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Catalytic Activity of Anti-Ground State Antibodies, Antibody Subunits, and Human Autoantibodies

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ABSTRACT

Catalytic antibodies may be produced over the natural course of antibody-affinity maturation by placement of chemically reactive residues in antibody-active sites by somatic hypermutation or V-D-J-gene rearrangement. This hypothesis has received support from recent observations on the chemical reactivity of antibodies to vasoactive intestinal peptide (VIP), DNA, and steroid- and dinitrophenyl-esters. Recent studies reveal that monoclonal antibodies raised against the ground state of VIP can accelerate the cleavage of peptide bonds. The light-chain (L-chain) subunit of human autoantibodies display increased hydrolytic rate and diminished VIP-binding affinity compared to the parent antibody, consistent with increased turnover owing to weaker binding of the substrate ground state. These observations reveal an essential limitation of catalytic antibodies, i.e., large turnover rates may be associated with diminished substrate specificity.

The hydrolysis of VIP by IgG purified by affinity chromatography from asthma patients and nonasthmatic controls was compared. IgG from the majority of asthma patients displayed VIP-hydrolyzing activity. V_{max} values for IgG from asthmatics tended to be higher than those from the nonasthmatic group.

In principle, catalysis by antibodies may be an important mediator of immunological defense, regulation, and autoimmune dysfunction. The verification of these possibilities will require studies that utilize efficient assays of antibody catalysis during experimental immunization and autoimmune disease, as well as mechanistic investigation of catalysis by antibodies and their subunits.

Index Entries: VIP; catalytic antibodies; autoantibodies; light chains; ground state recognition; asthma.

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INTRODUCTION

The extraordinary rate of sequence diversification in antibody-combining sites over the course of maturation of the immune response may theoretically permit the evolution of a catalytic function in antibodies, assuming that catalytic antibody-producing lymphocytes survive immune selection mechanisms. The probability with which catalytic antibodies arise in nature cannot be determined with any degree of confidence at this time. It is clear, however, that at least some naturally formed antibodies are catalytic, e.g., human autoantibodies (*1*) and murine antibodies to vasoactive intestinal peptide (VIP) (*2*), DNA-cleaving autoantibodies (*3*), and esterase-like antibodies raised against unactivated haptens (*4,5*). Homologies between the light chains (L-chains) of antibodies and serine proteases, in particular the sequence surrounding the active site serine, are described (*6*). It is well known that antibodies to transition-state analogs can catalyze chemical reactions with limited energy requirements (*7*). However, these antibodies often display unexpected chemical activity and specificity (*8,9*), suggesting that transition-state stabilization resulting from simple shape complementarily does not explain the catalytic activity of these antibodies. It is noteworthy that a systematic comparison of catalytic antibody formation in response to substrates and transition-state analogs is not available. In view of the chemical activity of antibodies raised to unactivated substrates, it is important to verify experimentally the purported advantage of immunization with transition-state analogs.

MATERIALS AND METHODS

The reagents and experimental techniques were as described elsewhere (*1,2,10–12*).

RESULTS AND DISCUSSION

Autoantibody-Mediated VIP Hydrolysis

The biochemical properties of VIP autoantibodies from three human IgG preparations were characterized in detail (designated HS-1, HS-2, HS-3) (*1,12*). These IgG preparations hydrolyzed radiolabeled VIP ([Y^{10}-^{125}I]VIP), estimated as the TCA-soluble radioactivity or as fragments separated by RP-HPLC. The hydrolysis increased linearly with increasing IgG, and the pH optimum for the reaction was 7–8. Background hydrolysis of VIP under the conditions of the assay was undetectable. Several types of experiments confirmed that the hydrolysis was mediated by antibodies; overloaded IgG preparations were electrophoretically pure; the hydrolytic

activity was bound by immobilized anti-human IgG and immobilized protein G, a bacterial protein that binds IgG; the activity was present in Fab fragments; specific antibodies prepared by affinity chromatography on immobilized VIP exhibited increased specific hydrolytic activity (*10*); the IgG and Fab displayed nanomolar K_m values indicative of tight substrate binding; also, unfractionated plasma from several human subjects negative for the antibodies did not display detectable VIP hydrolysis. Since the substrate concentration was held at low levels (<200 p*M*), the peptide hydrolysis assay is probably biased toward detection of high-affinity catalysts, accounting for the absence of detectable hydrolysis by proteases present in unfractionated plasma.

