Ontogeny of Proteolytic Immunity

IgM SERINE PROTEASES*

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We report the chemical activity of immunoglobulin μ and κ/λ subunits expressed on the surface of B cells and in secreted IgM antibodies (Abs) found in the preimmune repertoire. Most of the nucleophilic reactivity of B cells measured by formation of covalent adducts of a hapten amidino phosphonate diester was attributed to μ and κ/λ subunits of the B cell receptor. Secreted IgM Abs displayed superior nucleophilic reactivity than IgG Abs. IgM Abs catalyzed the cleavage of model peptide substrates at rates up to 344-fold greater than IgG Abs. Catalytic activities were observed in polyclonal IgM Abs from immunologically naïve mice and humans without immunological disease, as well as monoclonal IgM Abs to unrelated antigens. Comparison of several IgM Abs indicated divergent activity levels and substrate preferences, with the common requirement of a basic residue flanking the cleavage site. Fab fragments of a monoclonal IgM Ab expressed catalytic activity, confirming the V domain location of the catalytic site. The catalytic reaction was inhibited by the covalently reactive hapten probe and diisopropylfluorophosphate, suggesting a serine protease-like mechanism. These observations indicate the existence of serine protease-like BCRs and secreted IgM Abs as innate immunity components with potential roles in B cell development and Ab effector functions.

Antigen-specific IgG Abs1 in autoimmune and alloimmune disease are described to catalyze chemical reactions (1–7). However, no consensus has developed whether naturally occurring catalytic Abs represent rare accidents arising from adaptive sequence diversification processes or genuine enzymes with important functional roles. The major reason is that the turnover (kcat) of antigen-specific IgG Abs is low. Some catalytic Abs express catalytic efficiencies (kcat/Km) comparable to conventional enzymes, but this is due to high affinity recognition of the antigen ground state (reviewed in Ref. 8).

Certain enzymes cleave peptide bonds by a mechanism involving the formation of a transient covalent intermediate of the substrate and a nucleophilic residue present in the active site. The nucleophiles are generated by intramolecular activation mechanisms, e.g. the activation of Ser/Thr side chain hydroxyl groups by hydrogen bonding to His residues, and can be detected by covalent binding to electrophilic phosphonate diesters (9, 10). Using these compounds as covalently reactive analogs of antigens (CRAs), we observed that IgG Abs express nucleophilic reactivities comparable to trypsin (11). Despite their nucleophilic competence, IgG Abs display low efficiency proteolysis, presumably due to deficiencies in steps occurring after formation of the acyl-Ab intermediate, viz., water attack on the intermediate and product release. Occupancy of the B cell receptor (BCR, surface Ig complexed to α and β subunits along with other signal transducing proteins) by the antigen drives B cell clonal selection. Proteolysis by the BCR is compatible with clonal selection, therefore, only to the extent that the release of antigen fragments is slower than the rate of antigen-induced transmembrane signaling necessary for induction of cell division. Immunization with haptons mimicking the charge characteristics of the transition state (12) has been suggested as a way to surmount the barrier to adaptive improvement of catalytic rate constants. Catalysis by designer IgG Abs obtained by these means, however, also proceeds only slowly.

In mice and humans, the initial Ab repertoire consists of ~100 heritable VL and VH genes. Adaptive maturational processes expand the repertoire by several orders of magnitude. The initial BCR complex on the pre-B cell surface contains V-(D)-J rearranged Ig μ chains as a complex with surrogate L chains (reviewed in Ref. 13). Precise assignment of the B cell differentiation stage at which cell division becomes antigen-dependent is somewhat ambiguous, but it is generally believed that non-covalent antigen binding to the pre-BCR is not required for initial cell growth. κ/λ chains replace the surrogate L chain at the later stages of antigen-driven B cell differentiation, which is accompanied by diversification via somatic hypermutation processes and continued gene rearrangements (14, 15). V-(D)-J gene rearrangements allow development of specificity for individual antigens by IgM (16) but antigen binding affinities tend to be low compared with IgG Abs. Somatic mutations accumulating in the V domains following isotype switching to IgG promote high affinity antigen recognition. In some anatomic locations, IgM Abs can be extensively
and mutated and can display high affinity antigen binding (17). Loss of a membrane anchoring peptide at the C terminus of the H chain results in production of secreted IgM and IgG Abs.

Very little information is provided about the developmental aspects of Ab catalysis. Here, we report the nucleophilic reactivity of secreted IgM and the IgG subunits expressed on the surface of B cells. Cell surface μ and α chains were the major sites of covalent reaction of a haptan CRA with B cells, and the magnitude of nucleophilic and proteolytic activities of secreted IgM Abs was consistently superior to IgG Abs.

