (Kd for light chain, heavy chain, and intact IgG, respectively, 10.1, 6.8, and 1.9 nM) (33). In addition to the light chain, the heavy chain subunit appears to contribute noncovalent binding energy for Ab complexation with VIP but the heavy chain nucleophile does not seem to be sufficiently in register with the phosphonate group of the VIP-CRA to participate in the covalent reaction.

Additional evidence for irreversible and specific Ab recognition by the VIP-CRA is available from the catalysis assays. VIP-CRA adducts of the Abs obtained following the removal of unreacted VIP did not display catalytic activity. Catalytic cleavage of Pro-Phe-Arg-AMC by the recombinant light chain of the monoclonal Ab has been documented previously (15). This reaction is characterized by 57.5-fold higher Kd than the cleavage of VIP by the light chain and is attributed to cross-reactivity of the catalytic site with peptide substrates devoid of an antigenic epitope capable of participating in high affinity noncovalent binding. Pro-Phe-Arg-AMC cleavage by the light chain was inhibited more potently by the VIP-CRA than the hapten CRA. Similarly, the cleavage of VIP by polyclonal human autoantibodies to VIP was inhibited more potently by the VIP-CRA than the hapten-CRA.

Ab diversity poses an interesting challenge in achieving antigen-specific covalent inactivation of pathogenic Abs. Structural differences in the variable domains underlies Ab specificity for individual antigenic epitopes, and even Abs to small molecules presenting a limited surface area can contain structurally distinct binding sites (e.g. Refs. 34 and 35). Catalytic IgG preparations from patients with autoimmune disease cleave several backbone bonds in polypeptide (7, 24) and oligonucleotide (9) antigens. This may be due to the presence of multiple Ab species in polyclonal IgG preparations, each with a distinct scissile bond specificity. We have suggested previously that the nucleophiles enjoy some measure of mobility within Ab active sites that is not subject to restriction when noncovalent binding of Abs and antigens takes place (31, 32). To the extent that this hypothesis is valid, Abs with differing peptide bond specificity could react covalently with the VIP-CRA even if the phosphonate group is located somewhat imprecisely in the antigenic epitope. In this study, the placement of the phosphonate on the Lys20 side chain (as opposed to the peptide backbone) and inclusion of a flexible linker represent attempts to expand further the conformational space available for the covalent reaction. Complete inhibition of catalytic hydrolysis of VIP by polyclonal Abs that cleave several bonds between VIP residues 7 and 22 by the VIP-CRA was evident. Therefore, promising means to...
obtain antigen-specific covalent inhibition of diverse Abs include the exploitation of intrinsic conformational properties of Ab catalytic sites and the provision of enhanced access to the phosphonate group by manipulating the linker structure. In comparison, if Ab antigen binding is conceived as a rigid body interaction involving inflexible surface contacts, covalent inhibitor design must entail close topographical simulation of the transition state of each scissile bond and individual inhibitors must be developed to effectively inhibit different catalytic Abs. The importance of evaluating conformational factors in inhibitor design is supported by previous reports suggesting a split-site model of catalysis (31, 32) in which antigen binding at the noncovalent subsite imposes little or no conformational constraints on the catalytic subsite, allowing the catalytic residue to become positioned in the active site, allowing the catalytic residue to become positioned in the transition state of each scissile bond and individual inhibitors must entail close topographical simulation of the transition state of each scissile bond and enhancement of access to the phosphonate group by manipulating the linker structure.

As noted previously, catalytic Abs are proposed to contribute in the pathogenesis of autoimmune disease. Specific covalent inhibitors represent a novel means to help define the precise functional effects of the Abs. Such inhibitors may serve as prototypes for the development of therapeutic agents capable of ameliorating harmful Ab effects. In addition to inactivation of secreted Abs, reagents such as the VIPase Antibody Inhibitor may be useful in targeting antigen-specific B cells. The feasibility of this goal is indicated by evidence that CRAs bind covalently to Abs expressed on the surface of B cells as components of the B cell receptor (36). Ab nucleophilicity may be viewed as an indication of their competence in completing the first step in catalytic covalent synthesis, i.e. formation of an acyl-Ab reaction intermediate. This is supported by observations that the magnitude of Ab nucleophilic reactivity is correlated with their proteolytic activity (31). A recent study suggests that noncatalytic Abs also contain nucleophiles but are unable to facilitate steps in the catalytic cycle following covalent attack on the antigen, viz. water attack on the acyl-Ab intermediate and product release (31). Regardless of the physiological functions of nucleophiles expressed by noncatalytic Abs, their presence may allow CRA targeting of Ab populations with established pathogenic roles, e.g. anti-factor VIII Abs in hemophilia.

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