HUMAN B CELL ACTIVATION

Evidence for Diverse Signals Provided by Various Monoclonal Anti-IgM Antibodies

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Antiimmunoglobulin antibodies (Ab)[1] have been frequently used to stimulate B lymphocytes (1–5) since Sell and Gell's initial observation that antiallotype Ab induce the proliferation of rabbit lymphocytes (6). However, certain investigators have been unable to induce B cell proliferation with anti-Ig Ab (7, 8) while others have observed that these reagents inhibit the activation of B lymphocytes (9–11).

In some instances, these divergent findings could be attributed to possible differences in lymphocyte population subset (12), maturational stage (12–14), or cell cycle (15–17), or to the presence of accessory cells or factors (3, 5, 16, 18). In contrast, other evidence suggests that the nature of the polyclonal Ab itself is important in determining whether or not an Ab preparation is mitogenic to the B cell. For example, while in some experiments inhibitory effects are mediated by Fc regions of certain Ig subclasses (10, 19, 20), the fact that inhibition has also been observed with Ab preparations in which the Fc region has been digested (21, 22), suggests that the nature of the variable region influences the regulatory capabilities of anti-Ig reagents.

The hypothesis underlying the present studies is that the response of the B lymphocyte to anti-Ig Ab is influenced by the affinity and/or epitope specificity of the Ab. Since polyclonal antisera consist of indeterminant mixtures of multiple Ab with distinct affinities and differing specificities, this hypothesis can be examined more directly by the use of monoclonal Ab (mAb). A number of distinct anti-IgM mAb were assayed for the ability to stimulate human B cell proliferation in the presence and absence of T cell supernatant. In addition, defined combinations of these mAb were tested to determine whether the signals delivered by combinations of mAb differ from the signals provided by individual mAb.

The data obtained suggest that the ligand-binding characteristics of individual anti-IgM mAb influence the presence or absence of B cell proliferation and the

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[1] *Abbreviations used in this paper:* Ab, antibody; BCGF, B cell growth factor; mAb, monoclonal antibody; mIg, membrane immunoglobulin; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PVC, polyvinylchloride; RIA, radioimmunoassay; SE diff, standard error of the difference; TRITC, tetramethylrhodamine.

degree of the response obtained. Furthermore, the data show that combinations of certain individually nonmitogenic mAb induce pronounced B lymphocyte proliferation. Moreover, at a high ligand concentration, certain mAb mixtures, but not individual mAb, deliver signals for B cell activation that obviate the need for ancillary signals provided by T cell supernatants.

Materials and Methods

Preparation of Murine Anti-Human Hybridomas. mAb VIIIE11, 1G6, and XG9 were obtained in two fusions of the nonsecreting murine myeloma Sp 2/0 with spleen cells from BALB/c mice by following standard immunization and fusion protocols (23, 24). The same two human IgM myeloma proteins were immunogens for both fusions. mAb Mu 53 and P24 were generously donated by Dr. David Posnett and the late Henry Kunkel and were derived similarly from BALB/c mice immunized with an aggregated human μ chain and intact human IgM mAb, respectively. Anti-IgM mAb 5D7 was fortuitously derived from spleen cells of BALB/c mice immunized with a partially purified human IgG3 myeloma protein preparation which contained some contaminating IgM. Anti-IgM mAb HB57 was purchased from the American Type Culture Collection (J. Kearney, donor).

CBDA-4E5 anti–human IgD mAb and PMG3A-4C6 anti–human lambda light chain mAb were prepared as described for anti-IgM mAb except that an IgDλ myeloma protein and an IgG3λ myeloma protein, respectively, were used as immunogens. HB45 anti–human kappa light chain mAb was obtained from the American Type Culture Collection (J. Kearney, donor). 22c6 and SG171 mAb bind to monomorphic determinants on human Ia molecules (25, 26).

Isolation and Purification of mAb. Anti-IgM–producing hybridomas were detected in a solid phase, enzyme-linked immunosorbent assay (ELISA) (horseradish peroxidase–goat anti-mouse Ig assay kit; New England Nuclear, Boston, MA) and cloned at limiting dilution. Clones were screened for binding to 9 human IgM and 14 human myeloma proteins of other Ig classes. Selected hybrids were propagated in irradiated (350 rad), pristane-primed BALB/c mice. Ascitic fluid was cleared by centrifugation at 17,300 *g* for 0.5 h, made 0.1% wt/vol with sodium azide and stored at 4°C.