EXPERIMENTAL PROCEDURES

Splenocyte-CRA Binding—Synthesis of compounds I-IV and confirmation of their chemical identity have been published (11, 18). Compounds I, III, and IV are diphenyl phosphonate esters reactive with nucleophilic sites (9, 10, 18). Biotin incorporated in these compounds was from Sigma. DFP was from Sigma. BALB/c mice (5–6 weeks, female, Jackson Laboratories) were euthanized by cervical dislocation and splenocytes were subjected to deconvolution (5 iterations) for each probe (FITC; emission at 525 nm, exc 470 nm; DAPI; emission at 578 nm). Flow cytometry was performed employing an Olympus IX-70 inverted microscope and Applied Precision Delta work station (SoftWoRx™ software; Ref. 19). Stained cells were subjected to multiple acquisitions at a thickness of 0.25 m, and the images were stacked. The images were subjected to deconvolution (5 iterations) for each probe (FITC; λex 488 nm, Λem 525 nm; DAPI; λex 350 nm, λem 470 nm; phycoerythrin; λex 585 nm, Λem 620 nm). Flow cytometry was performed in the Baylor Medical College Core Facility (EPICS XL-MCLs Beckman-Coulter flow cytometer, EXP320 software). Instrument calibration to minimize cross-detection of PE and FITC was done using cells stained individually with these fluorochromes. Forward and side scatter measurements allowed exclusion of dead cells from the gated cell population. CRA-stainable cells were identified as the population showing staining above the level observed for compound II staining. CRA-stainable CD19+ cells were estimated by subtraction of background observed using the isotype-matched Ab. Cell extraction was by treatment with the detergent CHAPS (12 mM, 2 h at 4 °C). The extract was centrifuged (10,000 × g, 30 min), the supernatant diluted with PBS to 1 mM CHAPS and then subjected to affinity chromatography using goat polyclonal Abs (IgG) to mouse μ, δ, ε, and κ chains (Caltag) immobilized on protein G-Sepharose columns (100 μl of settled gel; 0.5 × 5 cm columns; Amersham Biosciences). For this purpose, the Abs (50 μg) were mixed with the protein G gel in a column (15 min, 4 °C) in PBS containing 1 mM CHAPS (PBS-CHAPS), the gel allowed to settle, the unbound fraction collected and the columns washed with PBS-CHAPS. The cell extract (1.4 ml; diluted to 1 mM CHAPS; from 3 × 108 cells) was passed through the column, the column washed with PBS-CHAPS (9 volumes) and bound proteins were eluted with 100 mM glycine-HCl, pH 2.7 (8 volume columns) and subjected to reducing SDS-polyacrylamide gel electrophoresis (4–20%, Bio-Rad). Protein-CRA adducts were visualized by staining nitrocellulose electrobLOTS of the gels with streptavidin-peroxidase as in (11). For immunoblotting, the blots were stained with goat polyclonal IgG (mouse Ab; 10 μg/ml or as stated; Molecular Probes, Eugene, OR) and PE-conjugated isotype-matched rat Abs to an irrelevant Ab (c23.4 (anti-VIP; C); 3 cycles; Ref. 20) followed by affinity chromatography on the anti-human IgM column. IgG was purified on protein G-Sepharose columns (21) using as starting material the unbound fraction from the anti-IgM cell columns or cell-free ascites. FPCPL gel filtration of IgG was as described in (21) while that a Supergel with 1 ml was employed. IgM precipitates were prepared by digesting IgM (300 μl, 1 mg/ml) with agaroase-conjugated pepsin (0.6 ml of gel, 30 min, 37 °C) in 100 mM sodium acetate, pH 4.5, 150 mM NaCl, 0.05% NaN3, 0.1 mM CHAPS) as recommended by the manufacturer (Pierce). The unbound fraction was dialyzed against buffer B, purified by FPLC gel filtration on a Superose 12 column and dialyzed against 50 mM Tris-HCl, pH 7.7, 0.1 mM glycine, 0.1 mM CHAPS. Total protein was determined by the biinchoninic acid method (Pierce). Immuno blotting of SDS-gels containing murine Ab was as in the preceding section. Human Ab gels were immunoblotted using peroxidase-conjugated goat anti-human μ, anti-human κ and anti-human λ Abs (Sigma).

