ANTI-IgM-MEDIATED B CELL SIGNALING

Molecular Analysis of Ligand Binding Requisites for Human B Cell Clonal Expansion and Tolerance

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The perturbation of membrane immunoglobulin (mIg)[1] on B lymphocytes can have diverse effects on B cell physiology. Immature B cells and certain activated B cell populations are particularly susceptible to inhibition (tolerance) after mIg cross-linking (1–4), while mature, resting B cells are characteristically stimulated to DNA synthesis (4–6). Because both stimulatory and inhibitory signal transduction appear to involve similar early biochemical reactions (1, 6), it is of some interest that the ligand dose requirements for achieving these distinct functional phenomena have generally been found to differ by one or more orders of magnitude (3, 7–10). This suggests that the ligand binding requisites for triggering B cell tolerance may be significantly different from those for directly triggering B cell clonal expansion.

In this report, we have attempted to rigorously evaluate the binding requisites for eliciting the activation, or alternatively, inactivation of B lymphocyte DNA synthesis through mIg. With the use of a large panel of anti-human IgM mAbs, we examine how the site specificity, the affinity, and the valency of epitopes bound on mIgM affect the capacity for ligand-induced regulation of DNA synthesis in the appropriately sensitive human B lymphocytes. The data demonstrate that the IgM binding requisites for inducing inhibition of B cell DNA synthesis significantly differ from those for stimulation. These studies may be revelant to the control of human B cell clonal expansion by antiidiotype antibodies and rheumatoid factors.

Materials and Methods

Murine Anti-Human IgM mAbs and Fragments. All 10 murine anti-human IgM mAbs used in these studies have been described previously (11, 12). With the exception of 5D7 (IgG2a), all are of the IgG1 isotype. Fab' fragments of most anti-IgM mAbs were obtained by reduction of the corresponding $F(ab')_2$ fragments derived from pepsin digestion (13) (exceptions are mAbs 5D7 and IF11, which formed predominately Fab' upon exposure to pepsin). Un-

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[1] *Abbreviations used in this paper:* mIg, membrane Ig; SI, stimulation index; TBS, Tris-buffered saline.

digested intact antibody was removed by passage of digests over protein A-Sepharose (11). Nonbound $F(ab')_2$ fragments (or Fab' fragments in the cases of mAbs 5D7 and IF11) were further purified by sieving through a calibrated Sephacryl S-200 column eluted in 0.01 M Tris-HCl, 0.15 M NaCl, pH 7.3 (TBS), and run at <3 ml/cm^2/h. Analysis of protein in the concentrated (≥1 mg/ml) single sharp peak by 5–20% gradient SDS-PAGE under nonreducing conditions revealed only one major band with an average mol mass of 110 kD (or 52.4 kD for the 5D7 and IF11 mAbs). Production of Fab' fragments from $F(ab')_2$ involved reduction with 10 mM cysteine (Sigma Chemical Co., St. Louis, MO) and alkylation for 1 1/2 h at room temperature in the dark with 30 mM iodoacetamide (Sigma Chemical Co.). Filtration through a S-200 column yielded a single peak with an average molecular mass of 52.4 kD by nonreducing SDS-PAGE.

Iodination of mAb Fab' Fragments. Proteins were labeled with carrier-free ^{125}Iodine (New England Nuclear, Boston, MA) by the chloramine-T method (14). Labeled protein and reactants were separated on a PD-10 filtration column (Pharmacia Fine Chemicals, Piscataway, NJ) (previously equilibrated with 10% BSA-PBS-A and washed extensively with PBS-A until the OD_{280} = 0). The percent TCA precipitable counts in the pooled protein peaks of all labeled preparations was >90%. Specific activity of the iodinated fragments was determined by protein quantitation with a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA) using bovine gamma globulin as the standard (range, 2–6 × 10^6 cpm/μg). Each Fab' preparation was then made 1% with respect to BSA.

IgM-bindable activity was determined by passing each labeled Fab' fragment (~26 ng = ~80,000 cpm) over a 1-ml column of IgM-Sepharose containing a vast excess of IgM (6 mg), or alternatively, a 1-ml column of mock-coupled Sepharose beads. Labeled protein retrieved from the control Sepharose columns as nonbound ranged from 98 to 100%, while the IgM-Sepharose-bindable protein ranged from 85 to 94%.

Most of the iodinated mAb Fab' fragments were also compared in their relative binding affinities for human IgM with unlabeled parent Fab'. This involved a competition solid-phase RIA (12) on polyvinylchloride wells using a limiting amount of human IgM as coating antigen (2 μg/ml in PBS-A). A constant, saturating concentration of ^{125}I-Fab' was added to the wells, together with defined amounts of cold Fab', to achieve various ratios of labeled to unlabeled Fab' (1:8, 1:4, 1:2, 1:1, 2:1, 4:1, 8:1). After a 16-h incubation at 4°C and washing, the amount of bound radiolabeled ligand was measured. The expected as well as observed cpm bound were compared and used as an indicator of the relative binding efficiency of the iodinated vs. unlabeled Fab'. In all cases tested, the observed and predicted values did not differ by >50%, indicating that the iodination procedure did not appreciably diminish the native Fab' binding affinity.

Pentameric and Monomeric Human IgM. Purified IgM myeloma protein Pan (12) was used as the source of human pentameric IgM. Monomeric units of Pan-IgM were prepared by reduction with 0.003 M 2-mercaptoethylamine (2-MEA) for one-half h and alkylation with 0.006 M iodoacetamide (15). Monomer IgM was isolated by sieving the material through a calibrated Sephacryl S-300 gel filtration column eluted with 0.1 M Tris-HCl, 0.15 M NaCl, pH 8.0, and run at <2.5 ml/cm^2/h. Analysis of the fractions from two main peaks and one very minor peak by 5–15% gradient SDS-PAGE, under both reducing and nonreducing conditions, showed the ascending limb of the second peak to consist of nearly pure IgM monomer (nonreduced, 212 kD), with a very slight amount of H-L (half-molecules of monomer; nonreduced, 97.7 kD). Final yield of purified IgM monomer was ~8% of theoretical maximum.

Cell Sources. Nonmalignant human B lymphocytes were obtained from splenectomies following trauma, surgery for a splenic cyst, and for Felty's syndrome (11). B cell–enriched populations were obtained by twice rosetting out T cells with neuraminidase-treated SRBCs as described (11). When evaluated for cell surface markers by FACS analysis after indirect immunofluorescent staining, these populations were found to contain >85% Ig^+ and Leu-16 $(CD\text{-}20)^+$ B cells, <1% 89b1 $(CD3)^+$ T cells (16), <2% P9 (Leu M3)$^+$ monocytic cells (16), and <4% cells positive for the activation-associated molecule bound by mAb 4F2 (17).