Anti-IgM mAb 5D7 (γ2a mouse isotype) and VIIIE11 (γ3 mouse isotype) were purified by affinity chromatography over protein A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). Ascitic fluid was first precipitated with 18% wt/vol Na_2SO_4. The resolubilized precipitates were dialyzed against 0.2 M PO_4 buffer, pH 8, before being loaded onto protein A Sepharose. The differential pH elution technique of Ey et al. (27) was used for purification; contaminating IgG1 was removed by washing with 0.1 M citrate buffer, pH 6, and the bound IgG2a or IgG3 hybridoma protein was eluted with 0.1 M citrate, pH 4.5. The remaining anti-IgM mAb of IgG1 mouse isotype (HB57, Mu53, 1G6, XG9, and P24) were purified by Na_2SO_4 precipitation and ion exchange chromatography over DEAE-Trisacryl (LKB Instruments, Rockville, MD), as described by Parham et al. (28), with slight modifications. Protein was loaded onto columns in 0.005 PO_4 M buffer, pH 8, and eluted by a linear salt gradient from 0.005 to 0.1 M PO_4 buffer, pH 8. All anti-IgM mAb eluted at a conductivity of $2.21–4.46 \times 10^3$ μS.

The purified anti-IgM mAb were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (29) under both native and denaturing conditions in a 5–12.5% acrylamide gradient gel. 20 μg of protein was applied in a volume of 10 μl. When stained with Coomassie Blue and analyzed on a densitometer, no deflections other than those accounted for by Igs were detected on both the nonreduced and reduced gels. Calculations based on densitometric tracings revealed levels of purity in all preparations of 90–95%. In addition, immunoelectrophoresis using goat anti–mouse serum and goat anti–mouse Ig as precipitating reagents showed only one precipitation line.

CBDA-4E5 anti–human IgD mAb (γ1 mouse isotype) was purified from hybridoma culture supernatants by affinity chromatography on a Sepharose column coupled with an

affinity-purified goat anti-mouse Ig. Both 22c6 and SG171 mAb (γ2a mouse isotypes) were purified from ascites by selective elution from protein A Sepharose.

Purification of Human Myeloma Proteins. Sources of monoclonal human IgM included serum either from patients with Waldenström's macroglobulinemia or from patients with mixed cryoglobulinemia who exhibited a monoclonal IgM spike. IgM proteins were purified by euglobulin fractionation (0.005 M phosphate buffer, pH 8.6) and either Sephacryl S-300 gel filtration or protein A Sepharose affinity chromatography to remove contaminating IgG. In addition, some IgM preparations were subjected to Pevikon block or agarose gel preparative electrophoresis as a preliminary purification step. IgG and IgD myeloma proteins were purified by either (*a*) absorption and selective elution from protein A Sepharose, (*b*) affinity chromatography over Sepharose columns conjugated with purified murine hybridoma Abs specific for human Ig isotypes, or (*c*) Na_2SO_4 precipitation followed by Pevikon block electrophoresis. IgA myeloma proteins were purified from serum by a sequence of steps that involved euglobulin precipitation to remove IgM, Na_2SO_4 precipitation to enrich for IgG and IgA, $ZnSO_4$ precipitation to separate IgG from IgA (30), and passage over protein A Sepharose to remove contaminating IgG.

Preparation of T Cell Supernatants Containing B Cell Growth Factor Activity. Peripheral blood mononuclear cells from 10 healthy volunteers were isolated by Ficoll-Hypaque density centrifugation and cultured at a concentration of 1.5×10^6/ml in Linbro 24-well plates (Flow Laboratories, McLean, VA). The culture medium used to prepare factor was identical to that used by Sredni (31) but included 1% phytohemagglutinin (PHA). After incubating for 3 d at 37°C, 6% CO_2, and 90% relative humidity, cells were pelleted at 2,000 rpm for 10 min and the supernatant was immediately frozen at −70°C. Shortly before being used in experiments, all thawed aliquots were dialyzed vs. culture media at 4°C for a minimum of 5 h, followed by sterile filtration. Preliminary experiments using an affinity-purified goat anti-human IgM Ab established that a 30% vol/vol dose of supernatant was optimal for enhancing proliferation of human B lymphocytes. The ability of this supernatant to enhance B cell proliferation induced by anti-IgM mAb was shown to not be due to PHA, in that concentrations of PHA ranging from 0.01 to 5% did not mimic the effect of the T cell supernatant when added to purified B cell cultures stimulated with anti-IgM mAb (data not shown).

Solid Phase Radioimmunoassays. The determination of mAb in hybridoma culture supernatants was performed by solid phase radioimmunoassay (RIA) using a modification of techniques reported previously (32). Briefly, 10 μg/ml of purified human myeloma protein was incubated on wells of a polyvinylchloride (PVC) plate for 1.5 h at 37°C. After three washes, diluted hybridoma Ab was added to myeloma-coated wells and incubated for an additional 1.5 h. Bound mAb was detected by incubating wells with ~25,000 cpm of tritium (^{3}H)-labeled, affinity-purified Ab specific for different murine Ig isotypes (32).