Secreted Ab-CRA Binding—Human serum Abs were from subjects without evidence of infection or immunological disease (2 females, 3 males; ages 23–45 years). Serum Abs were from BALB/c mice (purchased from Harlan, Indianapolis, IN, pooled from 150 mice; 8–12 weeks). Murine monoclonal IgM Abs used here are directed against major histocompatibility antigens (clones corresponding to catalog 8702, 8704, 9008, 9010, 9020; cell-free ascites; Cedarlane, Ontario, Canada). Monoclonal IgM Ywo is from a patient with Waldenström’s macroglobulinemia (20). All monoclonal IgM Abs contain κ chains. The 4 murine monoclonal IgG Abs used here were: clone c23.4 (anti-VIP; Ref. 6), clone c39.1 (anti-glucagon), ATCC clones HP6045 (anti-Fabb, γC) and ATCC clone HP6054 (anti-Ig λ chain). All monoclonal IgG Abs contain κ heavy chains and κ light chains. Serum or ascites fluid (1 ml) was mixed for 1 h with 1 ml of Sepharose BB-conjugated rat anti-mouse IgM Abs (settled gel; Zymed Laboratories Inc., San Francisco, CA) or agaroase-conjugated goat anti-human IgM Abs (Sigma) with IgM binding capacities 0.8 and 3 mg, respectively, in 50 mM Tris-HCl, pH 7.5, 0.1 mM CHAPS (buffer A). The unbound fraction was recovered and the gel washed with 20-buffer A volumes taking care that protein in the effluent had returned to undetectable levels prior to elution (λ280 < 0.001). Elution was with 100 mM glycine pH 2.7 (0.5 ml/fraction) into 25 μl of 1× Tris-HCl, pH 9.0. Further purification was on a Superose-6 FPLC gel filtration column (1 × 30 cm; 25 ml/min; Amersham Biosciences) in two different solvents: 50 mM Tris-HCl, pH 7.7, 0.1 mM glycine, 0.025% Tween-20 (buffer B), or 6 mM guanidinium hydrochloride in buffer B adjusted to pH 6.5 with HCl (buffer C). Prior to column fractionation, the affinity purified IgM was dialyzed against buffer C. Column calibration was with thyroglobulin (660 kDa), IgG (150 kDa), and albumin (67 kDa). IgM with 900 kDa eluted close to the void volume of the column. IgM was renatured following buffer C chromatography by dialysis against buffer B (21). IgM Ywo, a cryoglobulin, was purified from serum by repetitive warming (37 °C) and cooling (4 °C; 3 cycles; Ref. 20) followed by affinity chromatography on the anti-human IgM column. IgG was purified on protein G-Sepharose columns (21) using as starting material the unbound fraction from the anti-IgM cell columns or cell-free ascites. FPLC gel filtration of IgG was as described in (21) while that a Supergel with 1 ml was employed. IgM precipitates were prepared by digesting IgM (300 μl, 1 mg/ml) with agaroase-conjugated pepsin (0.6 ml of gel, 30 min, 37 °C) in 100 mM sodium acetate, pH 4.5, 150 mM NaCl, 0.05% NaN3, 0.1 mM CHAPS) as recommended by the manufacturer (Pierce). The unbound fraction was dialyzed against buffer B, purified by FPLC gel filtration on a Superose 12 column and dialyzed against 50 mM Tris-HCl, pH 7.7, 0.1 mM glycine, 0.1 mM CHAPS. Total protein was determined by the biinchoninic acid method (Pierce). Immuno blotting of SDS-gels containing murine Ab was as in the preceding section. Human Ab gels were immunoblotted using peroxidase-conjugated goat anti-human μ, anti-human κ, and anti-human λ Abs (Sigma).

Proteolysis Assays—Cleavage of the amide bond linking amionothymylcumarin to the C-terminal amino acid in peptide-AMC substrates (Peptide International, Louisville, KY or Bachem, King of Prussia, PA) was measured in 50 mM Tris HCl, pH 7.7, 0.1 mM glycine, 0.025% Tween-20 at 37 °C in 96-well plates by fluorimetry (λex 360 nm, λem 470 nm). Authentic amionothymylcumarin was used to construct a standard curve. Kinetic parameters were obtained by fitting rate data obtained at increasing concentrations of peptide-AMC substrates to the Michaelis-Menten-Henri Equation 1.

\[ v = \frac{V_{\text{max}} [S]}{K_{\text{m}} + [S]} \]  

(Eq. 1)

Progress curves in the presence of inhibitors were fitted to Equation 2,

\[ \frac{[\text{AMC}]_{\text{max}}}{[\text{AMC}]_{\text{max}}} = 1 - e^{-k_{\text{cat}}r} \]  

(Eq. 2)

where \([\text{AMC}]_{\text{max}}\) is the AMC concentration in the absence of inhibitor. \(I_{\text{50}}\) (concentration yielding 50% inhibition) was obtained from Equation 3,

\[ \% \text{ inhibition} = 100/(1 + 10^{(\text{IC}_{50} - \text{log}[\text{inhibitor}])}) \]  

(Eq. 3)

with the curve forced through 0.

a S. Paul, unpublished observations.
RESULTS

Irreversible CRA-B Cell Binding—Hapten CRAs such as compound I (Fig. 1) react irreversibly with nucleophilic sites in conventional serine proteases and Abs (9–11,18). To evaluate the nucleophilic reactivity expressed on the surface of B cells in the preimmune repertoire (viz., the repertoire developed spontaneously without purposeful immunological challenge), viable splenocytes from BALB/c mice were treated with hapten CRA I. The control compound II is identical in structure to hapten CRA I, except that the phosphonate group is not esterified, which results in loss of covalent reactivity with nucleophilic residues (11,18). Treatment with hapten CRA I resulted in staining of most of cells at levels greater than compound II, with a minority of the cells displaying intense staining (11 ± 2, n = 3 experiments; determined by counting 400 lymphocytes using a UV microscope). All of the CRA I-stained cells displayed lymphocytic morphology, with no evident staining of monocytes or the occasional basophil. No loss of viability of the cells was evident following incubation with CRA I or compound II, as determined by trypan blue exclusion. Flow cytometry confirmed the microscopy results. Seventy-nine percent of the CRA I-treated cells displayed fluorescence intensities exceeding the compound II-treated cells, including a minority subpopulation with very high fluorescence intensity (14%; subpopulation 2 in Fig 2A). In three repeat experiments, the proportion of CRA I-stained cells that were positive for the B cell marker CD19 was 82 ± 4% (Fig 2B). Deconvolution microscopy indicated that the fluorescence pattern due to hapten CRA I binding was nearly coincident with the anti-CD19 Ab fluorescence pattern (Fig. 2, C–E). Most of the CRA fluorescence was restricted to the surfaces of the B cells (Fig. 2F).

