Conformation-Specific Monoclonal Antibodies Directed against the Calcium-Stabilized Structure of Human Prothrombin†

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ABSTRACT: Prothrombin undergoes a conformational transition induced by metal ions. For preparation of monoclonal antibodies directed against the metal-stabilized conformer of human prothrombin, murine somatic hybrids were prepared that elaborate antibodies against various determinants on the prothrombin surface. Eight anti-prothrombin-producing clones were characterized. All eight antibodies bound human prothrombin and prothrombin fragment 1 but failed to bind prethrombin 1, α-thrombin, or bovine prothrombin. Four monoclonal antibodies bound abnormal prothrombin. These antibodies bound prothrombin with greater affinity than fragment 1, emphasizing the conformational distinctions between the protein and its fragment. One antibody bound prothrombin only in the presence of $CaCl_2$; this antibody did not bind abnormal prothrombin. Three functional groups of anti-prothrombin monoclonal antibodies were identified: (1) antibodies specific for the metal-stabilized prothrombin conformer that do not bind abnormal prothrombin; (2) antibodies that bind prothrombin and abnormal (des-γ-carboxy) prothrombin independent of calcium; (3) antibodies whose binding to prothrombin is independent of calcium and do not bind to abnormal prothrombin. Conformation-specific monoclonal antibodies directed against the metal-stabilized conformer of prothrombin bound to prothrombin only in the presence of metal ions. Half-maximal antibody–antigen binding was observed at calcium concentrations of 0.10 mM. In addition, Mn(II), Mg(II), and Co(II) supported binding. The interaction of antibody with prothrombin–Ca(II) was characterized by a single class of binding sites with a K_A of 2.3×10^9 M^{-1}. These studies emphasize the potential of conformation-specific antibodies that are directed against the liganded form of a macromolecule. Moreover, they demonstrate that the property of conformation specificity may be associated with a monospecific homogeneous antibody population derived from a single hybrid clone. Thus, the polyclonal conformation-specific antibodies previously studied do not have an inherent and absolute requirement for multispecificity, i.e., directed against different determinants on the protein surface.

Conformation-specific antibodies have been used as probes of protein structure to complement chemical and physical methods in determining the relationship of protein structure and function. Immunochemical approaches have been applied to the study of conformational motility of polypeptides (Sachs et al., 1972; Furie et al., 1975; Hurrell et al., 1977), pathways of protein folding (Chavez & Scheraga, 1977; Creighton et al., 1978), distinction of abnormal and mutant proteins (Young et al., 1975; Blanchard et al., 1979), and the effects of ligand binding on protein conformation (Furie et al., 1975; Furie & Furie, 1979; Dean & Schechter, 1979; Tai et al., 1980; Madar et al., 1980).

Immunochemical methods using subfractions of polyclonal antibodies have been previously applied to the study of the vitamin K dependent blood coagulation protein, prothrombin (Furie & Furie, 1979; Tai et al., 1980; Madar et al., 1980; Furie et al., 1978)). Prothrombin contains γ-carboxyglutamic acid residues that function in metal binding (Nelsestuen et al., 1974; Stenflo et al., 1974; Sperling et al., 1978; Furie et al., 1979) and are necessary for prothrombin activation under physiologic conditions. Metal ions induce a conformational transition that has been monitored by changes in circular dichroism (Bloom & Mann, 1978), intrinsic fluorescence (Nelsestuen, 1976; Prendergast & Mann, 1977), and immunochemical methods (Furie & Furie, 1979; Tai et al., 1980; Madar et al., 1980; Furie et al., 1978). These studies have described distinct antigenic differences in bovine prothrombin in the presence or absence of calcium with antibodies raised in rabbits and fractionated by affinity chromatography. These antibodies have been used to localize the metal-induced conformational transition to the NH_2-terminal region of prothrombin (Tai et al., 1980; Furie et al., 1978; Madar et al., 1982). The concentration of metal ions that cause half-maximal antigenic changes has been shown to be comparable to that determined by physical methods to monitor the conformational transition (Furie & Furie, 1979). By use of immunochemical methods, the metal-induced conformational transition can be measured even in complex biological fluids.

A fundamental and unanswered question relates to the molecular basis of antibody recognition of a particular three-dimensional form of a macromolecule (Furie et al., 1982). One hypothesis suggests that antibody binding to a specific protein conformer stabilizes that conformer and facilitates recognition and binding by a second antibody to a second antigenic site. In turn, a third, fourth, fifth, etc. antibody each bind to distinct sites stabilized by antibody binding. In this cooperative binding, a series of weak, conformation-specific antibodies directed against multiple determinants on the protein surface yield an average antibody–antigen interaction of high affinity and specificity for the ligand-stabilized conformation. Alternatively, a single antibody may have absolute specificity for a ligand-stabilized conformer with which it binds with high affinity. These hypotheses, one of which necessarily employs polyclonal antibodies, might be distinguished by the preparation of conformation-specific monoclonal antibodies. The present study indicates that the characteristic of conformation specificity can be a property of monoclonal antibodies. This communication describes the

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production and characterization of conformation-specific hybridoma-produced monoclonal antibodies that are directed against an antigenic surface that is stabilized by the presence of metal ions.