Affinity Purification of Autoantibodies

Purification of IgG on VIP coupled via NH_2 groups to CNBr-Sepharose in a high-salt buffer permitted up to 3000-fold enrichment of the specific hydrolytic activity (*10*). The hydrolytic specificities of IgG and affinity-purified antibody from HS-1 were identical (cleavage at Q^{16}-M^{17}). Isoelectric focusing of the late-eluting HS-3 antibodies revealed five closely spaced bands, similar to the pattern observed with monoclonal antibodies (Fig. 1). The multiplicity of bands may be explained by:

1. The existence of several different antibodies;
2. Charge heterogeneity owing to posttranslational modifications (e.g., heavy chain [H-chain] glycosylation); or
3. Antibody subunit fragmentation and rearrangement by S—S reduction or exchange reactions.

Catalytic Efficiency

VIP hydrolysis by IgG and affinity-purified antibodies from HS-1 was saturable and displayed Michaelis-Menten kinetics (*1,12*). Saturation analyses of binding and hydrolysis indicated antibodies with a single K_d and K_m, suggesting interaction of VIP with one species or several species with near-identical binding properties. Compared to classical proteases, the antibodies turn over slowly. However, the antibodies bind VIP tightly, and their kinetic efficiencies (k_{cat}/K_m) consequently approach those of proteases (Table 1). For example, the kinetic efficiency of specific HS-1 IgG in Table 1 is $1.7 \times 10^4\ M^{-1}\ s^{-1}$, a value only 10,000-fold smaller than the upper limit for a diffusion-limited, kinetically perfect enzyme.

The affinity of cellular receptors for VIP is in the low-nanomolar range (*13*). Effective clearance mechanisms for VIP may be assumed, therefore, to require catalysts displaying low K_m. Although VIP is degraded by conventional enzymes, like trypsin, with micromolar to millimolar K_m (*14*),

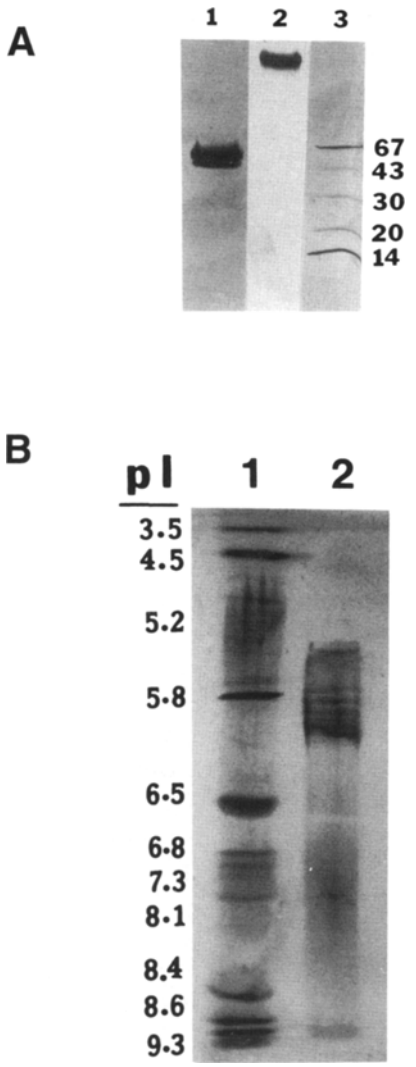

Fig. 1. (**A**) Nonreducing SDS-gel electrophoresis of silver-stained IgG purified from HS-3 by protein G-Sepharose chromatography (lane 2), Fab prepared by papain digestion of HS-3 IgG, protein A-agarose chromatography and gel filtration on a Superose-12 column (lane 1), and marker proteins (lane 3; mass in kilodaltons is indicated). (**B**) Silver-stained isoelectric focusing gel of specific VIP antibodies (pH 3–10.5) from HS-3 (lane 2); markers (lane 1).