To identify the nucleophilic molecules on the cell surface, purified B cells were labeled with CRA I, detergent extracts of the cells were boiled and then analyzed by SDS-electrophoresis. Only limited CRA-containing proteins were evident (Fig. 3A). As expected, silver staining revealed the presence of heterogeneous species, reflecting the complex protein constitution of the cells. The mass of the predominant CRA adduct band was 70 kDa, and this band was stainable by anti-μ Ab (Fig. 3B). Smaller amounts of CRA-containing bands were evident at 25, 40, 50, 55–60, 90–135, and 140 kDa. The bands at 55–60 kDa and 140 kDa were stainable by the anti-μ Ab, and the bands at 25 and 50 kDa were stainable with anti-κ/λ Ab. The anomalous μ and κ/λ bands at mass range different from the full-length monomer proteins presumably represent unreduced oligomers, breakdown products and truncated B cell Ig products, as also observed in previous studies of secreted Abs and B cell extracts (22–24). The minor bands at 40 and 90–135 kDa that were not stainable with Abs to μ, γ, κ/γ (Fig. 3B) and δ chains (not shown) presumably represent non-Ig proteins. No CRA-containing adduct corresponding to Ig γ chains were detected. Immunoblotting of the cell extracts identified a band at 50 kDa stainable with anti-γ Ab, but the band was visible only in highly overexposed gels, suggesting that only small amounts of γ chains were present in the extract.

Confirmation that the CRA I adducts contain Ig subunits was by affinity chromatography on columns of immobilized Abs to μ, δ, γ, and κ/λ chains followed by SDS-electrophoresis (Fig. 3C). CRA-containing μ and κ/λ bands were evident in eluates from the anti-μ and anti-κ/λ columns. Recovery of CRA-containing μ chains in the eluate from the anti-κ/λ column can be explained by the presence of disulfide bonded light and heavy chain complexes on the cell surface. No CRA-containing bands were evident in eluates from the anti-γ and anti-δ columns (not shown), but this can not be interpreted to reflect deficient γδ chain nucleophilic reactivity, as these proteins are expressed only at low levels in B cells from immunologically naïve mice. To determine the proportion of overall cellular CRA staining attributable to complexation with Ig subunits, the B cell extract was fractionated on a single affinity column composed of immobilized Abs to μ and κ/λ chains. Eighty percent of the total CRA content of the cells was adsorbed by the column (not shown), determined by densitometry of the biotin-containing bands in the unbound fraction and the extract loaded on the column. Taken together, these observations indicate that most of the CRA staining of intact B cells is attributable to irreversible binding to surface Ig, with the μ chain accounting for most of the covalent reactivity.

Nucleophilic Reactivity of Secreted IgM—The initial velocity for formation of hapten CRA I adducts by IgM purified from the
Fig. 2. Hapten CRA I reactivity with spleen cells. A, flow cytometry of murine splenocytes (naïve BALB/c mouse) stained with biotinylated hapten CRA I (gray line) and compound II (black line); both compounds 100 μM, 4 h; streptavidin-FITC (50 μg/ml), 25,000 cells were counted. B, anti-CD19 Ab staining (gray line; phycoerythrin conjugate) of hapten CRA I-labeled cells; streptavidin-FITC 1 μg/ml). Black line shows staining with the phycoerythrin conjugate of the isotype matched control antibody. C–F, deconvoluted (3 iterations) fluorescence acquisitions showing two B cells labeled with CRA I (streptavidin–FITC, 1 μg/ml, panel C) and phycoerythrin-conjugated anti-CD19 Ab (panel D). E shows a merged rendition of the FITC and phycoerythrin probes. F is a three-dimensional wire frame model of the FITC emission patterns compiled from 30 individual sections and then subjected to split screen extraction. Blue counterstain, 4′,6-diamidino-2-phenylindole.

Fig. 3. Immunohistochemical identification of hapten CRA I-labeled Ig subunits in B cells extracts. A, SDS-gel electrophoresis lanes showing extract of B cells labeled with hapten CRA I (100 μM, 4 h) following staining with silver (lane 1) and peroxidase-conjugated streptavidin (lane 2). Migration of marker proteins shown on left. B, SDS-gel immunoblots of hapten CRA I labeled B cell extract stained with Abs to μ (lane 2), κ (lane 4), and γ (lane 6) chains. C, streptavidin-peroxidase stained SDS-gels showing hapten CRA I-labeled proteins recovered by affinity chromatography of splenocyte extract on immobilized anti-μ (lane 7), anti-κ (lane 8), and anti-γ Abs (lane 9).