Materials and Methods

Human prothrombin, factor X, and factor IX were purified from human plasma by barium citrate absorption and elution, DEAE-cellulose[1] chromatography, and heparin–Sepharose chromatography (Rosenberg et al., 1975; Miletich et al., 1978). Fragment 1 and prethrombin 1 were prepared by digestion of prothrombin by thrombin; traces of fragment 1 and prothrombin in prethrombin 1 were removed by rabbit anti-prothrombin–Ca(II)–agarose affinity chromatography (Blanchard et al., 1983). α-Thrombin, the gift of Dr. John Fenton, was inhibited with (*p*-amidinophenyl)methanesulfonyl fluoride (Laura et al., 1980). Abnormal (des-γ-carboxy) prothrombin was isolated from plasma of a patient treated with sodium warfarin, as previously described (Blanchard et al., 1983). Bovine prothrombin was purified by standard methods (Furie et al., 1978). All proteins migrated as a single band upon electrophoresis in polyacrylamide gels containing dodecyl sulfate.

Human prothrombin was radiolabeled with ^{125}I by the lactoperoxidase method (Morrison, 1980). ^{125}I-Labeled prothrombin was functionally active and migrated as a single band upon gel electrophoresis.

Commercially available reagents included the Fab fragment of sheep anti-mouse Ig conjugated to β-galactosidase (Bethesda Research Laboratories), mouse Ig heavy- and light-chain typing sera (Miles), and bovine serum albumin (fraction V, Sigma). $Na^{125}I$ and lactoperoxidase were obtained from New England Nuclear.

Preparation of Hybridomas. Balb/c mice were immunized with an initial subcutaneous injection of 75 μg of human prothrombin in complete Freund's adjuvant. Mice were then immunized biweekly with 50 μg of prothrombin in incomplete Freund's adjuvant for 2–6 months. Following 2 months without immunization, mice were injected with 50 μg of prothrombin in 0.15 M NaCl intravenously for the 3 consecutive days prior to fusion.

Spleen cells (5×10^7) from immunized mice were fused with the Sp2/0 plasma cell line (5×10^6) in 28% poly(ethylene glycol) 4000 (Sigma) by the method of Köhler & Milstein (1975). The fused cells were suspended in hypoxanthine-, aminopterin-, and thymidine-containing (HAT) medium. The suspension was distributed into microtiter trays (Costar) at 3×10^5 cells/well. Supernatants were assayed for anti-prothrombin antibody after several weeks. Selected positive cultures were cloned by the limiting dilution method (McKearn, 1980). Cell lines producing anti-prothrombin antibodies were stored at −70 °C in 95% bovine fetal calf serum and 5% dimethyl sulfoxide.

Solid-Phase Radioimmunoassay of Anti-Prothrombin. Polystyrene tubes (12 × 75 mm) were coated with purified goat anti-mouse immunoglobulin (20 μg/mL) in 0.05 M borate buffer, pH 8.5, for 4 h at 4 °C. After being washed, the tubes were coated with 5% bovine serum albumin in 0.04 M Tris-HCl, pH 7.2–0.14 M NaCl. Hybridoma supernatants (100 μL) were added to the tubes and incubated at 37 °C for 18 h. For comparison, Sp2 cell supernatant and mouse anti-prothrombin antiserum were used as negative and positive controls, respectively. After an extensive washing with 0.04 M Tris-HCl, 0.14 M NaCl, and 10 mM $CaCl_2$, pH 7.2, ^{125}I-labeled prothrombin (0.28 μg) was added to each tube. After incubation at 37 °C for 24 h, the tubes were washed with water and assayed for ^{125}I in a Beckman γ 8000 scintillation spectrometer.

Solid-Phase Enzyme-Linked Immunoabsorbent Assay (ELISA) of Anti-Prothrombin. Microtiter plates (96 wells; Immunolon 2, Dynatech) were coated with human prothrombin at 20 μg/mL in 0.05 M borate, pH 8.5, for 16 h at 4 °C. The plates were washed exhaustively with 0.05 M Tris-HCl, pH 7.2, 0.14 M NaCl, 5 mM $CaCl_2$, 0.05% NaN_3, and 2% bovine serum albumin in the same buffer added to the wells for 30 min at 24 °C. After an extensive washing with 0.05 M Tris-HCl, pH 7.2, 0.14 M NaCl, 5 mM $CaCl_2$, and 0.05% NaN_3, 50 μL of tissue culture supernatant or mouse polyclonal anti-prothrombin antiserum was added to the plate and incubated at 37 °C for 1 h. The plates were extensively washed with 0.15 M Tris-HCl, pH 7.2, 0.14 M NaCl, 1.5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 0.05% Tween 20, and 0.05% NaN3. Fab fragments of sheep anti-mouse Ig (50 μL) conjugated to β-galactosidase in the above buffer were added. After the plates were incubated at 24 °C for 2 h, they were washed with the same buffer 3 times. *p*-Nitrophenyl D-galactoside [50 μL in 0.05 M sodium phosphate (pH 7.2), 1.5 mM $MgCl_2$, and 0.1 M β-mercaptoethanol] was added to each well and the reaction developed over 30–60 min at 23 °C. The absorbance at 405 nm was monitored on a Dynatech MR 580 Micro-ELISA auto reader.