Competitive Binding Radioimmunoassay. Analogously to the direct binding RIA, each purified anti-IgM mAb was coated onto PVC wells (in duplicate) by incubating 50 μl of a 10 μg/ml solution of mAb in each well at 37°C for 90 min. 0.5 h before the end of this initial coating, we prepared 1:1 mixtures composed of 1 μg/ml of a ^{3}H-labeled human IgM myeloma protein (sp act, 667,000 cpm/μg) and each of the soluble anti-μ mAb at concentrations of 0.08–50 μg/ml in fivefold dilutions. These mixtures were incubated in a 24-well Linbro plate at 37°C until the end of the coating incubation, at which time all mAb-coated wells were washed and 50 μl of each inhibition mixture was added to wells coated with each anti-μ mAb. After a final 3 h incubation, unbound ^{3}H-IgM was discarded and plates were washed and assayed for bound ^{3}H-IgM. Inhibition curves were plotted by determining the percent inhibition obtained when various concentrations of soluble anti-IgM mAb were used as an inhibitor. Percent inhibition values were calculated as follows: $100 \times [1 - (\text{cpm } ^3\text{H-IgM bound in presence of soluble mAb inhibitor})/(\text{cpm } ^3\text{H-IgM bound in absence of soluble mAb inhibitor})]$.

Immunofluorescence Studies. For all immunofluorescence studies, 2×10^5 cells in phosphate-buffered saline/bovine serum albumin (PBS-BSA)-azide (assay buffer) were incubated with 100 μl of 1,000 or 10 μg/ml of purified mAb in a 96-well V-bottomed microtiter plate (Linbro, Flow Laboratories) for 1 h at 4°C. mAb were tested at 1,000

μg/ml to ensure saturation binding of potentially low-affinity Ab (28). All tested mAb had previously been ultracentrifuged at 108,000 *g* for 80 min at 4°C. After washing four times with assay buffer (200 μl/wash), tetramethylrhodamine (TRITC)-labeled $F(ab')_2$ goat anti-mouse Ig was incubated with the cells for 20 min on ice before washing. Cells used for staining included: (*a*) Daudi, a lymphoblastoid B cell line (μ,κ); (*b*) two membrane IgM (mIgM)-positive chronic lymphocytic leukemias (μ,κ and μ,λ); (*c*) an mIgM-positive hairy cell leukemia; and (*d*) a B type chronic lymphocytic leukemia, expressing no detectable mIgM. For flow cytometric analyses (FACS IV; Becton Dickinson Immunocytometry Systems, Mountain View, CA), cells were analyzed by both forward narrow angle light scatter and fluorescence emission upon excitation at 514 nm (33). After gating for viable cells on the basis of the scatter signal, the fluorescence of 10,000–15,000 cells was compared with that obtained when cells were stained with 100 μl of 1,000 or 10 μg/ml irrelevant control Ab: MOPC-245 for γ1 mAb, LPC-1 for γ2a, and FLOPC-21 for γ3 mAb. Data were collected using logarithmic amplification and plotted as the number of cells (ordinate) vs. log relative fluorescence intensity (abscissa). All gain and offset parameters were kept constant between different mAb.

Isolation and Purification of B Lymphocytes from Human Spleen Fragments. Residual spleen fragments were obtained from both a normal spleen removed during an operative procedure and a spleen removed during surgery as treatment for Felty's syndrome. The fragments were diced and pressed through a fine stainless steel sieve. After sedimenting twice (1 *g*) to eliminate debris, the resulting single-cell suspension was layered on Ficoll-Hypaque and centrifuged (2,000 cpm, 20 min) at room temperature. The interface layers were harvested, washed three times with PBS, and frozen in liquid nitrogen. When assayed for B cell, T cell, and monocyte markers by visual indirect immunofluorescence with a mixture of anti-μ, anti-κ, and anti-λ mAb, anti-Leu-1, and P9 and R17 anti-monocyte mAb (34), respectively, cells from both spleen sources were found to be 31–38% Ig^+, 35–40% Leu-1^+, and <2% positive for monocytic markers.

Immediately before an experiment, T cells were removed by rosetting twice with neuraminidase-treated sheep red blood cells (35). The resulting spleen cell population was found to be >85% Ig^+, <1% Leu-1^+, and <1% $P9^+$ or $R17^+$. In addition, the cell surface activation antigen bound by mAb 4F2 (36) was expressed weakly on <1% of these T cell–depleted splenocytes.