Pooled serum of immunologically naïve BALB/c mice was 40-fold greater than by IgG (Fig 4A); values are sums of velocities for the reactions occurring at the two Ab subunits expressed per unit concentration of intact Abs). The velocity difference is 8-fold when expressed per unit combining site concentration3 (10 and 2 combining sites, respectively, in IgM and IgG). Three CRA-containing bands were observed in reducing SDS-gels of the IgM reaction mixtures at 70, 50, and 25 kDa (Fig. 4B). The 70 and 25 kDa bands were stainable with anti-μ and anti-κ/λ Abs, respectively. The 50-kDa band was stainable with anti-μ Ab and presumably represents a μ breakdown product. Two CRA-containing bands corresponding to γ and κ/λ chains were observed in reducing gels of the IgG reaction mixtures. Similar results were obtained with a panel of 6 randomly selected monoclonal IgM Abs (5 murine and 1 human) and 4 monoclonal IgG Abs (all murine). The monoclonal IgM Abs uniformly displayed superior rates of irreversible CRA I binding compared with the IgG Abs (Fig 4C; mean ± S.E.: 62.6 ± 24.4 × 104 and 1.9 ± 0.4 × 104 AAU/μM Ab/hour, respectively; p < 0.01, Mann-Whitney U test, 2-tailed). Consistent with the polyclonal Ab experiments, the μ chain accounted for most of the covalent binding in the polyclonal and monoclonal IgMs, but smaller levels of binding at the κ/λ chain subunit were also observed for every Ab preparation (for clarity, μ chain and the corresponding κ/λ chain data points from individual IgM preparations are connected in Fig. 4C; data are expressed per micromolar subunit concentration to allow ready comparison). The four monoclonal IgG Abs contain γ2a heavy chains, and all monoclonal IgM/IgG Abs contain κ light chains. No attempt was made to determine the nucleophilic reactivity of various γ chain isotypes. However, the polyclonal Ab data indicate that the average nucleophilic reactivity of the IgG isotype mixture in blood is lower than the IgM reactivity. A similar argument can be presented in regard to antigenic specificity. The 5 murine IgM Abs and 4 IgG Abs were raised by experimental immunization and bind different antigens (MHC antigens, VIP, glucagon, Ig subunits; Refs. 6 and 25 and specifications provided by the manufacturers). The sixth monoclonal IgM was from a patient

3 However, all 10 IgM valencies are usually not filled (e.g., Ref. 44).
with Waldenström's macroglobulinemia with unknown antigenic specificity (20). The monoclonal IgM Abs uniformly displayed superior reactivity to IgG Abs, suggesting that divergent antigenic specificities do not account for the reactivity difference.

One of the monoclonal IgM Abs, Yvo, was employed to help define the structural requirements favoring hapten CRA covalent binding. Compound II, which contains the unesterified phosphonate, did not form adducts with the IgM at incubation times up to 3 h (reaction conditions as in Fig. 3B). Similarly, the neutral hapten CRA III devoid of the amidino group and the hapten CRA IV with weak leaving groups (methyl instead of phenyl groups) failed to form detectable adducts with this IgM Ab. These reactivity characteristics are similar to those of IgG Abs reported previously (11).

Secreted IgM Catalytic Activity—The catalytic activity of polyclonal IgM and IgG prepared from pooled mouse serum was initially measured using Glu-Ala-Arg-AMC as substrate (Fig. 5A). Cleavage of the amide bond linking the AMC to the C-terminal Arg residue of this peptide has been validated as a surrogate for peptide bond hydrolysis by IgG Abs (21). Cleavage of Glu-Ala-Arg-AMC by polyclonal murine and human IgM fractions proceeded at rates 344-fold and 237-fold greater, respectively than the IgG fractions from the same sera (computed from initial velocity data; expressed per unit intact Ab concentration). If all 10 IgM valencies and both IgG valencies are filled, the velocities for individual combining sites of murine and human IgM are 69- and 47-fold greater than the corresponding IgG velocities. Consistent with the irreversible binding data in the preceding section, Glu-Ala-Arg-AMC cleavage by murine polyclonal IgM was inhibited by hapten CRA I (Fig. 5B) and the serine protease inhibitor diisopropylfluorophosphate (not shown; 63 and 93% inhibition at 30 and 100 μM DFP, respectively after 12 h). The deviation of the progress curve from linearity in the presence of CRA I suggests an irreversible inhibition mode (26). Progressively increasing inhibition of the murine IgM activity (9–100%) at increasing hapten CRA I concentrations (10–300 μM) was evident (IC_{50} 42 μM; not shown). Similar results were obtained using human polyclonal IgM as the catalysts (IC_{50} value for hapten CRA I inhibition, 111 μM).

**FIG. 4.** Irreversible hapten CRA I binding to IgM and IgG Abs. A, progress curves for polyclonal murine Ab-CRA adduct formation. AAU, Arbitrary area units. Reaction conditions: IgM 0.2 μM or IgG 1 μM (equivalent combining site concentration); hapten CRA I 0.1 mM. Values are means of intensities of the H chain-CRA and L chain-CRA bands for IgM (●) and IgG (○; means of closely agreeing duplicates). B, examples of reducing SDS-gel lanes showing CRA-Ab subunit adducts at 2 h. Lanes 4 and 5, Streptavidin-peroxidase-stained blots showing adducts of IgM subunits and IgG subunits, respectively. IgM subunits stained with Coomassie Blue, anti-μ chain Ab and anti-κ/λ chain are shown in lanes 1, 2, and 3, respectively. C, comparative initial velocities of hapten CRA I adduct formation at the subunits of IgM and IgG. Each point represents a different Ab. For comparison, data points corresponding to the μ and κ/λ chains of individual IgM Abs are connected. Abs studied: polyclonal human IgM, polyclonal mouse IgM, 5 monoclonal murine IgM Abs (clones 8702, 8704, 9008, 9010, 9020), monoclonal human IgG, polyclonal mouse IgG and 4 monoclonal IgG Abs (clones c23.4, c39.1, HP6045, HP6054). *p < 0.05 versus μ chain group in each case (Student’s t test, 2 tailed).