In studies evaluating the effect of metal ions on antibody–prothrombin interaction, an additional step in the ELISA was included. After nonspecific binding of antibody was blocked with bovine serum albumin, the plates were washed with 10 mM EDTA in 0.05 M Tris-HCl, pH 7.2–0.14 M NaCl and 0.05 M Tris-HCl, pH 7.2–0.14 M NaCl sequentially. Antibody solutions in 0.05 M Tris-HCl (pH 7.2)–0.14 M NaCl containing varying concentrations of Ca(II), Mg(II), Mn(II), or Co(II) were added to the plates and incubated for 60 min at 37 °C. After several washings, bound mouse immunoglobulin was detected and quantitated as described above.

Determination of Antibody Specificity. For determination of the antigenic specificity of monoclonal antibodies, a variation of the solid-phase enzyme-linked immunoabsorbant assay for anti-prothrombin was used. Competitors, at indicated concentrations, were added to a constant amount of antibody. Fifty microliters of this mixture was added to microtiter wells coated with prothrombin. The interaction of antibody with the competitor in solution instead of the immobilized prothrombin was monitored as a decrease in the amount of mouse immunoglobulin bound to the solid-phase antigen.

Ouchterlony immunodiffusion was performed by standard methods. Monoclonal antibody solutions (20 μL) and commercial typing sera (5 μL) were applied to appropriate wells prepared in 1% agarose, 0.05 M Tris-HCl, pH 7.5, and 0.02% NaN_3.

Antibody Purification. Hybrid clones producing anti-prothrombin antibodies of interest were injected intraperitoneally into Balb/c mice by standard methods (McKearn, 1980). After 2–3 weeks, ascitic fluid was removed. Specific antibodies were purified from this fluid by affinity chromatography. Ascitic fluid (2 mL) was applied to a 6.5 × 2 cm column of prothrombin–agarose equilibrated in Tris-HCl, pH 8.1, 1 M NaCl, and 7 mM $CaCl_2$. The eluate was monitored at 280 nm. The column was washed free of unbound protein; bound

[1] Abbreviations: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; Ig, immunoglobulin; EDTA, ethylenediaminetetraacetic acid.

Table I: Specificity of Anti-Prothrombin Monoclonal Antibodies[a]

clone	type	relative binding				
		prothrombin	fragment 1	abnormal prothrombin	prethrombin 1	*p*-APMS-thrombin
RL 1·1	IgG_1, κ	1	0.50	<0.001	<0.001	<0.001
RL 1·3	IgG_{2a}, κ	1	0.11	<0.001	<0.001	<0.001
RL 1·4	IgG_1, κ	1	0.22	<0.001	<0.001	<0.001
RL 1·5	IgG_1, κ	1	0.40	0.07	<0.001	<0.001
RL 1·6	IgG_{2b}, κ	1	0.14	<0.001	<0.001	<0.001
RL 1·7	IgG_1, κ	1	0.19	0.05	<0.001	<0.001
RL 1·8	IgG_1, κ	1	0.33	0.025	<0.001	<0.001
RL 1·9		1	0.50	<0.001	<0.001	<0.001

[a] The relative binding affinity was determined by comparing the concentration of competitor with the concentration of prothrombin required to inhibit 50% of antibody–antigen (solid-phase) interaction.

protein was eluted with either 0.05 M Tris-HCl, pH 7.2, 0.5 M NaCl, and 70 mM EDTA or 4 M guanidine hydrochloride. Antibodies were dialyzed against 0.05 M Tris-HCl, pH 7.2–0.14 M NaCl and stored at −15 °C.

Results

Development of Anti-Prothrombin Antibody Producing Clones. Of 496 wells plated with the cell-fusion suspension, growth was observed in 95% of the wells following selection with HAT medium. Anti-prothrombin antibody production was assayed with two separate assays. In the solid-phase ELISA mouse anti-prothrombin antibodies bound to immobilized prothrombin were detected. In the solid-phase radioimmunoassay, mouse anti-prothrombin antibodies that bound to immobilized goat anti-mouse immunoglobulin were detected by the binding of ^{125}I-labeled prothrombin. Figure 1 shows the results of the initial screening of wells for anti-prothrombin antibody binding activity. Although the ELISA and radioimmunoassay correlate well for about 60% of the assayed wells, some wells showed greater reactivity in one assay than the other. The reason for this is unclear, but may relate in part to the antigenic specificity of the antibody and to the differences in exposure of certain antigens on prothrombin depending on the assay used. Furthermore, the initial ELISA did not include added calcium; the radioimmunoassay contained 10 mM $CaCl_2$. The well labeled in the figure with a star yielded the conformation-specific antibody directed against the calcium-stabilized form of prothrombin (RL 1·3).

Nine of these cultures were cloned by the method of limiting dilution, resulting in 42 anti-prothrombin-positive clones. Eight of these subclones were chosen for further investigation.

Characterization of Monoclonal Antibodies. The immunoglobulin type and subclass of the antibodies were determined by Ouchterlony immunodiffusion with precipitating antisera. The clones elaborated anti-prothrombin monoclonal antibodies of the IgG class (Table I). Seven of the antibodies had κ light chains, and one was indeterminant. Most of the immunoglobulins were of the IgG_1 subclass. These results give further evidence for the clonal character of each of these cultures.