Leukemic B cell specimens were contributed by Drs. Ilona Szer, Stephanie Seremetis, and Janet Cuttner. The Bia and Lan clonal cell populations were obtained by leukophoresis of

patients with chronic lymphocytic leukemia (CLL). Kon and Lub cells were from patients with hairy cell leukemia (HCL) and have been described elsewhere (16). At the time of sampling, all donor patients had peripheral white blood cell counts ≥ 262,000 cells/mm^3 with little or no contamination with nonleukemic cells, as confirmed by indirect immunofluorescence staining with T cell, monocyte, and B cell-specific mAb probes (16). FACS analysis of these leukemias with Ig heavy and light chain-specific mAbs and a fluorescein-conjugated goat F(ab')$_2$ anti-mouse Ig probe revealed the following membrane Ig phenotypes: Bia (μ, δ, λ), Lan (μ, δ, κ), Kon (μ, δ, κ), and Lub (μ, δ, κ). The B lymphoblastoid cell line Daudi (μ, δ, κ; American Type Culture Collection, Rockville, MD) was also used for some ligand-binding experiments. With the exception of Daudi, cells from each source were frozen at the same time in multiple aliquots and stored in the vapor phase of liquid nitrogen.

Cell Culture Conditions and T Cell Factors. T cell-depleted splenocytes and leukemic B cells were cultured at 2–2.5 × 10^5 cells per well in a volume of 0.2 ml as previously described (11, 16). DNA synthesis was assessed by uptake of an 18-h pulse of 1 μCi [^{3}H]thymidine (72.5 Ci/mmol; New England Nuclear, Boston, MA). Where raw data are illustrated, mean (X) cpm ± SEM from three identical cultures are reported. Data indicated as Δ cpm represent the difference between the cpm in test vs. control cultures. Index of stimulation (SI) values represent the quotient obtained by dividing the cpm measured in test wells by the cpm observed in control cultures.

Activated T cell supernatants containing growth factors for B cells were obtained from cultures of PHA-stimulated mixtures of peripheral blood cells of allogeneic individuals as described previously (11, 16). In some experiments, a partially purified B cell growth factor (BCGF) preparation was used (Cellular Products, Inc., Buffalo, NY).

Propidium Iodide Analysis. The DNA content of individual nuclei from cultured Kon leukemic cells was measured with the FACS IV using propidium iodide (PI) as a marker (5). Briefly, cells were centrifuged and stained with 50 μg/ml PI (Sigma Chemical Co.) in 0.1% sodium citrate and 0.1% NP-40 detergent (Sigma Chemical Co.). Approximately 10,000 nuclei from each sample were analyzed. Debris was excluded from analysis by gating based upon forward light scatter. The amount of DNA was assessed by the level of fluorescence emission at 608–655 nm after excitation at 488 nm. Segregation of cells in G_0 (G_1) vs. S, G_2, and M was based upon slope extrapolation as described (18). G_0 (G_1) cells were considered to be those falling between the gates set by linear extrapolation of the ascending and descending limbs of each diploid cell peak to baseline. Cells above the upper gate of the diploid peak were considered to be in S, G_2, or M.

Equilibrium-Binding Analysis. Assays used the phthalate oil method of separating cell-bound from free ligand (19). Before assay, cells were enriched for viability (generally >95%) by centrifugation through Ficoll/Hypaque at 4°C. The number of cells used varied, depending upon the mIgM density of the cell population, the specific activity of the mAb Fab' fragment, and its affinity for IgM. Assays with Bia cells used 1.3–3.6 × 10^7/ml; those with Dau = 1.2–2.5 × 10^7; Lan = 0.6–1.8 × 10^7/ml; Lub = 2.5–11.9 × 10^7/ml; and KE37 = 2.5 × 10^7/ml. Before each experiment, iodinated mAb Fab' fragments were ultracentrifuged at ~100,000 *g* for 8 min (Beckman Airfuge, Beckman Instruments, Inc., Fullerton, CA) with the top one-half used for analysis.

The equilibrium-binding experiments were performed as follows: 2:3 serial dilutions of iodinated mAb Fab' fragments in assay medium (DMEM + 1% BSA + 15 mM sodium azide with 50 mM 2-deoxy-D-glucose to block potential ligand internalization [20]) were added to cells in medium precoated 400-μl Eppendorf tubes to a final vol of 200 μl. Triplicates were run when label was used alone, and duplicates were used for assessing background binding (see below). The tubes were slowly rotated at 4°C until equilibrium was reached at 2 h, i.e., when a twofold increase in reaction time resulted in a <10% increase in bound ligand (21). After incubation, 150 μl of the cell mixture was gently layered over 150 μl of precooled phthalate oils (2:1 vol/vol mixture of dibutyl/dioctyl phthalate [Kodak]) in 7 × 50 mm (400 μl) polyethylene microfuge tubes (Beckman Instruments, Inc.) and centrifuged in a microfuge (Beckman Instruments, Inc.) at 12,000 *g* for <1 min at 22°C. Separation of the cell pellet from the nonbound ligand in the aqueous layer was achieved by freezing the tubes at −70°C and sawing off the tube tip. The cell-bound cpm in the pellet was determined with a gamma

counter. The data were analyzed by the LIGAND computer program (22) to give the equilibrium binding constants (K_a in 1/mol or M^{-1}) and the number of epitopes bound by mAb Fab' ligand, in addition to the percent coefficient of variance of these values (22).

Nonspecific binding of label to cells was always determined in two different ways: (*a*) by assaying the binding of ligand to cells in tubes containing a 100:1 pM ratio of pentamer IgM to Fab' ligand and (*b*) by measuring the binding of iodinated Fab' fragments to the T-lymphoblastoid cell line, KE37. Both gave comparable background levels. In addition, in many experiments, unlabeled Fab' was substituted for pentameric IgM, yielding similar results.

Immunoelectron Microscopy. Techniques detailed elsewhere were used (23, 24). Briefly, freshly cleaved mica sheets, onto which a thin film of carbon was coated, were serially floated onto: a 1% tryptophan solution; H_2O; mAb $F(ab')_2$ fragments, IgM monomer, or their immune complexes in 0.15 M ammonium acetate; 0.3 M ammonium acetate; and a 2% uranyl formate staining solution. Monomeric IgM and $F(ab')_2$ antibody were diluted in 0.5 M acetate to yield a 1:1 ratio at 1–3 μg/ml total protein. All reagents were placed as large droplets onto a Teflon ring slide and kept at 4°C. The polished down side of a copper grid was gently placed upon the carbon film floating on top of the stain. After insuring that the carbon membrane adhered to the grid, the grid-membrane complex was quickly removed from the surface of the staining well, and excess stain was removed by capillary action. In such a procedure, the carbon membrane folds back upon itself and thus traps a thin film of stain between two folds of the protein-coated film on the grid. The grids were examined on a JEOL CEM CX-100 electron microscope and photographed at 100,000 diameters magnification. Negatives were printed at a total magnification of × 260,000 for analysis.