**FIG. 5.** Proteolytic activities of IgM and IgG Abs. A, cleavage of Glu-Ala-Arg-AMC (400 μM) by polyclonal murine IgM (●), polyclonal human IgM ( ■), polyclonal murine IgG (○) and polyclonal human IgG (□). IgM, 5 μM; IgG, 160 μM. B, inhibition of polyclonal murine IgM (5 nM) catalyzed Glu-Ala-Arg-AMC (400 μM) cleavage by hapten CRA I ( ●, 30 μM; □, 100 μM). ●, progress curve without inhibitor. Values are means of triplicates ± S.D.

Contamination of IgM with conventional proteases was studied by methods employed previously to validate IgG and Ab light chain enzymatic activities (21, 27). The IgM obtained by affinity chromatography on the anti-μ column displayed essentially identical levels of catalytic activity as the 900-kDa IgM fraction obtained by further purification by FPLC gel filtration (Fig. 6A). This fulfills the criterion of purification to constant specific activity required for assignment of enzymatic activity to IgM. Next, we examined IgM treated with 6 M guanidine hydrochloride to dissociate any noncovalently associated contaminants. For this purpose, the affinity purified IgM was subjected to three cycles of gel filtration in 6 M guanidine hydrochloride (Fig. 6B) and the 900 kDa fraction from the final gel filtration cycle was renatured by dialysis. Time-dependent Glu-Ala-Arg-AMC cleavage by IgM subjected to these procedures was observed (Fig. 6C).

Substrate selectivity of the polyclonal IgM preparations and 6 monoclonal IgM Abs was studied using a panel of 10 peptide-
Proteolytic IgM Antibodies

Fig. 6. IgM purity. A, purification of polyclonal murine IgM to constant specific activity. ○, IgM purified by anti-μ affinity chromatography; ●, affinity purified IgM subjected to further fractionation by FPLC gel filtration. IgM, 5 nM; Glu-Ala-Arg-AMC, 200 μM. B, denaturing gel filtration profiles (Superose 12 column) of polyclonal murine IgM conducted in 6 M guanidine hydrochloride. The IgM fractions under the bar from the first cycle of denaturing chromatography (−−−−) were pooled and subjected to 2 additional cycles of denaturing gel filtration. IgM recovered from the third chromatography cycle (−−−−−) was analyzed for catalytic activity in panel C. C, progress curve for cleavage of Glu-Ala-Arg-AMC (200 μM) by IgM (2.5 nM) purified by 3 cycles of denaturing gel filtration in panel B.

TABLE I
Cleavage preference of IgM Abs

| Substrate | Polyclonal, human | Polyclonal, murine | Yvo | Designations |
|-----------|------------------|--------------------|-----|--------------|
| AE-AMC    | ND*              | ND                 | ND  | 8702         |
| AAA-AMC   | ND*              | ND                 | ND  | 8704         |
| IIW-AMC   | ND*              | ND                 | ND  | 9008         |
| AAPF-AMC  | ND*              | ND                 | ND  | 9010         |
| EKK-AMC   | ND*              | ND                 | ND  | 9020         |
| VLK-AMC   | 1.6 ± 0.1        | ND                 | ND  |              |
| EAR-AMC   | 35.4 ± 0.7       | 86.4 ± 12.2        | ND  |              |
| IEGR-AMC  | 0.8 ± 0.1        | ND                 | ND  |              |
| PFR-AMC   | 5.6 ± 0.2        | 20.5 ± 4.4         | ND  |              |
| GP-AMC    | ND*              | ND                 | ND  |              |

* ND, not detectable (<0.125 μM/h/μM Ab).  
* NT, not tested.

AMC conjugates. The rates shown in Table I were computed as slopes of the progress curves. Only substrates containing a basic residue at the cleavage site were hydrolyzed by the IgM Abs. No hydrolysis was detected with substrates containing acidic and neutral residues at the cleavage site. All 6 monoclonal IgM Abs displayed catalytic activity, but the activity levels for different Abs were not identical (varying, for example, over a 24-fold range with Glu-Ala-Arg-AMC as substrate). The Abs displayed different substrate selectivity profiles. For example, the ratio of Glu-Ala-Arg-AMC and Ile-Glu-Gly-Arg-AMC cleavage rates varied from 0.9 to 30.0 for the 5 murine monoclonal IgMs, and the human monoclonal IgM cleaved the former substrate at a robust rate without cleaving the latter substrate detectably (Fig. 7). Hydrolysis of Gly-Gly-Arg-AMC and Gly-Gly-Leu-AMC by IgM 9020 was compared to confirm the requirement for a basic residue at the cleavage site. These substrates are identical except for the Arg-AMC/Leu-AMC linkage, eliminating the possibility of confounding remote residue effects. Cleavage of Gly-Gly-Arg-AMC was detectable, but cleavage of Gly-Gly-Leu-AMC was not (12.6 ± 0.6 and < 0.13 μM/μM Ab/hour, respectively).