The antigenic specificity of the monoclonal antibodies from RL 1·1, 1·3, 1·4, 1·5, 1·6, 1·7, 1·8, and 1·9 was evaluated by a competitive assay based on the solid-phase ELISA method (Figure 2 and Table I). Antibodies from these clones bound immobilized human prothrombin; free prothrombin competed with bound prothrombin for antibody. By use of this competition assay the interaction of antibodies with prothrombin fragment 1, abnormal (des-γ-carboxy) prothrombin, thrombin, prethrombin 1, and bovine prothrombin were examined. All of the monoclonal antibodies bound fragment 1, the NH_2-terminal third of prothrombin, and none bound prethrombin 1, the COOH-terminal two-thirds of prothrombin. The antibodies did not bind to thrombin that had been treated with (*p*-amidinophenyl)methanesulfonyl fluoride. Antibodies derived from RL 1·5, 1·7, and 1·8 cross-reacted significantly with abnormal prothrombin while the others did not. However, none of the antibodies cross-reacted equivalently with fragment 1 and prothrombin. Significantly higher concentrations (from 2-fold to 10-fold) of fragment 1 compared to prothrombin were required to inhibit 50% of antibody-immobilized prothrombin interaction. None of the monoclonal antibodies bound to bovine prothrombin (data not shown).

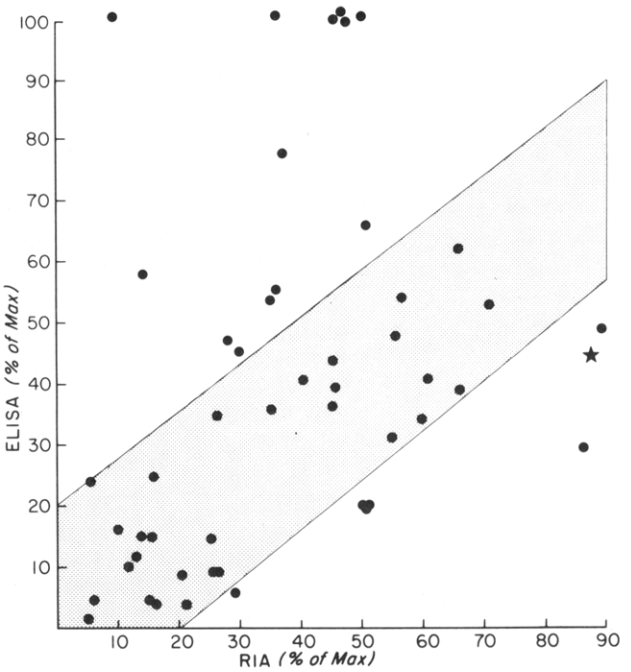

FIGURE 1: Anti-prothrombin activity in primary hybrid wells. Anti-prothrombin antibody activity, measured by a radioimmunoassay, is shown on the *x* axis. In this system, goat anti-mouse Ig immobilized on a tube bound mouse anti-prothrombin Ig. The binding of ^{125}I-labeled prothrombin to this matrix permitted quantitation of anti-prothrombin. $CaCl_2$ (10 mM) was employed. Anti-prothrombin antibody activity, measured with an ELISA, is shown on the *y* axis. In this system, prothrombin is immobilzed in a microtiter well. Mouse anti-prothrombin that binds to the immobilized prothrombin is detected by using a sheep anti-mouse Ig–β-galactosidase conjugate. The shaded region defines those wells that contain anti-prothrombin activity that reacts similarly in both assays. Clone RL 1·3 was derived from the primary well indicated by the star.

Monoclonal antibodies derived from the culture supernatants of the eight clones were examined for prothrombin binding activity in the presence and absence of calcium. Three clones, RL 1·3, 1·4, and 1·9, produced conformation-specific antibodies. As shown in Figure 3A, monoclonal antibodies from RL 1·3 bound prothrombin in the presence of 5 mM $CaCl_2$ but