Results

Domain and Epitope Specificity of Murine Anti-Human IgM mAbs. Table I lists the murine anti-human IgM mAbs used in this study and shows the μ chain domain specificity as well as the likely number of unique epitopes bound by these mAbs.

Evaluation of Anti-IgM mAb Affinity for Human mIgM. Results from equilibrium-binding experiments, performed at 4°C with four different IgM^+ clonal B cell

TABLE I

Human IgM Domain and Epitope Specificity of Murine mAbs

mAb	μ chain domain specificity*	Epitope designation‡
XG9	Cμ1	a
P19	Cμ1	b
HB57	Cμ2	c
P24	Cμ2	c(c')
Mu18	Cμ2	d
Mu53	Cμ2	d(d')
5D7	Cμ4	e
4-3	Cμ4	f
1G6	Cμ4	g
IF11	Cμ4	h

* IgM domain specificity was established by inhibition RIAs using both enzymatically derived IgM domain fragments and domain-deletion mutant proteins (12).

‡ mAbs were defined as binding to the same or distinct epitopes based upon competition RIAs (12). The designations c (c') and d (d') indicate that these mAbs cannot be unequivocally distinguished as binding to the same or very proximate sites as the mAbs binding to c and d epitopes, respectively.

TABLE II

Analysis of Anti-IgM mAb Affinity for B Cell Membrane IgM

mAb Fab'	K_a (M^{-1}) × 10^{-7} measured on various B cell populations*				Mean K_a (M^{-1})	K_d (M)	EC_{50}† (μg/ml) theoretical
	Bia	Dau	Lan	Lub			
HB57	64.72 ± 7.74 (6)§	48.78 (5)	51.34 (12)	48.85 (4)	5.34×10^8	1.87×10^{-9}	0.1
5D7	15.15 (11)	12.21 (5)	11.39 (11)	10.84 ± 0.13 (8)	1.24×10^8	8.06×10^{-9}	0.4
XG9	4.73 ± 0.41 (5)	6.09 ± 0.83 (13)	10.04 (4)	1.03 ± 0.31 (34)‖	6.95×10^7	1.44×10^{-8}	0.8
Mu18	4.48 (5)	4.04 (4)	5.45 (6)	4.66 (5)	4.66×10^7	2.15×10^{-8}	1.1
4-3	1.63 (9)	1.85 (12)	2.52 (1)	1.84 (5)	1.96×10^7	5.10×10^{-8}	2.7
Mu53	1.30 (5)	1.96 (12)	2.01 (3)	1.45 (9)	1.68×10^7	5.95×10^{-8}	3.1
1G6	0.72 (6)	0.65 (11)	0.77 (9)	0.67 ± 0.08 (15)	7.03×10^6	1.42×10^{-7}	7.4
P19	~0.17 (9)	~0.31 (10)	~0.74 (9)	~0.25 (26)	$\sim 3.68 \times 10^6$	2.72×10^{-7}	14
P24	ND	~0.14 (67)	~0.27 (28)	ND	$\sim 2.05 \times 10^6$	4.88×10^{-7}	25
IF11	ND	~0.02 (26)	ND	ND	$\sim 2.00 \times 10^5$	5.00×10^{-6}	260

* The data represent the K_a values from one to three independent determinations on each clonal B cell population. In instances where ≥1 determination was made, the mean value ± SEM of the K_a as well as the mean of the percent coefficient of variation is shown.

† EC_{50} here refers to the calculated concentration of anti-IgM Fab ligand that should bind 50% of the total available mIgM epitopes, under conditions of Fab ligand excess, based on theoretical considerations previously described (26). $EC_{50} = (K_d \text{ in mol/liter}) (5.2 \times 10^4 \text{ g/mol}) (10^6 \text{ μg/g}) (10^{-3} \text{ liters/ml})$.

§ Percent coefficient of variation of the Scatchard plot as determined by the computer program LIGAND (22).

‖ The K_a value for mAb XG9 Fab' on the Lub B cell population was consistently found to be significantly lower than that measured on all other B cell populations. For this reason, this variant affinity was not used in the calculation of the mean affinity for XC9 mAb.

populations and analyzed by the method of Scatchard (22, 25), are shown as K_a and reciprocal K_d values in Table II. HB57 exhibited the greatest average K_a while mAb IF11 the least. K_a obtained with the different cell populations were comparable with one another (average variation of 25.2 ± 4.8% [X ± SEM]). This was similar to the day-to-day average variation of separate determinations made on the same

TABLE III

Evaluation of the Valency of the mAb-definable Epitopes on Human Membrane IgM

mAb Fab'*	Number of epitopes per cell‡			Normalized number of epitopes per mIgM molecules§				Proposed valency per mIgM molecule
	Bia	Lan	Lub	Bia	Lan	Lub	Mean ± SEM	
		$\times 10^{-4}$						
XG9	2.3 ± 0.1 (3)‖	24.2 (4)	2.4 ± 0.4 (30)	2.0	2.0	2.0	2.0	2
HB57	1.9 ± 0.5 (2)	17.3 (2)	2.7 (1)	1.7	1.4	2.2	1.8 ± 0.2	2
Mu18	1.3 (3)	12.7 (3)	2.1 (3)	1.1	1.1	1.8	1.3 ± 0.2	2 (1?)
Mu53	2.6 (4)	20.7 (3)	3.4 (7)	2.3	1.7	2.8	2.3 ± 0.3	2
5D7	3.8 (3)	64.4 (3)	8.8 ± 2.1 (3)	3.3	5.3	7.2	5.3 ± 1.1	6(4?)
4-3	2.0 (7)	16.5 (1)	2.6 (4)	1.8	1.4	2.2	1.8 ± 0.2	2
1G6	1.8 (5)	16.8 (7)	3.3 ± 0.7 (11)	1.6	1.4	2.8	1.9 ± 0.4	2

* Because of lesser reliability of cell binding data from low affinity ligands, mAbs P19, P24 and 1F11 have been excluded from this analysis.

‡ The number of epitopes per cell is equal to the number of Fab' molecules bound per cell at saturation as derived from the Scatchard plot.

§ The number of epitopes per mIgM molecule was calculated by normalizing all n $\times 10^{-4}$ values to that of XG9 Fab' on the basis that this latter Cμ1-specific mAb binds to two epitopes per mIgM molecule. The formula used was the following: normalized number of epitopes per mIgM molecule for mAb x = [(number of bound epitopes per cell observed with x) × 2]/number of bound epitopes per cell observed with mAb XG9.