The constant domain scaffold in the 5 murine monoclonal IgM Abs is identical. Observations of divergent catalytic activity levels and substrate selectivities suggested that the catalytic site is located in the V domains. To confirm this, IgM Yvo was digested with immobilized pepsin and Fab fragments were purified by gel filtration as the 55 kDa protein peak (Fig. 8A). Concentration-dependent cleavage of Glu-Ala-Arg-AMC by the Fab fragment was observed (Fig. 8B). Next, we considered the possibility that pepcid released from the column could be responsible for the observed Fab activity. The pH optimum of pepcid is 1.5–2.7 depending on the substrate (28). The catalysis assays were repeated in 0.1 M glycine, pH 2.7, 1 mM CHAPS. At Fab concentrations affording readily detectable catalytic activity at neutral pH (Fig. 8B), no detectable cleavage of Glu-Ala-Arg-AMC by the Fab was evident at pH 2.7. The cleavage site preference of pepsin (hydrolysis on the C-terminal side of aromatic and hydrophobic residues) is dissimilar to the basic residue preference of IgM Abs. Purified pepsin did not cleave...
Glu-Ala-Arg-AMC under conditions yielding readily detectable catalysis by the Fab (675 nM pepsin; other reaction conditions as in Fig. 8B). These data indicate that pepsin contamination is not a factor in the observed Fab activity.

Determination of reaction rates for 4 IgM preparations at increasing Glu-Ala-Arg-AMC concentrations indicated typical enzymatic kinetics (polyclonal murine and human IgM, monoclonal IgM 9020 and IgM 9008). The rates were saturable at excess substrate concentration and consistent with the Michaelis-Menten-Henri kinetics (Table II). Observed $K_m$ values were in the high micromolar range, reminiscent of the recognition characteristics of conventional proteases. Catalytic antibodies that are adaptively specialized to recognize individual antigens, on the other hand, display $K_m$ values in nanomolar to low micromolar range, e.g. IgG $c23.5$ shown in Table II cleaves the autoantigen VIP with $K_m$ 0.4 nM (from Ref. 6). Apparent $k_{cat}$ values for the IgMs exceed those reported previously for IgG (21).

**DISCUSSION**

IgM Abs, the first class of Abs produced by B cells, displayed superior nucleophilic and catalytic reactivities compared with IgG Abs. This contrasts with the noncovalent antigen binding function of Abs, which improves adaptively over the course of the immune response. The nucleophilic and catalytic IgM activities were identified in unimmunized mice and healthy humans. Preferential IgM recognition of the positive charged group adjacent to the phosphonate group of the hapten CRAs and the scissile bond in peptide substrates presumably reflect an intrinsic property of the preimmune Ab repertoire. This noncovalent recognition motif enables low affinity interactions with the peptide substrates, whereas traditional noncovalent epitope-paratope binding is characterized by high affinity antigen recognition (nanomolar range $K_m$).

Hapten CRA I was validated previously as a probe for nucleophilic reactivities expressed by serine proteases, including IgG Abs (11, 28). The extent of irreversible CRA binding activity correlates approximately with the catalytic activity (11, 29). In the present study, hapten CRA I adducts were located in close proximity to CD19 on the surface of B cells. The latter protein fulfills a signal transducing role as a component of the BCR throughout B cell development (30). Immunochemical and affinity chromatography studies suggested that the majority of the B cell surface staining is attributable to covalent binding by Ig subunits, with the $\mu$ chain providing the dominant contribution, and $\alpha/\lambda$ chains, a smaller contribution. This is consistent with the superior nucleophilic reactivity of the $\mu$ chain subunit of secreted IgMs. The control phosphonic acid hapten, which stained the cells poorly, does not react with nucleophiles due to the poor electrophilicity of the phosphorus atom (11, 18). Monoclonal BCRs were not included here, but all six monoclonal IgM Abs examined expressed nucleophilic reactivity, suggesting that the reactivity may also be expressed by a significant proportion of BCRs. A minority of the B cells was stained intensely by the CRA. These cells are of interest as a potential source of catalysts in future studies. Observations that both Ig subunits express nucleophiles are consistent with the ability of light and heavy chains to independently catalyze the cleavage of peptide bonds in the absence of their partner subunit (31). Site-directed mutagenesis studies have indicated a serine protease-like catalytic triad in the light chain of an IgG Ab (32) and the heavy chain of other IgG Abs is reported to contain nucleophilic Ser residues (e.g. Ref. 33).