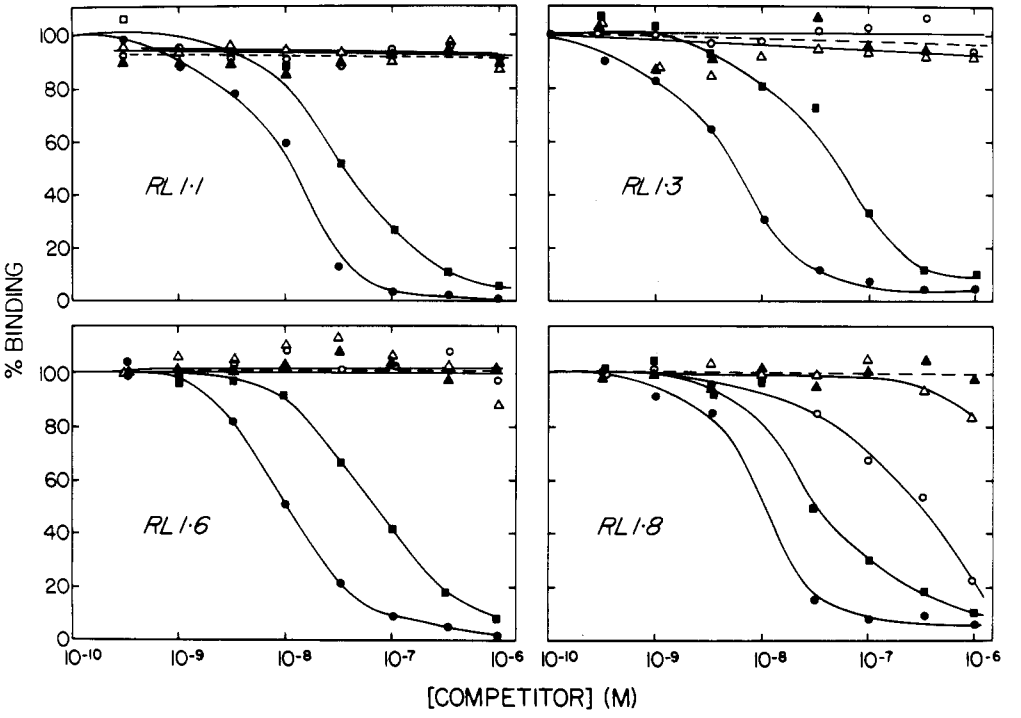

FIGURE 2: Antigenic specificity of monoclonal antibodies to prothrombin. The binding of various polypeptides to antibody was evaluated by a competition ELISA system. The ability of competitor to bind antibody in solution and preclude antibody binding to prothrombin immobilized on the plate surface was quantitated as the percentage of maximal binding of the antibody to the plate in the absence of competitor. Prothrombin (●), fragment 1 (■), abnormal (des-γ-carboxy) prothrombin (○), prethrombin 1 (▲), and (*p*-amidinophenyl)methanesulfonyl fluoride-inhibited α-thrombin (△) were evaluated. The results of antibody derived from clones RL 1·1, 1·3, 1·6, and 1·8 are illustrated. Data from all of the clones are presented in Table I.

showed no significant binding in the presence of 7 mM EDTA. In contrast, antibodies from RL 1·5 bound prothrombin similarly in the presence of $CaCl_2$ or in the presence of EDTA (Figure 3B). These experiments indicate that the monoclonal antibodies from clone RL 1·3 are specific for the conformer of prothrombin stabilized by calcium ions.

Purification of Antibodies. Monoclonal antibodies from clones RL 1·3 and RL 1·5 were isolated from ascitic fluid of mice injected with each of these clones. Ascitic fluid was applied to a prothrombin–agarose column in buffers containing $CaCl_2$. Antibodies from the RL 1·3 ascitic fluid could be eluted from the prothrombin–agarose column with 70 mM EDTA, as expected (Figure 4A). No additional anti-prothrombin antibody was eluted with 4 M guanidine hydrochloride. The purified RL 1·3 monoclonal antibodies were used in subsequent experiments. Anti-prothrombin antibodies from the RL 1·5 ascitic fluid could not be eluted from the prothrombin–agarose with 70 mM EDTA (Figure 4B). Antibodies were eluted with 4 M guanidine hydrochloride and were found to be fully active against prothrombin after dialysis into 0.05 M Tris-HCl, pH 7.2–0.14 M NaCl.

Characteristics of Antibody–Prothrombin Interaction. The binding of purified RL 1·3 antibody with prothrombin was studied by using the solid-phase ELISA method (Frankel & Gerhard, 1979). The amount of anti-prothrombin bound to the plate was determined from a standard curve prepared on the basis of the binding of antibody of known concentration to wells coated with excess prothrombin. On the basis of these data, a Scatchard plot was prepared (Figure 5). The binding constant, K_a, was observed to be $2.3 \times 10^9\ M^{-1}$. The binding curve was linear over the concentration range studied. This indicates a single population of antibody combining sites, anticipated for a monoclonal antibody preparation.

The relationship of the antigenic determinants against which monoclonal RL 1·3 antibody and polyclonal rabbit anti-prothrombin–Ca(II) antibody (Blanchard et al., 1983) are directed was evaluated in a competition immunoassay. RL 1·3 antibody was displaced from prothrombin by anti-pro-

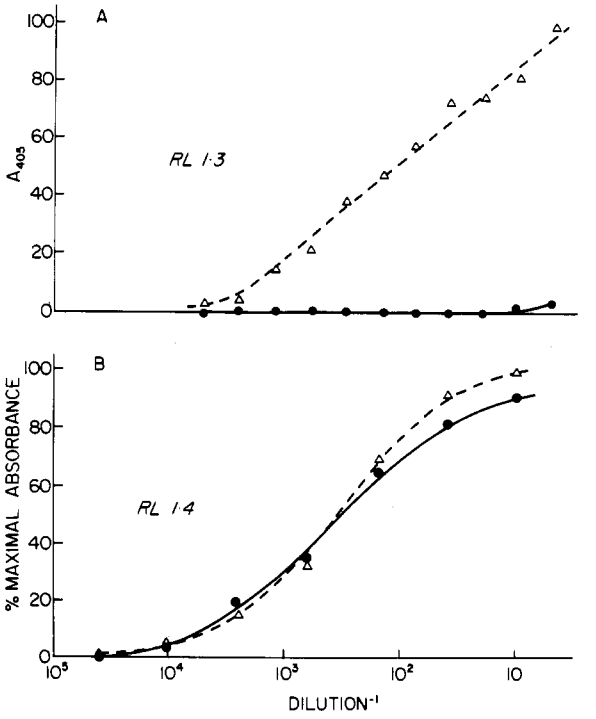

FIGURE 3: Effect of calcium on anti-prothrombin antibody–prothrombin interaction. Culture supernatants from clones RL 1·3 and RL 1·5 were studied for anti-prothrombin binding activity in the presence of $CaCl_2$ (△) or EDTA (●) by the ELISA direct-binding system. The amount of monoclonal antibody bound to the plate, proportional to the absorbance at 405 nm, is plotted against the dilution of the culture supernatant. $CaCl_2$, 5 mM; EDTA, 7 mM.