Cell Culture Conditions for Inducing B Cell Stimulation. T cell–depleted splenic lymphocytes were cultured in flat-bottomed wells of a 96-well plate (Linbro) at 2×10^5 per well in a volume of 0.2 ml. Medium used for culture consisted of 50% Iscove's modified Dulbecco's medium and 50% Ham's F-12 nutrient mixture supplemented with bovine insulin, progesterone, transferrin, 2-mercaptoethanol, trace elements, and gentamicin (37) and 15% fetal calf serum (Rehatuin, lot No. W60006; Armour Pharmaceutical, Phoenix, AZ). After 56 h of incubation, proliferation was assessed by an additional 16 h incubation with 1 μCi/well [^{3}H]thymidine (72.5 Ci/mmol; New England Nuclear, Boston, MA). Wells were harvested onto paper discs with a multiple automatic cell harvester, and incorporation of [^{3}H]thymidine was determined by standard liquid scintillation techniques. Where raw data is illustrated, cpm ± SEM from three identical cultures are reported; otherwise, when Δcpm values are shown, standard error of the difference (SE diff) between test triplicate and control triplicate cultures are given.

Calculation of T Cell Supernatant Enhancement Ratio. For each set of triplicate cultures, an index representing the degree of T cell supernatant–dependent proliferation was calculated by computing the quotient of the Δcpm obtained in cultures in the presence of T cell supernatant vs. the Δcpm obtained for cultures in its absence. Δcpm values were used if and only if they were >2.5 times the standard deviation of the respective control cultures. Larger index values indicate that a greater degree of proliferation was obtained in the presence of the supernatant.

Results

Specificity of Anti–Human IgM mAb. Table I is a compilation of five experiments demonstrating the specificity of mAb HB57, 5D7, VIIIE11, Mu53, 1G6,

TABLE I

Demonstration of Specificity of Anti-Human IgM Monoclonal Antibodies by Solid Phase Radioimmunoassay

mAb	Murine isotype	Percent cpm ^{3}H-anti-mouse Ig bound*								
		Human myeloma proteins coated onto assay wells								
		Gre ($\mu\lambda$)	Pan ($\mu\kappa$)	Lac ($\gamma1\lambda$)	Tsc ($\gamma2\lambda$)	Dia ($\gamma3\kappa$)	Tuc ($\gamma4\kappa$)	And ($\alpha1\lambda$)	Cla ($\alpha2\kappa$)	Whi ($\delta\lambda$)
HB57	$\gamma1$	31.8	32.5	2.3	1.1	0.3	1.3	0.3	1.3	0.9
5D7	$\gamma2a$	35.0	35.0	1.0	0.5	0.4	1.0	1.0	ND§	1.5
VIIIE11	$\gamma3$	7.3‡	13.6	1.9	0.9	0.2	0.9	0.5	0.7	1.0
Mu53	$\gamma1$	18.5	27.4	0.4	0.9	0.2	0.4	0.2	0.3	0.3
1G6	$\gamma1$	20.2	21.7	0	0.1	0.8	0	0.1	0	ND
XG9	$\gamma1$	18.2‡	42.8	2.4	0.6	0.2	0.3	0.6	1.1	0.5
P24	$\gamma1$	14.9	18.1	0	0.6	0.4	0.4	0	0.3	ND
Anti-κ	$\gamma1$	3.8	29.6	4.3	0.8	33.3	28.6	0.4	23.0	1.1
Anti-λ	$\gamma3$	20.6	1.9	20.7	21.2	3.5	3.5	20.7	4.3	16.7

* ^{3}H-labeled anti-mouse isotype reagents (32) were used as probes to detect bound mAb. ~25,000 cpm of ^{3}H-Ab was added to each well. All data were corrected for background binding. The results represent a compilation of several experiments.

‡ Gre μ heavy chain preparation was used to coat wells in this experiment.

§ Not done.

XG9, and P24 for human IgM. The binding observed to all non-IgM myeloma proteins was at least one to two orders of magnitude lower than that to the IgM myeloma proteins Gre or Pan. The binding of anti-light chain mAb is included as controls for both the presence and the relative purity of the coating myeloma proteins. The low levels of inappropriate specific binding observed were consistent with minor contamination of the myeloma preparations by background nonmyeloma Ig. Similar results obtained with seven other IgM, five other IgG, and two more IgA myeloma proteins, not illustrated, confirmed the specificity assignment of the mAb for human IgM.

Selective Binding of Anti-IgM mAb to Epitopes Expressed on IgM-bearing B Cells. The fluorescence distribution histograms shown in Fig. 1 demonstrate that the anti-IgM mAb bound to determinants exposed on the surface of a B cell lymphoblastoid line expressing mIgM. The mAb did not bind to a B type chronic lymphocytic leukemia that had been previously found to lack detectable mIgM. Distribution histograms identical to those in Fig. 1 were observed when the mAb were tested on three additional leukemias that express mIgM (data not shown). The histograms illustrated were obtained using 1,000 μg/ml of mAb; similar results were also obtained when 10 μg/ml of each mAb was used for staining (data not shown).