VIPases displaying high affinity for the peptide have not been identified. VIP-binding and hydrolyzing autoantibodies are found in healthy athletes (*15*), and VIP is released during muscular exercise (*16*). A hypothesis worthy of further study is that catalytic cleavage of VIP by antibodies is a physiological clearance mechanism for the peptide.

Table 1
Kinetic Properties of VIP Antibodies

Antibody source	Antibody type[1]	K_m^2	V_{max}^3
HS-1	Total IgG	0.037	3.5×10^{-4}
HS-1	Specific IgG	0.110	7.3×10^{-1}
HS-1	Specific L-chains	4.1	67.2
HS-2	Specific IgG	1.85	1.5
HS-3	Total Fab	0.038	1.0×10^{-2}
HS-3	Total L-chains	0.380	1.9×10^{-1}

[1]The antibodies were purified by protein G-Sepharose chromatography (total IgG) followed by papain fragmentation and gel filtration (total Fab) or chromatography on VIP-Sepharose (specific IgG). Specific L-chains were purified by three cycles of chromatofocusing and total L-chains, by chromatography on immobilized anti-H- and anti-L-chain Ab.
[2]μM.
[3]pmoles/min/μg protein.

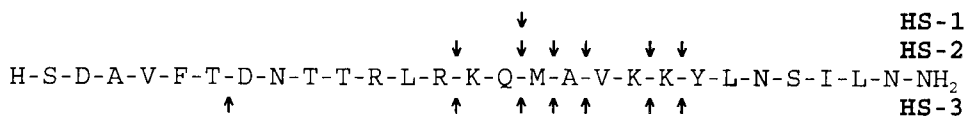

Fig. 2. Peptide bonds cleaved by VIP antibodies (arrows) from three human subjects (HS-1, HS-2, and HS-3). Data for HS-1 antibodies are from (*1*) and those for HS-2 and HS-3, from (*12*).

Scissile Bonds

Two polyclonal antibody preparations hydrolyzed VIP at several bonds linking chemically dissimilar amino acids and one antibody preparation, at a single, unique bond (Fig. 2). This suggests that the antibody hydrolytic repertoire is considerable. The conservative conclusion is that several antibodies with unique specificity are responsible for the observed cleavages. The more likely possibility is that a single antibody cleaves at multiple bonds, since kinetic data suggested limited heterogeneity. It is possible, for instance, that the hydrolytic specificity of the antibodies is determined primarily by recognition of the peptide backbone, rather than amino acid side chains in the substrate.

Preliminary information on the relationship between the substrate-binding and hydrolytic specificity of the antibodies is available (*11*). Screening for binding of synthetic fragments of VIP to polyclonal and monoclonal antibodies revealed that VIP(15–28) was the dominant binding site. A Gln^{16}-Met^{17} cleaving antibody recognized VIP(15–28) nearly as well as the full-length VIP (residues 1–28). The free energy of binding of this antibody

to a shorter fragment, VIP(22–28), was 41% that of binding to full-length VIP. Although VIP(22–28) is located four residues distant from the scissile bond, it inhibited antibody-mediated hydrolysis of full-length VIP. Residues distant from the scissile bond may contribute significantly in antibody recognition. This is consistent with the large size of antibody-binding sites, which can accommodate 15–22 antigen residues (*17*).

Antigen contact occurs mainly at complementarity determining regions (CDRs). Framework residues contribute in correct CDR folding and may also participate directly in antigen binding (*18*). The residues involved in binding and catalysis by anti-VIP antibodies may not be identical, but they are likely close to each other either in the CDRs or in closeby framework regions.