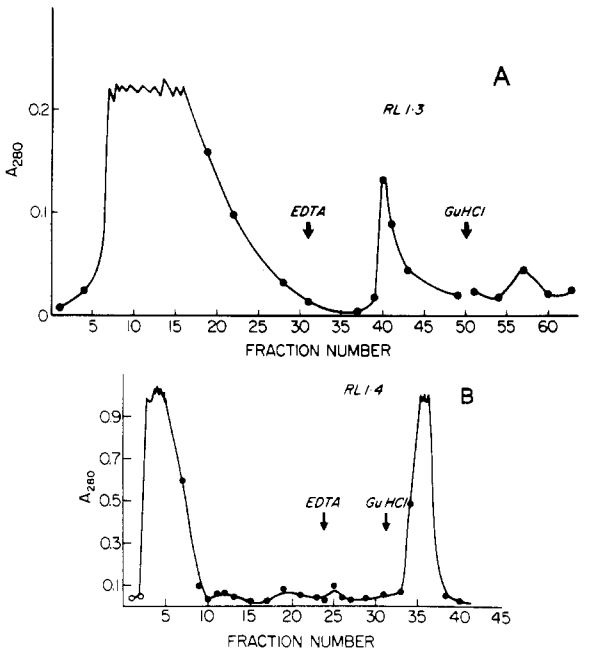

FIGURE 4: Purification of monoclonal antibodies by affinity chromatography. Anti-prothrombin antibodies were affinity purified from ascites on a column containing prothrombin–agarose. The column was equilibrated with Tris-HCl, pH 8.1, 1 M NaCl, and 7 mM $CaCl_2$. The ascitic fluid (2 mL) was applied to the column. After the optical density returned to the base line, the conformation-specific antibodies were eluted with 70 mM EDTA. After the base line was reestablished, the remaining bound antibodies were eluted with 4 M guanidine hydrochloride. (A) Ascites prepared from clone RL 1·3, which synthesizes a monoclonal antibody specific for the metal-stabilized conformer of prothrombin. (B) Ascites prepared from clone RL 1·5, which synthesizes antibody that binds prothrombin regardless of the presence or absence of calcium ions.

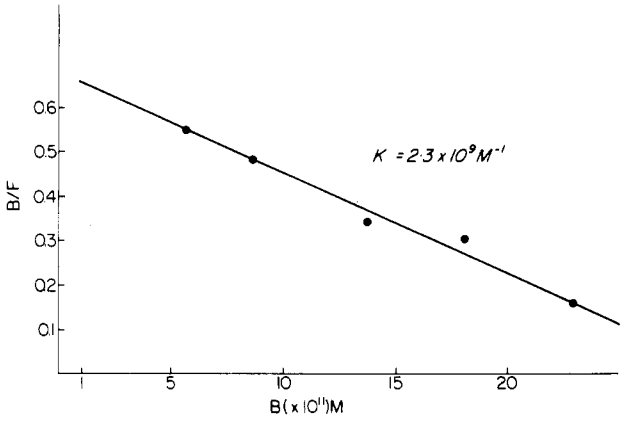

FIGURE 5: Scatchard analysis of the binding of prothrombin-purified anti-prothrombin monoclonal antibody derived from clone RL 1·3. The solid-phase ELISA system was employed. B/F, concentration of bound antibody divided by the concentration of unbound anti-antibody; B, concentration of bound antibody. $K_a = 2.3 \times 10^9\ M^{-1}$.

thrombin–Ca(II), indicating that these antibodies compete for similar or overlapping sites in the fragment 1 domain.

Interaction of Conformation-Specific Antibodies with Prothrombin. The interaction of antibodies derived from the RL 1·3 clone and prothrombin was studied over a wide range of calcium concentrations. The ELISA method was employed. For elimination of contaminating calcium, plates containing immobilized prothrombin were washed with a buffer containing EDTA and then exhaustively washed with 0.05 M Tris-HCl, pH 7.2–0.14 M NaCl prepared with metal-free water. As shown in Figure 6, antibody binding to prothrombin