Recognition of Distinct Epitopes on Human IgM by Anti-IgM mAb. A cross-inhibition assay was performed to evaluate which anti-μ mAb are directed to distinct determinants on human IgM. mAb that recognize the same epitope with comparable affinities should each reciprocally inhibit the binding of ^{3}H-IgM to the other.

The data from Table II demonstrate that: (*a*) mAb 1G6 bound to a unique

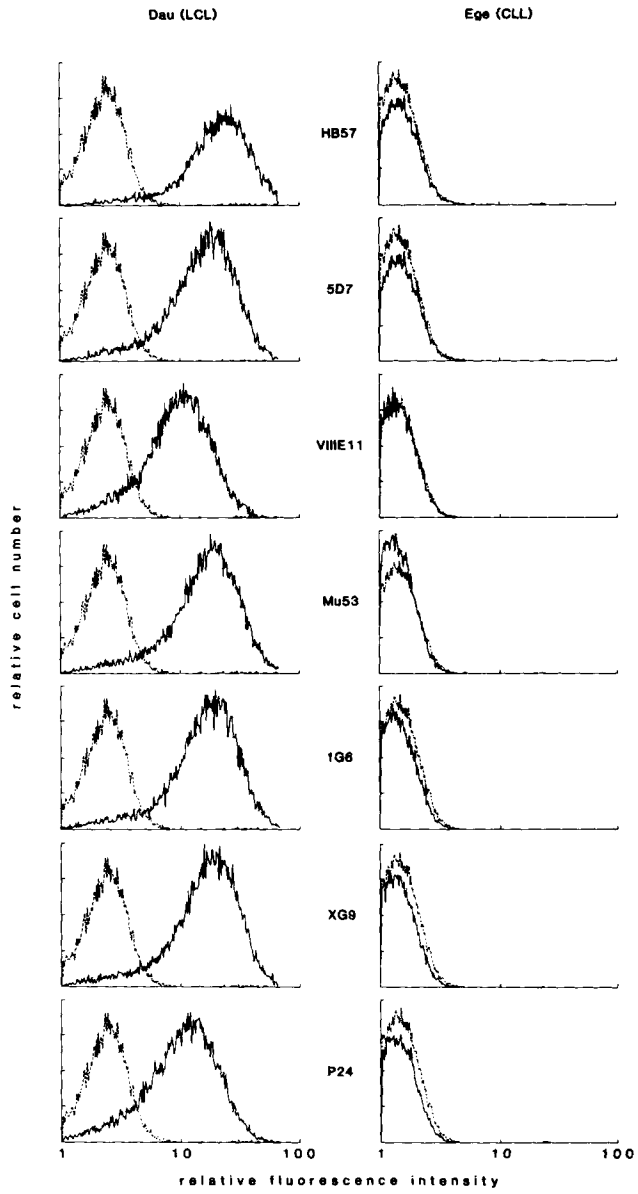

FIGURE 1. Anti-IgM mAb recognize B cell membrane IgM. FACS distribution histograms with logarithmic amplification for both an mIgM-positive lymphoblastoid cell line (Dau) and an mIgM-negative chronic lymphocytic leukemia (Ege) are shown. Cells were incubated with 1,000 μg/ml of ultracentrifuged mAb followed by a TRITC-F(ab')$_2$ goat anti-mouse Ig reagent. The binding of each mAb (—) is compared with the binding of an appropriate control Ab (- - -): MOPC-245 is the IgG1 mouse isotype control, LPC-1 is the IgG2a control, and FLOPC-21 is the IgG3 control. The binding of all anti-μ mAb to the mIgM-positive cell line was >1 log greater than the control Abs. In addition, no significant binding was evident when either anti-μ or control Abs were tested on an mIgM-negative cell line.