‖ In instances in which >1 equilibrium binding experiment was performed, the average n ± SEM are given. Numbers in parentheses indicate the percent CV of the n value(s).

cell population of 21.3 ± 5.6%. One mAb, XG9, consistently had a significantly lower affinity on Lub leukemic cells (1.03 ± 0.31 × 10^7 M^{-1}) than on the remaining three cell populations (mean K_a = 6.95 ± 1.59 × 10^7 M^{-1}). K_a values for the lower affinity mAbs (P19, P24, and IF11) were determined with less precision in part because only Dau and Lan, expressing high-density mIgM, yielded useful data and in part because fewer data points fulfilled the requirement of being 100-fold above the background binding with excess IgM (21).

Others have previously noted that increases in temperature can either increase or decrease the measured equilibrium binding constant (27, 28). The available data do not allow us to determine whether the mAb intrinsic affinities measured at 4°C are identical or different from those that occur at 37°C.

Analysis of the Stoichiometry of mAb Fab' Binding to mIgM. The total number of IgM epitopes per cell bound by the Fab' fragments at ligand saturation (n) (22, 29, 30) is shown in Table III. With the clear exception of mAb 5D7, and the possible exception of mAb Mu18, all of the evaluated mAbs bound to a comparable number of epitopes within each of the cell populations tested. 5D7 consistently bound to an epitope expressed with a two- to threefold greater frequency. This difference is significant since replicate determinations with 5D7, made on the same cell population but on separate days, resulted in an average variation of 35 ± 7% (X ± SEM, range = 2–63%). The multiple epitopes bound by mAb 5D7 Fab' appeared to be identical since all derived Scatchard plots were generally linear and conformed to a one-site model when evaluated by the LIGAND computer program (22) (data not shown).

The right hand portion of Table III lists normalized values for the number of epi-

topes bound per mIgM molecule, based upon the assumption that the Cμ1-specific mAb XG9 binds to two determinants per molecule. This was considered a valid supposition because the two Cμ1 domains are spaced widely apart on the Fab arms of monomeric IgM (31) and because immunoelectron microscopic data, to be presented later, supported the existence of a XG9-binding determinant on each Fabμ arm. By this analysis, the Cμ2-specific mAbs HB57 and Mu53, and the Cμ4-specific mAbs 4-3 and 1G6, apparently bind to divalently expressed determinants on mIgM, the epitopes presumably arising from each of the two μ chains that make up the intact IgM molecule. The Cμ4-specific mAb 5D7 appeared to recognize an epitope with a valency of 4–6 per mIgM molecule (i.e., 2–3 per μ heavy chain). The Cμ2-specific mAb Mu18 was consistently calculated to bind to <2 but >1 epitopes per mIgM molecule. This latter finding may reflect steric hindrance or induced changes in conformation that reduce Mu18 Fab access to the second epitope.

Relationship of Anti-IgM Domain Specificity to Mitogenic Potential for Human B Splenocytes. Fig. 1 indicates that, in the presence of 30% T cell supernatant as a source of ancillary growth factors, neither of the Cμ1-specific mAbs, directed to two distinct epitopes, were able to elicit substantial B cell DNA synthesis at any dose tested (concentration range: 2–1,000 μg/ml). An additional seven Cμ1-specific mAbs that have been defined as binding to the same epitope or a contiguous determinant to that of mAb XG9 (12) also are nonmitogenic or only weakly mitogenic for human B cells (11; data not shown). In contrast, all but one of the Cμ2-binding mAbs tested induced substantial DNA synthesis, although the doses required to induce proliferation varied considerably. Of the four Cμ4-directed mAbs, 5D7 was exceptionally mitogenic at low ligand concentrations; mAb 4-3 required high ligand concentrations for significant B cell stimulation; and mAbs 1G6 and IF11 were only very weakly mitogenic at the highest doses tested.

The above data indicate that while soluble anti-IgM mAbs of Cμ1-domain specificity are impaired in their capacity for triggering human B cell DNA synthesis, the binding of mAbs to either Cμ2 or membrane-proximal Cμ4 can result in significant B cell proliferation. Since not all mAbs to Cμ2 and Cμ4 elicited the same degree of stimulation, binding characteristics other than IgM domain specificity must play a critical role in determining mitogenic potential.

Relationship of Anti-IgM mAb Affinity to Potential for Triggering B Cell DNA Synthesis. Fig. 2 describes the capacity of individual mAbs of differing affinities to stimulate in vitro B cell DNA synthesis at either low (10–20 μg/ml) or high (100–300 μg/ml) concentrations. The mean SI achieved by divalent antibodies is plotted as a function of their intrinsic Fab affinities. At low concentrations, only the two highest affinity anti-IgM mAbs, i.e., HB57 and 5D7 (K_a = 5.3 and 1.2 × 10^8 M^{-1}, respectively) induced significant DNA synthesis (Fig. 2 *A*). Greater concentrations compensated for diminished ligand affinity and allowed for induction of DNA synthesis by antibodies with intrinsic affinities of 0.37–4.66 × 10^7 M^{-1} (Fig. 2 *B*). mAbs with K_a of <0.37 × 10^7 M^{-1} were nonstimulatory, even at high ligand concentrations. With the notable exception of mAb XG9, a direct relationship was observed between mAb binding affinity and stimulatory potential. A least squares linear regression analysis (excluding mAb XG9 and the lowest affinity antibody IF11), yielded a regression line of r = 0.985.

Monogamous Binding Interactions of mAb XG9 with Monomeric IgM. Immunoelec-

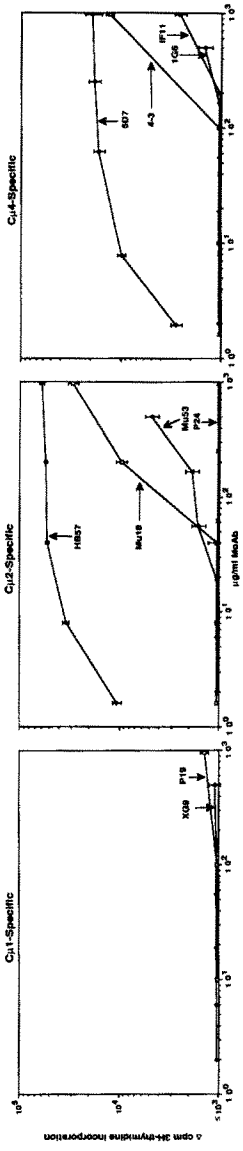

FIGURE 1. Human B cell proliferation elicited by domain-specific anti-IgM mAbs. T cell–depleted splenic B lymphocytes were cultured with the indicated concentrations of anti-IgM mAb in the presence of 30% T cell supernatant. The data are expressed as Δ cpm ± SE diff from triplicate cultures at 72 h and were obtained from three representative experiments in which the average background proliferation in the absence of mAb was 2,120 ± 529 cpm (X ± SEM). While neither Cμ1-specific mAb induced substantial B cell DNA synthesis, there was diversity in the ability of both Cμ2- and Cμ4-binding mAbs to elicit proliferation.