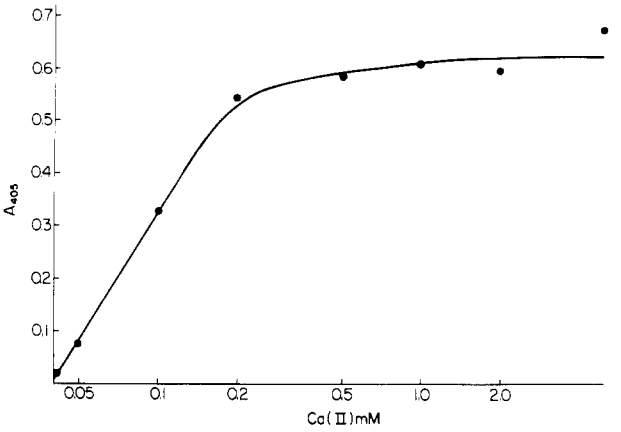

FIGURE 6: Calcium-dependent interaction of prothrombin with monoclonal antibodies derived from clone RL 1·3. The ELISA system was employed. By use of RL 1·3 anti-prothrombin monoclonal antibodies and prothrombin immobilized on the plate, the amount of antibody that bound to the plate (proportional to the absorbance at 405 nm) was studied as a function of the $CaCl_2$ concentration in solution. Half-maximal antibody binding was observed at a calcium concentration of 0.10 mM.

was calcium dependent. Maximal binding was observed at 0.9 mM $CaCl_2$ and half-maximal binding observed at 0.10 mM $CaCl_2$. This correlates well with previous data obtained with polyclonal anti-$(12–44)_N$ antibodies (Furie & Furie, 1979) and anti-prothrombin–Ca(II) antibodies (Tai et al., 1980).

The effect of other cations on antibody–antigen interaction was evaluated by the same methods. In contrast to metal-free buffer, buffers containing Mg(II), Mn(II), Co(II), and Ca(II) support antibody–antigen interaction. Half-maximal antibody binding was similar for Ca(II), Mg(II), and Co(II). Lower concentrations of Mn(II) were effective in supporting antibody–prothrombin interaction. These data are in good accord with previous data obtained with polyclonal antibody fractions (Furie & Furie, 1979).

Discussion

Monoclonal antibodies provide certain advantages in the application of immunochemical techniques to the study of macromolecular structure. Although conformation-specific monospecific polyclonal antibodies have proved useful, the quantity of antibody isolated has been limited and the isolation procedure protracted. These antibodies are generally purified from complex antiserum by immunoabsorption techniques that often rely on affinity chromatography. Specific subpopulations can be obtained that are directed against antigens of functional or structural importance. These antibodies are heterogeneous with regard to charge, antigen affinity, and antigenic determinant. By use of hybridoma methodology (Köhler & Milstein, 1975), homogeneous monospecific antibody may be selected for unique binding properties to a protein, nucleic acid, or cell surface. They can be produced by facile methods and can be isolated in large quantity.

In the current work, we have described the properties of antibodies from eight separate clones and have focused our attention on the antibodies produced by two clones of special interest. We have provided circumstantial evidence for the clonal nature of each of these cell lines: (1) a statistical analysis based upon the limiting dilution method for cloning cells; (2) the secretion of antibody with a single heavy-chain subclass and a single light-chain type; (3) the presence of a single class of high-affinity antigen binding sites on antibodies derived from clone RL 1·3. The eight antibodies studied show this evidence for monoclonality. However, definitive studies

by the determination of the primary structure of the hypervariable region of the light and heavy chains have not been performed. By comparison of the antibody subclass, antigen specificity, and antigen affinity, it would appear that each of these clones yields a unique antibody.

The eight antibodies that were characterized are directed against the fragment 1 region of prothrombin, a domain containing the γ-carboxyglutamic acid residues. This region represents about one-third of the primary structure of prothrombin. This region is known from our earlier studies to be highly immunogenic. Of the anti-prothrombin antibodies isolated from rabbit anti-bovine prothrombin antiserum, about two-thirds of the antibodies were directed against fragment 1 (Furie et al., 1978). The fact that eight of eight monoclonal antibodies to prothrombin bind to fragment 1 is much higher than expected. We have shown that polyclonal anti-prothrombin antibodies directed against prethrombin 1 interact with prothrombin immobilized on microtiter plates.[2] Therefore, the absence of monoclonal antibodies to the prethrombin 1 region of prothrombin is not due to problems of the screening ELISA system used.

Monoclonal antibodies to prothrombin bound prothrombin preferentially over fragment 1. The amount of fragment 1 needed to inhibit 50% of antibody binding to the plate varied from 2-fold to 10-fold the amount of prothrombin needed. In our prior studies with polyclonal rabbit anti-$(12–44)_N$ antibodies (Furie et al., 1978) and anti-prothrombin–Ca(II) antibodies (Tai et al., 1980), these immunochemical reagents reacted equivalently with bovine prothrombin and fragment 1. The current data emphasize that the three-dimensional structure or the conformational flexibility of human prothrombin and fragment 1 is not equivalent. A full discussion of this concept, as it relates to factor X, is presented elsewhere (Keyt et al., 1982). It is likely that the monoclonal antibodies, directed against a single discrete portion of the antigenic surface of the protein, can detect subtle changes in the protein surface to which polyclonal antibodies are relatively insensitive. The binding of heterogeneous polyclonal antibodies to a protein is studied as an averaged phenomenon, and small populations emphasizing certain facets of antigenic differences may be lost. These results emphasize the special utility of monoclonal antibodies as probes of protein structure. Although fragment 1 has been a useful model for the γ-carboxyglutamic acid containing domain of prothrombin, these studies raise questions as to whether all information gleaned from fragment 1 may be extended to prothrombin.