TABLE II

Cross-inhibition RIA to Analyze Epitope Specificity of Anti-IgM Monoclonal Antibodies

Wells coated with 10 μg/ml of:	Concentration (μg/ml) of soluble mAb needed for 50% inhibition of ^{3}H-IgM binding to well-bound mAb*						
	HB57	5D7	VIIIE11	Mu53	1G6	XG9	P24
HB57	0.19	115	175	9	43	35	21
5D7	90	0.64	>1,000	>1,000	23	>1,000	200
VIIIE11	>1,000	>1,000	0.52	>1,000	>1,000	0.28	>1,000
Mu53	0.79	>1,000	>1,000	2.2	>1,000	>1,000	230
1G6	>1,000	>1,000	>1,000	>1,000	1.0	>1,000	>1,000
XG9	>1,000	>1,000	0.75	>1,000	>1,000	0.40	>1,000

* PVC wells were coated with 10 μg/ml of purified anti-IgM mAb. The ability of various concentrations of soluble anti-IgM mAb (0.04–25 μg/ml) to inhibit the binding of ^{3}H-IgM (20,501 cpm added per well at a 0.5 μg/ml concentration) was evaluated. All tests were run in duplicate; the standard deviation of cpm bound from averaged wells were all <15% of mean value. The cpm of ^{3}H-IgM bound to mAb-coated wells in the absence of soluble inhibitor were as follows: HB57 (5,055 cpm), 5D7 (6,469), VIIIEII (9,463), Mu53 (8,253), 1G6 (9,611), XG9 (9,058), and P24 (867).

epitope on human IgM as shown by the fact that no other mAb was capable of competing with it in a reciprocal fashion. (*b*) mAb VIIIE11 and XG9 bound to the same or very similar determinant since they strongly competed with each other. This epitope is probably distinct from that recognized by all other mAb since no other mAb competed reciprocally. (*c*) mAb 5D7 presumably binds to a unique epitope since it competed only minimally with HB57 and was inhibited only by 1G6, in a nonreciprocal fashion. (*d*) mAb HB57 and Mu53 could have specificity for the same or very neighboring site, as they were able to reciprocally compete at low concentrations. However, unlike mAb Mu53, the binding of HB57 to ^{3}H-IgM could be inhibited to some degree by all the mAb. (*e*) P24 and HB57 appear to recognize the same or very proximate epitopes, since they yielded identical patterns of inhibition when used to compete with all other insolubilized mAb.

Results obtained from inhibition assays in which P24 mAb was insolubilized on wells are not included in Table II because the amount of ^{3}H-IgM bound to such wells was too low to allow reliable inhibition curves to be made. This could reflect either a low affinity of P24 mAb for IgM or a diminution in its binding capacity when the mAb is immobilized onto PVC wells. It should be noted that soluble P24 mAb was very effective at binding to IgM-coated wells at mAb concentrations as low as 2.5 ng/ml (data not shown).

Differential Ability of Anti-IgM mAb to Induce Proliferation of Human Splenocytes. Fig. 2 illustrates the results from three experiments demonstrating differences in the ability of the anti-IgM mAb to induce human splenic B cell proliferation in the presence of T cell supernatant. Over a dose range of 0.5–1000 μg/ml, mAb HB57 always induced the greatest proliferation response. While mAb 5D7 induced a considerably lower degree of stimulation, it gave a similarly shaped dose response profile to that of mAb HB57. With both, a long plateau of maximal stimulation was observed using concentrations of 1,000 to nearly 20 μg/ml; mAb concentrations as low as 2 μg/ml resulted in proliferation significantly above background.