Catalysis and Binding Activity of Antibody Subunits

Catalysis by an antibody occurs if it stabilizes the transition state more than the ground state. Catalysis is rendered more difficult if the catalyst binds (stabilizes) the ground state strongly. On the other hand, the substrate specificity of a catalyst derives from its ability to distinguish ground states, not transition states. The greater the substrate-binding affinity of the antibody, the greater its specificity is likely to be. A strong substrate-binding catalytic antibody may be predicted to display, therefore, a limited turnover and a low K_m (high-binding affinity).

This prediction was borne out by a comparison of the kinetic parameters for intact Fab and purified L-chains (Table 1). VIP was hydrolyzed by the L-chains more rapidly than intact antibody (*19*). Increased turnover is apparently gained at the expense of specificity, evidenced by increased K_m. These observations suggest a relatively inflexible active site in intact antibody that provides a very good fit for the substrate. In comparison, L-chains may possess a more flexible substrate-binding site better suited for catalysis and rapid product release. The L-chains appear to retain sufficient substrate-binding affinity to ensure specific catalysis. As in the case of intact antibodies, different L-chains displayed different K_ms ranging from low-nanomolar to low-micromolar values.

We have observed high-affinity VIP binding by a reduced and alkylated L-chain (Fig. 3) from a monoclonal antibody (K_d 10.1 n*M*, compared to 1.8 n*M* for the parent antibody; *20*). Excess albumin as well as several short and midsized peptides unrelated to VIP did not inhibit the binding of radioiodinated VIP by the L-chains. A nonimmune L-chain did not bind VIP. These observations indicate that the binding is sequence-specific and can be attributed to the variable regions of the L-chain. Based on the cDNA sequence of the subunits of this antibody (obtained by the reverse transcriptase-PCR and dideoxynucleotide sequencing), the H-chain and L-chain CDRs were deduced to consist of 26 and 32 amino acids, respectively (Gao, Q. S. and Paul, S., unpublished). CDR3 of the H-chain is widely believed to be an important determinant of antibody specificity.

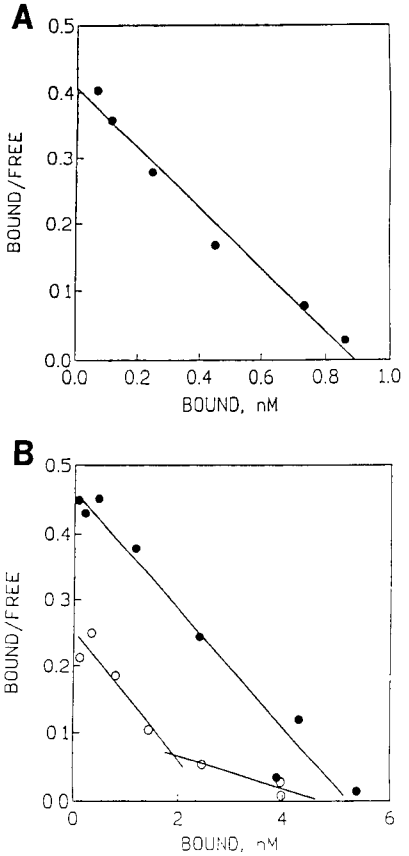

Fig. 3. (**A**) VIP-binding affinity of intact c23.5 antibody and (**B**) its purified H (○) and L chain subunits (●). Binding of [^{125}I-tyr^{10}]VIP (0.16 n*M*) by antibody (1.7 n*M*), L-chain (221 n*M*), or H-chain (417 n*M*) in the presence of increasing concentrations of unlabeled VIP (0.2 n*M* to 1 μM) was determined. Antibody subunits were purified as described in (*20*). Data (means of duplicates) were analyzed using LIGAND (Elsevier Biosoft).

This CDR in the anti-VIP H-chain is short (four residues) compared to its length in most mouse antibodies (mean 8.7 residues; >95% of known H-chain CDR3s are composed of five or more residues, ref. *21*). We suggest that antigen interactions with the L-chain may be particularly significant when binding at the H-chain is limited because of size or steric factors. We consider it unlikely that the anti-VIP L-chain is unique in its high-affinity antigen-binding activity.