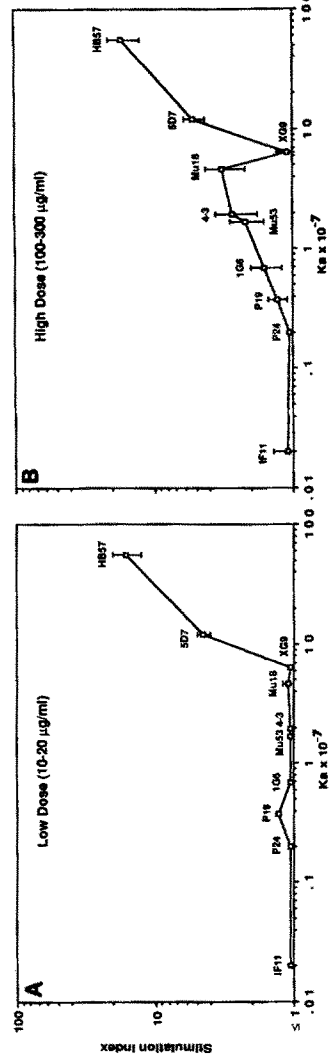

FIGURE 2. Relationship of mAb affinity for membrane IgM to mitogenic potential for human B lymphocytes at both low and high ligand concentrations. 2×10^5 T cell–depleted splenotypes were cultured with each mAb in the presence of 30% T cell supernatant. The SI used for each mAb represents the mean ± SEM of SI values obtained from between one and four separate experiments in which cells were cultured with 10–20 µg/ml of mAb, and from four to seven separate experiments in which cells were incubated with 100–300 µg/ml of mAb. These indices are plotted as a function of the mean K_a (M^{-1}) of the mAb Fab' fragments (see Table II). While only the highest affinity mAbs (HB57 and 5D7) were capable of inducing B cell DNA synthesis at limiting concentrations (10–20 µg/ml), at high ligand input (100–300 µg/ml) there was a direct linear relationship between mAb K_a and proliferation-inducing capacity. The lowest affinity mAbs (IF11, P24, and P19) were generally nonstimulatory, whereas mAbs of progressively higher affinity were increasingly more mitogenic.

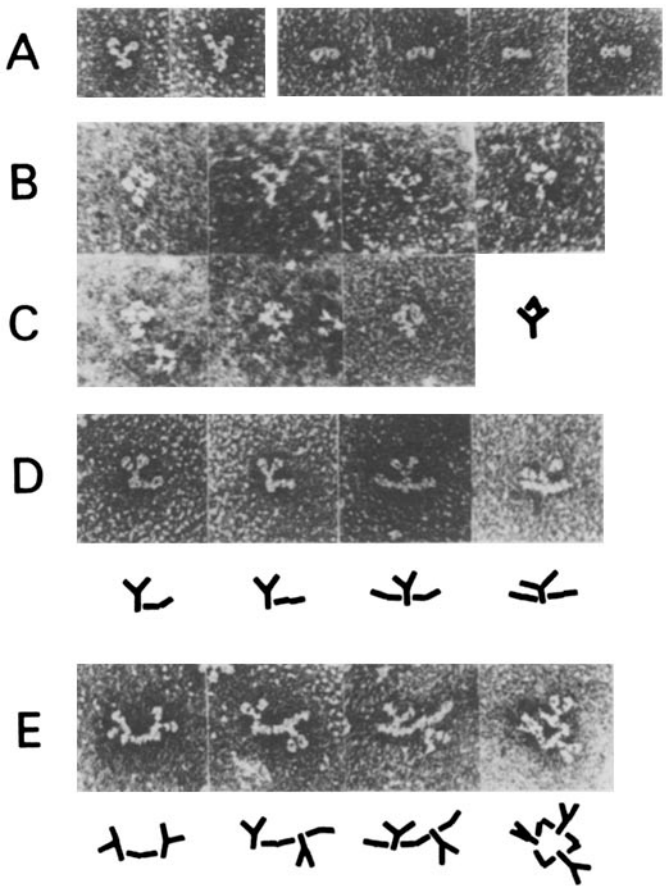

FIGURE 3. Immunoelectron photomicrograph of immune complexes formed between mAb F(ab')$_2$ fragments XG9 and 1G6 and human IgM monomer. (*A*) Panels *1* and *2* show the human monomeric IgM molecule alone; Panels *3* and *4*, and *5* and *6* show F(ab')$_2$ fragments of mAbs XG9 and 1G6, respectively. (*B* and *C*) Immune complexes formed between monomer IgM and F(ab')$_2$ fragments of mAb XG9; panel *C4* indicates the interpretation of these photomicrographs in schematic form, i.e., monogamous binding of a single mAb F(ab')$_2$ fragment to monomer IgM. (*D* and *E*) Immune complexes formed between monomer IgM and F(ab')$_2$ fragments of mAb 1G6 with interpretive schematic illustrations of those immune complexes.

tron microscopy was used to evaluate the possibility that Cμ1-specific XG9 mAb might bind to monomeric IgM in a monogamous fashion (30), i.e., both binding sites of a single XG9 Ab molecule engaged with epitopes on a single IgM molecule. Such a finding could explain the nonmitogenic nature of high-affinity mAb XG9, since a high proportion of monogamous binding would reduce the amount of mIgM crosslinking.

Fig. 3 (panels *B1*–4 and *C1*–3) show that XG9 F(ab')$_2$ can indeed bind monogamously to monomeric IgM (72% of the observed complexes). The remaining complexes had either one XG9 F(ab')$_2$ bound to two monomeric IgM molecules in a bigamous manner, or alternatively, XG9 F(ab')$_2$ molecules bound to IgM monomer via one arm only (data not shown). The monogamously bound XG9 F(ab')$_2$