The precise localization of the determinants against which the monoclonal antibodies are directed is difficult without a known three-dimensional structure and a series of variants with different primary structure. Nevertheless, the fact that the eight monoclonal antibodies cross-react poorly (1·5, 1·7, 1·8) or not at all (1·1, 1·3, 1·4, 1·6, 1·9) with abnormal prothrombin indicates the importance of γ-carboxyglutamic acid as part of the antigenic determinant or its contribution to the stabilization of antigen conformation. The absence of binding of antibodies to bovine prothrombin is curious and may relate to the negative selection of clones during the screening procedure in which the fetal calf serum (containing prothrombin antigen) inhibited the ELISA.

Antibodies generated from a single clone, RL 1·3, are specific for the metal-stabilized conformer of human prothrombin. This monoclonal antibody shares many of the functional features of polyclonal antibodies directed against the bovine and human prothrombin–Ca(II) complex (Tai et al., 1980; Madar et al., 1982; Blanchard et al., 1983). Antibodies from RL 1·3 bind prothrombin with high affinity but only in the presence of metal ions. Half-maximal binding of antibody and prothrombin was observed at a $CaCl_2$ concentration of 0.1 mM. Anti-$(12–44)_N$ (Furie & Furie, 1979) and anti-prothrombin–Ca(II) (Tai et al., 1980) antibodies made against bovine prothrombin indicated a half-maximal transition of the metal-induced conformational change at 0.2 mM $CaCl_2$. These values show good concordance.

The preparation of a conformation-specific monoclonal antibody directed against the metal-stabilized conformer of prothrombin bears significantly on the molecular recognition properties of this functional class of antibody. Given the above evidence for monoclonality, this antibody is homogeneous and directed against a single antigenic site on prothrombin. This site is formed only in the presence of metal ions. The fact that these antibodies bind such a metal-stabilized determinant with absolute specificity and high affinity proves that, unlike properties such as antigen precipitation, conformation-specific antibodies are not, of necessity, polyclonal and directed against multiple determinants on the protein surface. Rather, a monoclonal antibody with a unique antibody combining site can recognize and bind with high affinity a metal-stabilized antigenic determinant on a protein surface.

Conformation-specific monoclonal antibodies have significant potential for the study of conformational transitions in proteins in simple and complex biological solutions and on membrane surfaces. Given the monospecificity of the antibody, the antigenic determinant on the macromolecule against which the antibody is directed may be precisely identified. The recent work of Berzofsky et al. (1980, 1982) illustrates this approach elegantly as it has been applied to myoglobin as a protein antigen. Furthermore, monoclonal conformation-specific antibodies can facilitate studies of the structure of proteins in complex protein systems. This may be done in either solution or the solid phase, as on membrane surfaces. Hyafil et al. (1981) have employed a monoclonal antibody to uvomurolin, a calcium-binding membrane protein that may play an important role in cell–cell adhesion. In our own studies of abnormalities of vitamin K dependent carboxylation of prothrombin (Blanchard et al., 1981), the conformation-specific monoclonal antibodies described here will prove useful.

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Registry No. Prothrombin, 9001-26-7; Ca, 7440-70-2; Mn, 7439-96-5; Co, 7440-48-4; Mg, 7439-95-4.

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Analysis of the Rate-Limiting Step in a Ligand–Cell Receptor Interaction: The Immunoglobulin E System†

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ABSTRACT: Theory predicts that the kinetics of simple interactions between a ligand and a receptor bound on the surface of a cell will be affected by the occupancy of receptors on the same cell. In a diffusion-limited reaction the effect will be on the rate of dissociation but *not* on the rate of association until the cell is virtually saturated with ligand. If the rate of reaction is not diffusion limited, then the opposite holds; i.e., the forward velocities will be proportional to the concentration of vacant receptors, but the reverse reactions will not be. We examined the kinetics of reaction between immunoglobulin E (IgE) and its receptor and clearly demonstrated that the reaction is not diffusion controlled. The substantial ($\simeq$30-fold) increase in the forward rate constant observed for the reaction of IgE with solubilized receptors as opposed to cell-bound receptors is therefore not an artifact of calculation. Since the reverse rate constants show little difference, we postulate that the presence of other surface components (rather than conformational differences in the receptor) affects the reaction with the cells. As an aid to the analysis, the theory has been extended so that not only the rate constants but also the entire course of the reaction of ligand with cell receptors can be predicted for diffusion-limited vs. non-diffusion-limited interactions.

Recent theories of ligand–receptor interactions make a number of predictions bearing on cellular recognition that have not been previously tested. For example, if the reaction between ligand and cell surface receptors is diffusion controlled, the rate of binding is predicted to be the same as it would be if the surface were completely covered by receptors (Berg & Purcell, 1978), even when as little as a few tenths of a percent of the surface is in fact covered. An experimental consequence is that the forward reaction rate will be independent of the number of free receptors per cell throughout almost the entire reaction. Again, under such diffusion-limited conditions and with $\gtrsim 10^4$ unoccupied receptors, these theories indicate that the dissociation of labeled ligand from the cell surface will very likely be followed by rebinding to some other empty receptor

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