B-lymphocytes synthesize L-chains in excess over H-chains, and secretion of free L-chains by these cells has been demonstrated (*22*). Large amounts of L-chains accumulate in the extracellular fluids and tissues of patients with L-chain-secreting tumors (*23*). L-chains can mediate peptide-bond cleavage, activate complement components (*24*), and suppress antibody synthesis (*25*). The observation of high-affinity antigen binding and catalysis by L-chains warrants further study of their ability to simulate the antigen-specific functions of antibodies.

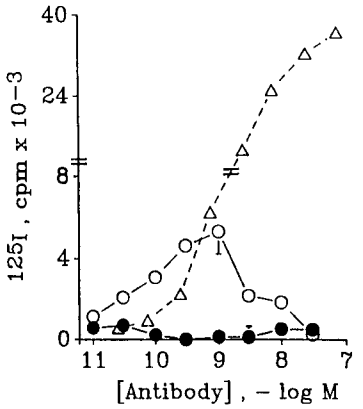

Fig. 4. [^{125}I-tyr^{10}]VIP hydrolysis by affinity-purified c23.5 antibody. Hydrolysis of [^{125}I-tyr^{10}]VIP (109 p*M*) incubated (9 h) with increasing c23.5 mAb (○) or irrelevant mouse myeloma $IgG_{2a}\kappa$ (●) was determined as trichloracetic acid-soluble radioactivity, and [^{125}I-tyr^{10}]VIP saturable binding by c23.5 mAb (△) as polyethylene glycol-precipitable radioactivity. Data are means of three replicates ± SD.

Antigens Capable of Provoking Formation of Antibody Catalysts

Unactivated Antigens

Two mouse monoclonal antibody (mAbs) (c23.5 and c23.4) and their Fab fragments generated by immunization with a VIP-keyhole limpet hemocyanin conjugate were observed to hydrolyze (Y^{10}-^{125}I)VIP (2). The specific activity (CPM hydrolysis/μg protein) of the intact c23.5 IgG peak isolated by protein G-Sepharose, Mono-Q, and hydroxylapatite chromatography was essentially constant. At mAb concentrations of 0.3 and 1 n*M*, VIP hydrolysis was consistent with Michaelis-Menten kinetics and was characterized by nanomolar K_m and k_{cat} about three to five orders lower than that of human antibodies (2). Hydrolysis of unrelated short peptides by the mAb was not observed, and several irrelevant monoclonal antibodies were without VIP-hydrolyzing activity.

Increasing concentrations of c23.5 IgG resulted in increasing (Y^{10}-^{125}I)VIP hydrolysis up to 19.7% of the available substrate, but a further increase in mAb (>1 n*M*) resulted in suppression of activity (Fig. 4). Binding of (Y^{10}-^{125}I)VIP displayed the expected increase over this range of concentrations. We are examining the following hypothesis to explain this dose–response relationship: There is a concentration-dependent equilibrium between two antibody forms $A \leftrightharpoons B$; both *A* and *B* bind VIP, but only *B* hydrolyzes the peptide. With increasing mAb concentrations, equilibrium between *A* and *B* is shifted to the left. Increasing complexation of VIP with *A* protects it from hydrolysis by *B*, producing a suppression of