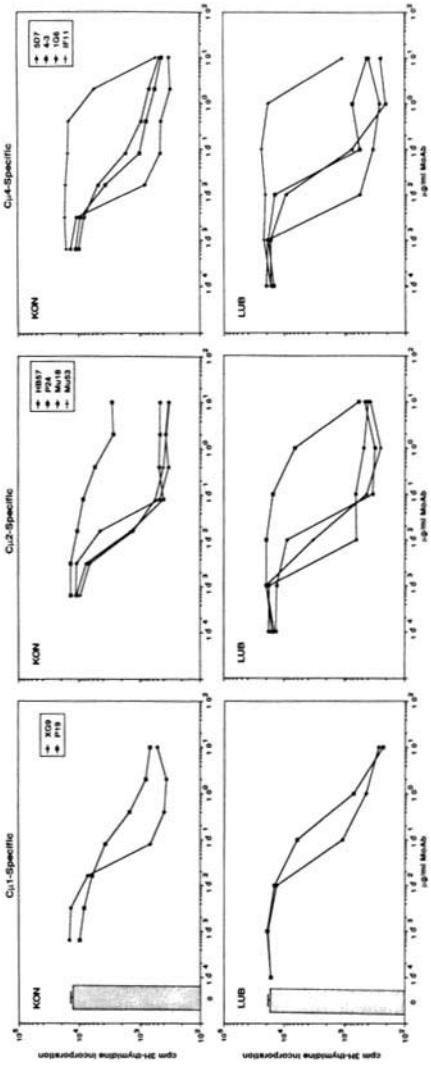

FIGURE 4. All anti-IgM mAbs can transduce inhibitory signals for leukemic B cell DNA synthesis. 2×10^5 Kon HCL B cells + 25% T cell supernatant or 2×10^5 Lub HCL cells in non-T supernatant supplemented medium, were cultured with the indicated concentrations of anti-IgM mAb. The data are expressed as cpm [^{3}H]thymidine incorporation on day 4 of culture. Stippled bars indicate the level of DNA synthesis obtained when these cells were cultured in the absence of mAb. All anti-IgM mAbs, irrespective of their domain specificity, were able to inhibit the DNA synthesis of these leukemic clones.

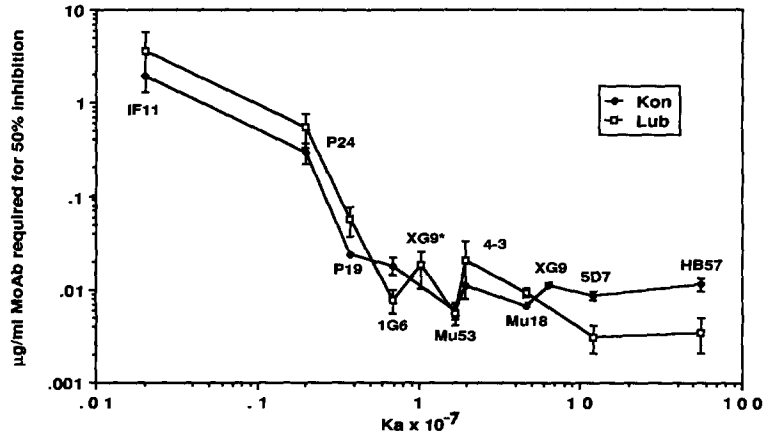

FIGURE 5. Effect of mAb affinity upon ability to inhibit leukemic B cell DNA synthesis. The concentration of intact mAb required for 50% inhibition of leukemic B cell [^{3}H]thymidine incorporation was calculated from dose-response profiles obtained from at least two separate experiments on both Kon and Lub HCL B cells (Fig. 4). The mean values are plotted as a function of mAb Fab' affinity in M^{-1}. In the case of Lub cells, mAb XG9 is indicated as having a lower affinity, XG9*, than on Kon cells (see legend Table II). The composite data indicate that the inhibition of leukemic B cell DNA synthesis by intact antibody is relatively independent of affinity, until an Fab' affinity threshold of $\sim 7 \times 10^6$ M^{-1}.

molecules appeared to have more compacted dimensions than when found in free, nonbound form. This has been previously noted with anti-κ mAb binding to mouse IgG molecules (23) and is considered to be due to geometric constraints that prevent the immune complex from lying on the same flattened plane.

Panels *D1-4* and *E1-4* of Fig. 3 show the contrasting types of complexes observed between monomeric IgM and the F(ab')$_2$ fragment of the Cμ4-specific mAb 1G6. The results clearly indicate the bivalent nature of the 1G6 epitope. Of the immune complexes formed, >75% had two 1G6 F(ab')$_2$ molecules bound per IgM monomer (panels *D3* and *4* and *E2-4*), whereas the remainder of observed immune complexes had only one F(ab')$_2$ bound per molecule of monomer (panels *D1* and *2* and *E1*). Monogamous binding of 1G6 F(ab')$_2$ to monomeric IgM was not detected.

Inhibition of Leukemic B Cell DNA Synthesis by mAbs Having Cμ1-, Cμ2-, and Cμ4-binding Specificities. The ligand-binding requisites for inhibitory signal transduction were evaluated with two human HCL B cell populations previously shown sensitive to anti-IgM-mediated negative signaling (16). These are: Kon, which can be induced to in vitro DNA synthesis by T cell factor-containing supernatant, and Lub, which synthesizes DNA spontaneously (Fig. 4). All mAbs, irrespective of their IgM domain specificity, were found to signal inhibition of Kon and Lub leukemic B cell DNA synthesis at ng/ml concentrations. This signaling required mIgM crosslinking and did not involve Fc-receptor binding (16).

Affinity Requirement for Anti-IgM-mediated Inhibitory Signaling. Fig. 5 shows that the diversity in the inhibitory potential of certain mAbs reflects their affinity for mIgM. mAbs that required significantly greater amounts of ligand to achieve 50% inhibition of leukemic B cell DNA synthesis, i.e., P19, P24, and IF11, had the lowest affinities for mIgM. While a direct relationship was noted between affinity of these three

TABLE IV

Binding Requisites for Stimulatory and Inhibitory Signal Transduction through Membrane IgM Differ in a Clonal B Cell Population

Anti-IgM mAb	Ab concentration	[^{3}H]Thymidine uptake (d5)*		Cells in S, G2, or M (d4)‡	
		No T supernatant	+ T supernatant	No T supernatant	+ T supernatant
	μg/ml	*cpm ± SEM*		*%*	
None	–	460 ± 40	19,929 ± 217	1.0	8.1
HB57	2	493 ± 13	813 ± 146	2.0	1.4
	200	40,596 ± 5,980	6,827 ± 410	10.7	4.2

* 2×10^5 Kon hairy cell leukemia B cells were cultured with or without HB57 anti-IgM mAb in the presence or absence of 25% T cell supernatant. Proliferation was assessed on day 5 after an 18-h incubation with [^{3}H]thymidine. The mean ± SEM from triplicate cultures are shown.

‡ Percent cells in S, G2, or M was determined from day 4 cultures by propidium iodide staining using the FACS as detailed in Materials and Methods.

ligands and inhibitory potential, concentration-dependent differences in inhibition by mAbs with Fab' binding affinities $\geq 7.0 \times 10^6\ M^{-1}$ were not evident. This suggests an affinity threshold for inhibitory signaling by anti-Ig ligands, above which increases in affinity have a minimal effect. Also notable in Figs. 4 and 5 is that mAb XG9 was not notably compromised in causing inhibition despite its established propensity for monogamous binding (Fig. 3).