The concentration response profiles of mAb Mu53 and VIIIE11 were similar

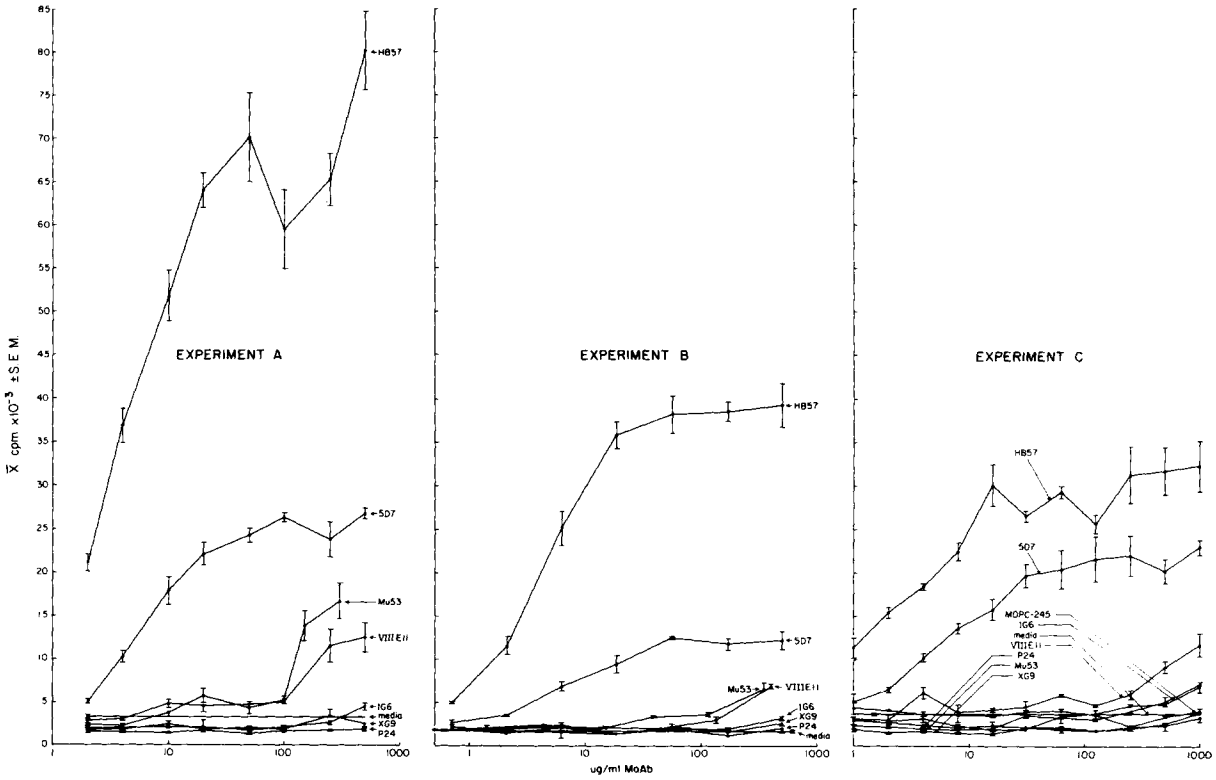

FIGURE 2. Individual anti-IgM mAb vary in ability to induce human splenic B cell proliferation. T cell–depleted human splenocytes from two separate donors (experiments A and B, donor 1; experiment C, donor 2) were cultured with various concentrations of purified anti-μ mAb in the presence of 30% T cell supernatant. The proliferative response observed in control cultures with the murine myeloma MOPC-245 is also illustrated. The data are expressed as $\bar{x}$ cpm ± SEM from triplicate cultures. Maximal B cell stimulation was always obtained with mAb HB57 and 5D7 (HB57 > 5D7). mAb VIIIE11 and Mu53 induced a lower level of proliferation that was only manifest at high mAb concentrations. 1G6, XG9, and P24 mAb usually induced an inhibition of background proliferation, indicated by the solid horizontal line.

to one another but distinct from those of HB57 and 5D7. A much greater concentration (>100 μg/ml) of these mAb was needed to induce a substantial degree of [^{3}H]thymidine uptake. Furthermore, the maximal level of stimulation obtained was always much lower than with HB57 or 5D7 mAbs. Concentrations of Mu53 and VIIIE11 ≤1,000 μg/ml did not produce a definable plateau in stimulation.

In contrast, mAb 1G6, XG9, and P24 did not induce stimulation at any concentration tested in five of six experiments. In one experiment, 1G6 induced proliferation comparable to that of Mu53. mAb XG9 and P24 always inhibited background proliferation. In addition, no significant proliferation over background was ever obtained with the irrelevant murine γ1 Ab, MOPC-245, at concentrations ≤1,000 μg/ml.

When experiments were performed using ultracentrifuged mAb preparations, the patterns of stimulation obtained were indistinguishable from those seen with

noncentrifuged preparations, suggesting that Ab aggregation was not a contributing factor to the differential responses observed. As demonstrated in Table III, cultures containing optimal stimulatory doses of ultracentrifuged anti-μ mAb HB57, 5D7, VIIIE11, and Mu53 were inhibited to background or below background levels of proliferation when 500 μg/ml soluble pentameric IgM was added. This suggests that mAb-induced proliferation depends upon the anti-μ–ligand interaction and not on a contaminating mitogenic moiety.

Marked Augmentation of B Cell Proliferation with Certain Combinations of mAbs. Fig. 3 illustrates that a 1:1 mixture of the two individually nonstimulatory mAb 1G6 and XG9 resulted in a marked enhancement of B cell activation. While the individual mAb did not induce >1,209 ± 462 cpm of [^{3}H]thymidine incorporation (Δcpm ± SE diff) at any dose tested, the combined mAb (250 μg/ml of each) induced 74,391 ± 4399 cpm. This level of stimulation was comparable to that elicited by the most stimulatory individual mAb, HB57. However, while maximal stimulation induced by mAb HB57 was achieved at 20 μg/ml, maximal stimulation induced by the 1:1 1G6-XG9 mAb mixture required >500 μg/ml of total Ab.

One-to-one mixtures of each of the anti-IgM mAb, in every paired combination, were tested for their mitogenicity. Mixtures of two mAb that induced more than twice the level of [^{3}H]thymidine incorporation obtained with either mAb alone were considered to exhibit the phenomenon of synergy. By this criterion, only those mixtures that included mAb 1G6 and in some instances mAb XG9 exhibited synergy. Most other combinations of mAb showed an additive response (data not shown).