peptide hydrolysis at elevated mAb concentrations. *B* may represent a variant conformer of intact mAb. Alternatively, *B* could be a dissociation product of the intact mAb. Gel-filtration analysis revealed peaks of hydrollytic activity at the positions of the antibody tetramer (150 kDa), H-L dimer (80 kDa), and L-chain monomer (30 kDa). The hydrolytic activity in the first two peaks was removed by immunoadsorption with antibody to mouse anti-IgG_{2a} H-chain as well as antibody to mouse L-chain (κ), but not control antibody to mouse IgG_1 H-chain. As expected, the anti-L antibody, but not the anti-H antibody removed the hydrolytic activity in the L-chain monomer fraction. Apparently, formation of the free H-L dimer and free L-chain is favored at low-antibody concentrations. The L-chain monomer peak displayed a high level of hydrolytic activity (specific activity $> 21.4 \times 10^3$ CPM/ng protein compared to 0.7×10^3 CPM/ng of the tetramer). VIP-binding activity in the H-L dimer and L-chain monomer peaks was not detected (L. Li and S. Paul, unpublished). These observations suggest the need for careful examination of spontaneous antibody fragmentation by S—S reduction and exchange, reactions that have been little studied until now, presumably because contamination of intact IgG with small amounts of subunits is unlikely to influence the binding activity of these preparations significantly.

Structurally Related Antigens

A rigid lock-and-key type of fit may be predicted to be nonconducive for catalysis. It may be speculated that imperfect surface alignment between a polypeptide and an antibody raised against a structurally related, but different antigen could afford sufficient conformational flexibility to permit facile catalysis. In this context, it is noteworthy that the strength of substrate interactions at the catalytic residues of the antibody may not be directly related to the macroscopic strength of the antigen–antibody binding reaction. It is possible, for example, that strong overall binding simply concentrates the antigen at the catalytic site and the binding of substrate by the antibody catalytic residues is substantially weaker.

Transition States (TS)

Since TS lifetimes are on the order of bond vibrational and rotational frequencies, anti-TS antibodies may not be formed frequently. Moreover, simple shape complementarily between antibody and a TS is probably insufficient for catalysis of demanding reactions like peptide-bond hydrolysis.

Unstable Polypeptides

Asn-X can undergo cleavage by an intramolecular mechanism involving attack of the peptide bond carbonyl by the side-chain amide of Asn (*26*). Antibody binding could force a conformation that facilitates the intramolecular cleavage. Although VIP is susceptible to slow autolysis in the absence of stabilizing protein, there is no detectable autolysis under the conditions used for studying antibody catalysis. Moreover, fragments

VIP	HSDAVFTDNYTRLRKQMAVKKYLNSILN
human GRF	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL
PHM	HADGVFTSSYRRILGQLSAKKYLESLM
Secretin	HSDGTFTSELSRLRDSARLQRLLQGLV
Glucagon	HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
PACAP	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK
HelospectinI	HSDATFTAEYSKLLAKLALQKYLESILGSSTSPRPPSS
Helodermin	HSDAIFTQQYSKLLAKLALQKYLASILGSRTSPPP

Fig. 5. VIP, GRF, and related peptides. Sequence identifies with VIP are underlined as are conserved basic residues.

of VIP that do not undergo detectable autolysis (e.g., VIP [15–28]) are hydrolyzed by antibodies.

Anti-antiproteases

An anti-idiotypic antibody to an anti-protease antibody may mimic the protease active site. Anti-idiotypic antibody raised against anticholinesterase displays cholinesterase activity (*27*). The limitation to this proposition is that since shape similarity between an anti-idiotypic antibody and the protease could presumably be achieved by many combinations of amino acids, the former need not necessarily display a chemical reactivity.

Asthma

Asthma is defined as spasmodic hyperresponsiveness of the airways. Decreased relaxation and enhanced contraction of airway smooth muscle could be responsible for the hyperresponsiveness (*28*). In the human airway, a nonadrenergic, noncholinergic system (NANC) is responsible for neurally mediated airway relaxation (*29*). There is substantial evidence that VIP is a mediator of NANC (*30*), including potent airway relaxation induced by VIP, presence of VIP in nerves supplying the airways, release of VIP during electrically stimulated airway relaxation, and inhibition of airway relaxation by anti-VIP antibodies in vitro. Nerves in asthmatic airways obtained at autopsy are deficient in VIP compared to healthy airways (*31*).