Binding Requisites for Stimulatory and Inhibitory Signal Transduction Differ in a Clonal B Cell Population. The above cumulative data suggest that the ligand binding requisites for triggering inhibition of DNA synthesis through mIgM are significantly less demanding than those for stimulating DNA synthesis. Because stimulation was evaluated with normal B lymphocytes and inhibition was evaluated with leukemic B cell clones, normal vs. malignant cell phenotype may have accounted for the distinctions. That this is not the case is shown in experiments with an unusual HCL clone, Kon, which can receive stimulatory, or alternatively, inhibitory signals from anti-IgM ligands, depending upon its state of activation (16). In the absence of T cell factors, Kon cells respond with very significant levels of DNA synthesis only to high concentrations of the highest affinity mAb, HB57 (Table IV) (16; data not shown). In striking contrast, the T cell factor–induced DNA synthesis of the clone is inhibited by low as well as high doses of all the anti-IgM ligands (Table IV; Fig. 4). These data with a clonal B cell population clearly indicate that the differences in ligand requisites for activating and inhibiting B cell DNA synthesis are not simply reflective of the malignant vs. normal nature of the cell populations used, but, rather, represent distinct mIg binding requirements for inducing the two functional outcomes.

Discussion

One principal conclusion of these studies is that the induction of human B cell DNA synthesis, a requisite for B cell clonal expansion, is primarily determined by the affinity of the mIgM crosslinking ligands. With one explainable exception, a direct relationship was noted between the intrinsic affinity of a mAb for mIgM and its mitogenic potential. At high ligand concentrations (100–300 μg/ml), the minimal affinity threshold for inducing significant B cell DNA synthesis in the presence

of ancillary T cell factors was $K_a = \sim 2 \times 10^7\ M^{-1}$. At lower ligand concentrations (10–20 μg/ml) this threshold was significantly greater, i.e., $K_a = \sim 1 \times 10^8\ M^{-1}$. This is as predicted given the law of mass action effect of concentration of reactants on complex formation (21). Because stimulatory signal transduction by anti-Ig antibodies requires bivalent binding of the ligand (6), and because bivalent mAb binding to cell membrane antigens is characterized by at least a 10–100-fold enhancement in avidity over that exhibited by univalent Fab' fragments (28, 29), it is anticipated that the minimal binding avidity of a soluble anti-IgM ligand required for inducing human B cell DNA synthesis at high concentrations is in the range of $2 \times 10^{8-9}\ M^{-1}$, and at low concentrations is in the range of $1 \times 10^{9-10}\ M^{-1}$. Our findings that affinity is a major contributing factor to ligand stimulatory capacity supports and extends in a quantitative way the findings of Goroff et al. (32) with anti-mouse IgD mAbs.

The only ligand that deviated from a direct linear relationship between intrinsic affinity and proliferation-inducing capability was mAb XG9. This relatively high-affinity antibody ($K_a = 6.95 \times 10^7\ M^{-1}$) was consistently nonmitogenic for normal human B cells, even at concentrations up to 1 mg/ml. Immunoelectron microscopy provided an explanation by revealing that mAb XG9 has a predilection for monogamous binding to the bivalently expressed Cμ1 epitopes on monomeric IgM. This tendency is probably due to the comparable spacing between Cμ1 epitopes and the bivalent combining sites of the mAb. It is highly likely that monogamous binding diminishes the stimulatory signaling potential of this high-affinity antibody by reducing its crosslinking potential.

The functional data shown here, as well as capping studies (our unpublished results), suggest that Cμ2- and Cμ4-specific antibodies have a lower probability of engaging in monogamous binding interactions with mIgM than Cμ1-specific antibodies. Monogamous binding may be precluded by the close interactions between homologous pairs of the Cμ2 and Cμ4 domains (31) as well as by the predicted topographically polar orientation of epitopes on each side of the globular paired domain structures. The findings of Goroff et al. (32) that high-affinity mAbs directed to the Fd portion of murine IgD were ineffective at capping and stimulating mouse B cell DNA synthesis, whereas high affinity mAbs to the Fc portion of murine IgD were good at inducing capping and very mitogenic, are also explainable in this context.

These findings have strong functional implications for antiidiotype antibodies, since monogamous binding appears to be characteristic of a substantial proportion of antiidiotype mAb interactions with both soluble and cell-bound Ig molecules (24, 28, 33). Those antiidiotype antibodies of the IgG class that bind monogamously to mIg should be significantly impaired in capacity for directly inducing B cell clonal expansion, regardless of their affinity for their respective epitopes.

Among the hypotheses initially considered for the diversity in mAb stimulatory signaling potential was the valency of mIgM epitopes recognized by each mAb. Differences in the epitope univalency (34) or divalency were expected to have significant effects on the extent to which mIgM molecules could be crosslinked, i.e., dimer formation vs. linear concatemer formation (30). The studies shown here on Fab binding stoichiometry to mIgM exclude this hypothesis, since with one exception, all of the anti-IgM mAbs appeared likely to bind bivalently expressed epitopes.

The Cμ4-specific mAb, 5D7, was unique in its recognition of an epitope with an apparent valency of 4–6 per mIgM molecule. Because the hybridoma cloning conditions, the unusual isotype of this mAb, and the unusual enzymatic fragmentation characteristics of 5D7 all attest to its monoclonality, we do not consider that the antibody preparation represents a mixture of antibodies to distinct determinants. Further characterization of the 5D7 epitope is in progress.

Of great significance in these studies was the fact that the conclusions regarding mIgM ligand-binding requisites for stimulation of B cell DNA synthesis did not apply to inhibition. First, consistent with other studies (3, 7–10), the ligand concentrations for maximal inhibition of B cell DNA synthesis were orders of magnitude lower than those for stimulation. Second, while inhibitory signaling was clearly affinity independent at univalent ligand affinities $>7.0 \times 10^6\ M^{-1}$, an upper affinity threshold for maximal stimulation of B cell DNA synthesis was not detected. Third, while a high-affinity Cμ1-specific mAb with a propensity for monogamous binding to mIgM was unable to initiate B cell DNA synthesis, the same antibody was quite competent at causing inhibition. These distinctions indicate that the ligand binding requisites for mIgM-mediated inhibition of B cell DNA synthesis are much less rigorous than those for B cell clonal expansion and explain the past observations of Leptin (35) with anti-mouse IgM mAbs. Although inhibitory signaling was here studied with leukemic B cells, these observations have physiologic significance for normal B cells since the mechanism for mIgM-mediated inhibition in malignant B cells is thought to mimic that responsible for at least one form of B cell tolerance (1, 2, 36).

Two major explanations appear plausible for the differences in binding requisites. First, stimulation of DNA synthesis may require a greater initial crosslinked receptor cluster, immunon (37), than inhibition. Thus, activation of susceptible cells may be achieved only with high-affinity ligands which, because of their likely lower dissociation rate (27) and prolonged occupancy, are more capable of stabilizing such clusters (see Fig. 6 *A*). Alternatively, the greater binding requisites for stimulation may reflect the duration of the stimulatory signaling process (Fig. 6 *B*). Our unpublished observations, as well as the work of others, have indicated that for S phase entry of anti-Ig–treated resting B cells, ligand must be present for an interval of 24–36 h (8, 38, 39), while inhibition of DNA synthesis may be achieved after significantly shorter ligand exposure (36). This difference becomes particularly relevant when one considers that, after an initial cycle of capping and endocytosis, B cells slowly replace mIg molecules and display very low-density mIgM for some time (38). Under these conditions, continued B cell signaling by mIgM crosslinking may be achieved only by high-affinity ligands that can remain univalently bound to mIgM for a period of time sufficient for distally located mIgM molecules to diffuse into proximity. This hypothesis places greater demands on the ligands only at later phases in B cell activation.