Functional roles for serine protease activities have been deduced in B cell developmental processes, but the molecules responsible for the activities have not been identified to our knowledge. The serine protease inhibitors DFP and a-1 antitrypsin inhibit mitogen induced B cell division (34, 35) and up-regulate the synthesis of certain Ab isotypes by cultured B cells (35). The DFP-sensitive enzyme is B cell-associated and prefers Arg-containing substrates (36). Serine protease inhibitors are reported to inhibit anti-IgM induced BCR signal transduction (37), and anti-IgM mediated B cell activation is correlated with the appearance of a serine protease activity on the cell surface (38). Undoubtedly, conventional serine proteases may contribute to B cell regulation, but it remains that the major CRA binding components on the B cell surface evident in the present study are the BCRs themselves. It is logical to hypothesize, therefore, that stimulation of BCR nucleophilic sites may influence B cell development. Such compounds include naturally occurring serine protease inhibitors and reac-

**TABLE II**

**Apparent kinetic parameters for IgM catalysis**

| Substrate, Glu-Ala-Arg-AMC (25–600 μM); IgM, 5 nM. Correlation coefficients for fits to the Michaelis-Menten Equation 1 were ≥0.96 in every case. |
|---|---|---|
| Antibody | $K_m$ | $k_{cat}$ |
| IgM, murine serum | 120 ± 22 x 10$^{-6}$ | 2.1 ± 0.1 |
| IgM 9010 | 144 ± 15 x 10$^{-6}$ | 1.9 ± 0.1 |
| IgM 9020 | 154 ± 28 x 10$^{-6}$ | 0.9 ± 0.1 |
| IgM, human serum | 120 ± 11 x 10$^{-6}$ | 2.8 ± 0.1 |
| IgG c23.5 | 0.34 x 10$^{-9}$ | 8 x 10$^{-4}$ |

* From Ref. 6. Substrate, VIP.
tive carbonyl compounds capable of irreversible binding to nucleophilic amino acids (39).

Observations of divergent levels of catalytic activity of monoclonal IgM Abs, their differing substrate preference and retention of the activity in the Fab fragments suggest that the catalytic site is located in the V domains. The catalysis assays were conducted in solution phase and at excess concentrations of the small peptide substrate. These conditions will not support binding of a single peptide molecule by more than one Ab valency. Dissociation of antigen bound reversibly at the individual combining sites may increase antigen availability for neighboring sites. However, such an effect will influence the rate of catalysis only when initial antigen concentration is limiting, and there will be no change in the observed V_{max}. Therefore, multivalent binding by non-interacting sites (avidity effects) is an unlikely explanation for the superior activity of decavalent IgM compared with the divalent IgG. The following explanations can be presented for the superior IgM activity. First, loss of catalytic activity may be attendant to V domain somatic diversification after isotype switching from IgM to IgG. Second, distinctive IgM constant domain characteristics may be important in maintaining the integrity of the catalytic site, in which case isotype switching itself may result in reduced catalytic activity. These explanations are not mutually exclusive. Both explanations are consistent with the argument that catalysis is a disfavored phenomenon in the advanced stages of B cell development (as efficient BCR catalysis will result in reduced BCR occupancy). We did not attempt to address these points experimentally in the present study. However, the monovalent Fab studies suggested that disruption of the constant domain architecture of IgM is deleterious for catalysis. The Fab preparations displayed ~10-fold lower activity than computed for the individual combining sites of pentameric IgM. Pepsin employed to prepare Fab cleaves μ chains on the C-terminal side of the CH2 domain (40), which is distinguished by its conformational flexibility (41). Alterations of antigen binding activity when the same V domains are expressed as full-chain IgMs and IgGs have been noted (42), but we are not aware of IgM-IgG V domain swapping experiments in the literature. Allosteric combining site activation due to filling of individual Ab valencies has previously been considered in the case of divalent IgG preparations (43). The temporal sequence of events as the individual IgM combining sites bind antigen has not been elucidated. At excess antigen, only 5 of the 10 IgM combining sites are thought to be filled (e.g. Ref. 44), suggesting that favorable allosteric effects on antigen binding, if present, must be restricted to conditions of limiting antigen concentrations.

Our screening experiments were restricted to a few IgMs and a few commercially available substrates. Additional studies are necessary to define the physiological substrates for IgM Abs and the upper limit for catalytic rates. However, certain conclusions can be reached from the available data. Apparent Km values measured as the V_{max}/k_{cat} parameter (K_{app} ~ K_m). As illustrated for the anti-VIP IgG in Table II, large gains in catalytic competence occur due to enhanced antigen binding affinity (reduced K_m). Certain polypeptides are recognized by IgM Abs present in the preimmune repertoire with high affinity, for example, the superantigens staphylococcal protein A and HIV gp120(20) are recognized by IgM Abs containing VH3 family domains with K_m in the nanomolar range (46, 47). Moreover, specific IgM Abs with improved affinity for individual antigens emerge by adaptive V domain maturation processes (16, 48). Similarly, future study of catalytic IgMs specialized to recognize individual autoantigens is of interest. IgM Abs from patients with autoimmune disease express glycosidase activity (49). Autoimmune humans and mice tend to synthesize catalytic Ig Abs at increased levels (50–53), and a proteolytic IgG preparation to VIP is shown to interfere with the physiological smooth muscle relaxant effect of VIP (54).

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4 This is valid if k_{cat} is the rate constant for dissociation of the antibody-antigen noncovalent complex, is >> V_{max}/K_{app}, the rate constant for chemical transformation of the noncovalent complex.

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