Other neuropeptides of the VIP family, polypeptide histidine methanine (PHM) and pituitary adenylate cylase-activating peptide (PACAP) (Fig. 5), could also serve as NANC mediators (*32,33*). PHM is cosynthesized and coreleased with VIP. PACAP is a newly discovered peptide found in airway nerves. Both peptides relax airway smooth muscle. Given

Table 2
VIP-Binding and Hydrolyzing Antibodies in Healthy and Asthma Subjects

Population	Frequency of binding,[1] %	Mean K_a[2]	Frequency of hydrolysis,[3] %	Mean V_{max}[4]
Healthy	16	0.13	36	0.36
Asthma	18	7.80	83	3.06

[1]N = 98 (healthy), 74 (asthma).
[2]$10^9 M^{-1}$; N = 8 (healthy), 7 (asthma).
[3]N = 19 (healthy), 35 (asthma).
[4]nM VIP/25 μg IgG/16 h; range, 0.05–0.88 (healthy), 0.95–8.25 (asthma); N = 4 each.

the importance of airway smooth muscle in the maintenance of life, it is reasonable that a great deal of redundancy should be present in mechanisms mediating airway relaxation.

Inflammation is an essential component of asthma. Mediators released from inflammatory cells (e.g., histamine, eicosanoids) are strong bronchoconstrictors. VIP is a known anti-inflammatory agent (*34*). It inhibits release of histamine and eicosanoid metabolites from mast cells and macrophages, and diminished VIP in asthma may permit a dominance of inflammatory mediator effects.

VIP Autoantibodies in Asthma

Two types of antibodies to VIP have been characterized in asthma patients. A minority of asthma patients and healthy subjects with a history of muscular exercise are positive for plasma VIP-binding antibodies (*15,35*). Compared to antibodies found in healthy athletes, those in asthma patients tend to bind VIP with higher affinity (Table 2). The second type of VIP antibodies display hydrolytic activity, determined by assay of (Y^{10}-^{125}I)VIP cleavage by protein G-Sepharose-purified IgG fractions. These were found frequently in asthma patients (Table 2). There was no correlation between the levels of VIP-hydrolyzing and VIP-binding activities in the IgG samples, suggesting efficient catalysis by antibody species present in minute quantities in the high-activity samples. The V_{max} values of four antibody preparations from asthmatic subjects tended to be greater than from four athletes. Based on these initial observations, we hypothesize that small amounts of catalytic antibodies found in asthma subjects can turn over rapidly. It appears that antibodies in asthmatics somehow acquire the characteristics of efficient VIP degradation or binding. A recent paper (*36*) shows that immunization of cats with VIP interferes with airway relaxation, consistent with a role for VIP autoantibodies in the pathophysiology of asthma. Decreased immunochemical staining for VIP has been observed in airway tissue from asthmatic subjects obtained at autopsy (*31*). It may be useful to speculate that the binding and catalytic

hydrolysis of VIP by antibodies may cause compensatory release from neurons, e.g., by disturbing feedback regulation of VIP release mediated by binding of the peptide to presynaptic receptors. This may eventually deplete the peptide stores in neurons.

Plasma autoantibodies to VIP are likely to reach the local microenvironment of the airway smooth muscle, nerves, and inflammatory cells, since blood-borne antibodies can permeate pulmonary vascular endothelium (37). Antibody synthesis by mucosal lymphocytes could be an additional contributory factor. Hydrolysis of VIP can be expected to inactivate the molecule permanently since VIP fragments relax airways minimally or not at all. In the case of catalytically nonproductive binding, the antibodies may compete with VIP receptors on target cells. Airway dysfunction could arise, therefore, from VIP hydrolysis or simple high-affinity peptide binding, provided sufficient concentrations of the antibody are present.

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DISCUSSION

S. Paul

Zouali: Were the catalytically active light chains from asthma patients' κ or λ chains?

Paul: The human preparations were a mixture of the two isotypes. The monoclonal mouse light chain is a κ-isotype.

Zouali: It would be important to know whether the activity is uniquely associated with either κ or the λ isotype.