Several recent observations from other laboratories lend support to this second explanation (Fig. 6 *B*). First, anti-Ig ligands that are ineffective at inducing the DNA synthesis of resting B cells have been documented to initiate a number of early activation-related biochemical changes in B cells that precede, or are concurrent with, an initial cycle of mIg capping (18, 40). Secondly, T cell factor–independent

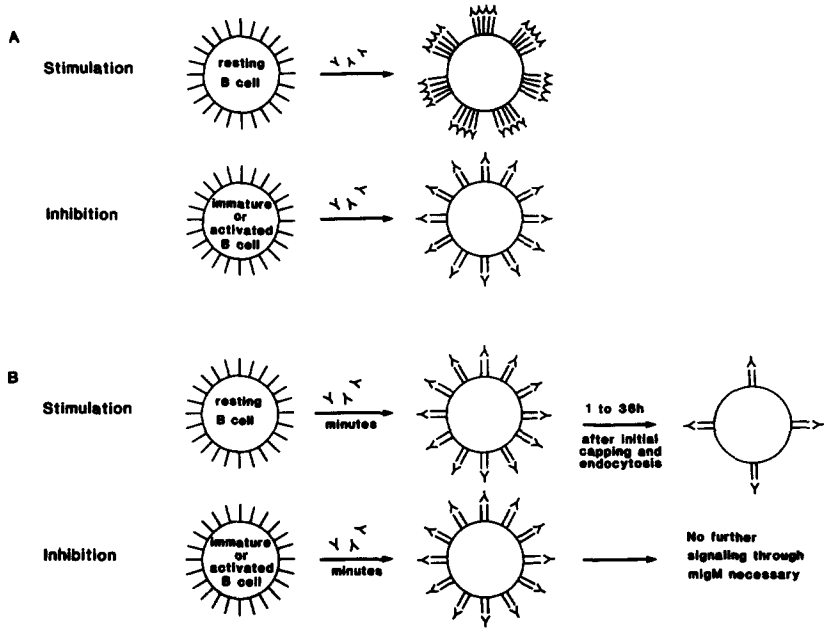

FIGURE 6. Alternative models for mIgM crosslinking events that lead to stimulatory or inhibitory signals for B cell DNA synthesis. (*A*) Stimulatory and inhibitory signal transduction may differ in the minimal requisite unit of crosslinked receptors. (*B*) Unit signal for activation and suppression of B cell DNA synthesis may be achieved by crosslinking similar numbers of receptors. However, the activation of DNA synthesis may require repeated cycles of mIgM crosslinking under conditions of extremely low receptor density.

signaling of B cell DNA synthesis by high concentrations of polyclonal anti-IgM antibodies requires high ligand concentrations at late, but not early intervals in the stimulation of murine B cells (8, 39).

Although it remains to be determined whether the binding requisites for inducing B cell tolerance are subtly different from those for inducing early activation related changes in resting mature B cells, the current data are compatible with the hypothesis that requirements for inducing *any* early biochemical phenomena in B cells with a comparable density of mIgM are the same. The inhibitory or stimulatory outcome of these early signals will depend upon the activation or differentiation state of the particular B cell population being studied (2, 3, 16). For early pre proliferative changes in stimulated resting B cells to lead to clonal expansion, one of two events is necessary. There is (*a*) further signaling through mIgM crosslinking by ligands that can form stable complexes of mIgM under conditions of low mIgM density, and/or there is (*b*) signaling by activated class II MHC-restricted helper T cells or their products (6, 41).

Our conclusion that tolerogenic signal transduction in susceptible B cells has much lower affinity requisites than signal transduction for B cell clonal expansion, would appear to contradict the conclusions of Riley and Klinman (42) in a murine experimental system. One major explanation may be that the former investigators evaluated B cell stimulation by antigens dependent upon cognate T cell help. The ligand binding requisites for sufficient B cell endocytosis, processing and presentation of

antigenic epitopes to T cells, and stimulation of early $G_0 \rightarrow G_{0^*}$ changes such as increased mIa levels, which enhance B cell-T cell collaborative interactions, are likely much less demanding than the binding requisites for direct ligand-induced cell cycle entry (18, 43).

The physiological implications of our studies would appear to have the most immediate relevance to the direct, cognate T cell-*independent* signaling role played by autologous rheumatoid factors or antiidiotype antibodies in the regulation of B cell immune responses. Although IgG antiidiotype antibodies with a propensity for monogamous binding (24, 28, 33) may be compromised in their capacity to directly signal the clonal expansion of B lymphocytes, they may be active in negatively regulating the maturation or differentiation of idiotype-positive B cell clones. Furthermore, since most human rheumatoid factors appear to be of relatively low affinity (44), it is expected that these ligands may manifest a more direct role in the feedback suppression of B cell responses than in B cell clonal expansion.

Summary

The ligand binding requisites for membrane IgM-mediated signaling of human B lymphocyte clonal expansion and B cell tolerance were investigated with a well-characterized set of soluble murine anti-human IgM mAbs. Evaluation of the impact of μ chain domain specificity, affinity, and binding stoichiometry for membrane IgM on antibody-induced regulation of normal and leukemic B cell DNA synthesis revealed that the ligand binding requisites for inducing or, alternatively, suppressing B cell DNA synthesis are significantly different. First, while the induction of S phase entry required μg/ml concentrations of ligand, orders of magnitude lower concentrations of ligand sufficed for inhibitory signaling. Second, while an upper affinity threshold for achieving maximal stimulation of B cell DNA synthesis was never detected, inhibitory signaling by bivalent ligands appeared to become relatively affinity independent at Fab binding affinities $>7.0 \times 10^6\ M^{-1}$. Third, while a Cμ1-specific mAb with an enhanced incidence of monogamous binding to mIgM was ineffective at inducing B cell DNA synthesis, the antibody was not significantly compromised in ability to initiate inhibitory signals. These differences could be observed in a clonal B cell population which positively or negatively responded to mIgM ligation depending upon its state of activation. The accumulated observations indicate that the ligand binding requisites for inhibitory signal transduction in human B lymphocytes are much less rigorous than those for stimulatory signal transduction and suggest that many physiologically relevant anti-Ig antibodies are more likely to function in the negative feedback regulation of B cell responses than in the direct triggering of human B cell clonal expansion.

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