Gabibov: I am interested in the specificity of the autoantibodies. One of your human subjects showed a higher specificity with cleavage at a unique peptide bond. In other patients, you observed a diverse specificity pattern. Is this the result of heterogeneity or the lability of active sites?

Paul: The mechanisms underlying the specificity profile remain to be explained. The expectation from all the work that we heard this morning and all of the previously published work was that catalytic antibodies would focus in on a small portion of the molecule and would cleave only one bond. We observed multiple cleavage site not only in the case of the human autoantibodies, but also the light chains derived from monoclonal antibodies. Catalyst heterogeneity cannot be excluded as a possible explanation at this time, since different antibodies or different conformations of the same antibody could exist in our preparations.

Gabibov: What is the physiological significance of the reaction of the VIP antibody with calmodulin-dependent MLCK?

Paul: I can only give you a speculative answer. MLCK is the rate-limiting enzyme in muscle contraction, and there has been a suggestion that its functions are altered in asthma. MLCK could be the original stimulus for formation of the VIP-binding antibodies. The reverse is also possible, i.e., the VIP autoantibodies may penetrate muscle cells and interfere with MLCK activity. It is too early to say what is going on.

Hansen: In talking about the polyclonal human antibodies, you indicated two times that there were some that showed hydrolysis without binding of VIP. With one of the monoclonal light chains, there was no binding, but there was hydrolysis. I do not know quite how you can have catalysis without binding.

Paul: Please realize that we routinely perform two types of assays. In one, we measure bound VIP, and in the other, hydrolyzed VIP. There was no correlation between the binding activity and the hydrolytic activity of IgG samples purified from human subjects. This simply means that some of the catalytically efficient antibodies were not detected by doing a binding assay, presumably because they are present at low concentrations or have too low an affinity for VIP. Concerning the monoclonal light chains, three forms were separated on a hydroxylapatite column, which may be because of chemical heterogeneity introduced by reduction and alkylation or alternate folding during renaturation. The three forms of light chains display different levels of binding activity. Hydrolytic activity was present in only one of these forms.

Tramontano: What is the K_m for the hydrolytic form of the light chain?

Paul: We have yet to do kinetic analyses on the hydrolytic light chain separated by hydroxylapatite chromatography. The K_m value for the light chain loaded on that column was in the range of 20 n*M*. The

refolding conditions appear to influence K_m, and we have observed values up to 3 μM in different preparations.

Zouali: When you immunized your mice with VIP-KLH, you obtained four monoclonals that were specific for VIP, but only one of them had catalytic activity. Why were the three others inactive? Were there differences in affinity?

Paul: There were five wells with binding activity and two wells with hydrolytic activity. The antibodies from the two wells with hydrolytic activity have a very similar sequence, suggesting that they may come from the same B-cell clone. So it is fair to say that one in four was hydrolytic. We are trying to understand why one of the four antibodies is catalytic, rather than why the other three are noncatalytic. The answer is likely related to the probability of deriving a catalytic site from a binding site. The K_m value of the hydrolytic antibody is in the nanomolar range, indicating that it has undergone affinity maturation. Systematic studies will be needed to determine the relative numbers of catalyzers and binders following experimental immunization, perhaps using the phage-display libraries.

Tramontano: When you refold the light chain and you get three fractions, is the appearance of that profile dependent on the folding process? Do you see variations in those peaks?

Paul: There is enormous variability associated with refolding. The protein concentration in the renaturation step is a key factor. We see reasonably high levels of specific activity in preparations that are refolded as low concentrations and lower activity at high protein concentrations. Inclusion of VIP during refolding also affects the activity.

Tramontano: Does the variation in activity correlate with the relative areas of those three peaks?

Paul: We have not analyzed a sufficient number of preparations on the hydroxylapatite column to make a firm conclusion.

Tramontano: If you observe a K_m of 20 nM, even though it is a mixture, why would that not be reflected in a binding activity of that fraction?

Paul: Yes, an antibody with a low K_m value is detected in the binding assay provided it is present at a sufficient concentration. The components of the buffer used in the binding assay do not permit VIP hydrolysis.