The ability of 1G6 and XG9 mAb to induce synergy in B cell stimulation is illustrated in Fig. 4, which shows that: (*a*) mAb 1G6 markedly enhanced the proliferation induced by mAb XG9, 5D7, and VIIIE11 but induced a minimal degree of enhancement with HB57 and Mu53 and none at all with P24. (*b*) mAb XG9 exhibited synergy with 1G6, 5D7, and Mu53, but did not manifest this phenomenon with HB57, VIIIE11, or P24. (*c*) When cultured with mAb Mu53, 1G6 and XG9 induced equivalent levels of [^{3}H]thymidine incorporation. In all other cases, the combination of a given mAb with 1G6 always induced a greater

TABLE III

Ability of Soluble Human IgM to Inhibit B Cell Proliferation Elicited by Ultracentrifuged mAb Preparations

mAb (μg/ml)	$\bar{x}$ cpm (±SEM)	
	Without inhibitor	With IgM inhibitor
HB57 (125)	67,908 (1,198)	4,196 (447)
5D7 (125)	34,754 (2,166)	2,174 (185)
VIIIE11 (500)	7,471 (962)	2,116 (322)
Mu53 (500)	7,763 (739)	1,772 (34)
Media	2,399 (199)	2,258 (75)

Purified splenic B cells were cultured with HB57, 5D7, VIIIE11, or Mu53 anti-IgM mAb at the indicated concentrations. All mAb were ultracentrifuged at 108,000 *g* for 80 min. The top one-third of each was used for culture. 500 μg/ml human IgM was used as inhibitor.

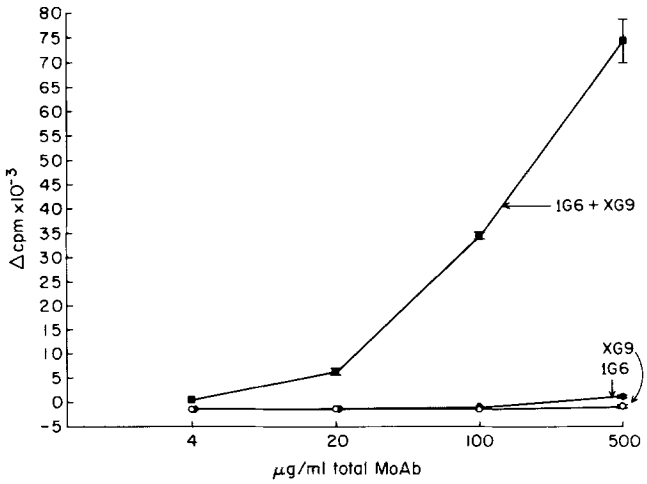

FIGURE 3. Synergy in B cell proliferation when anti-IgM mAb 1G6 and XG9 were cocultured. T cell–depleted splenic lymphocytes were cultured with 1G6 mAb (●) or XG9 mAb (○) or 1:1 mixtures of the two (■) at various mAb concentrations, in the presence of 30% T cell supernatant. The mAb concentrations indicated reflect the total Ab concentration in culture. Δcpm ± SE diff of culture triplicates are shown based upon 3,124 ± 225 ($\bar{x}$ cpm ± SEM) background [^{3}H]thymidine incorporation. Significant augmentation of B cell stimulation was observed when as little as 10 μg/ml of each mAb were cultured together. No significant stimulation over background was found when either 1G6 or XG9 mAb were cultured separately at doses up to 500 μg/ml.

degree of proliferation than the combination of the given mAb with XG9. (*d*) The inability of XG9 to induce synergy when cocultured with VIIIE11 is consistent with their apparent recognition of the same epitope on the IgM molecule (see Table II). However, these two mAb consistently induced different levels of proliferation when cultured with other anti-μ mAb. For example, the coculture of XG9 with 5D7 (at 100 μg/ml total Ab) elicited 54,534 ± 2,244 cpm of [^{3}H]thymidine uptake (XG9 alone, −1,433 ± 264; 5D7 alone, 20,955 ± 857), whereas the mixture of 5D7 and VIIIE11 elicited 34,438 ± 4,610 cpm of [^{3}H]-thymidine incorporation (VIIIE11 alone, 2,393 ± 1,032). (*e*) The lack of synergy observed in mixtures containing either 1G6 or XG9 together with P24 does not appear to be due to the delivery of a negative signal by P24. The proliferation obtained when P24 was cocultured with individually mitogenic anti-IgM mAb was not any lower than that obtained when these latter mAb were used singly (data not shown).

Absence of Synergy Between 1G6 Anti-IgM mAb and mAb Directed to Non-IgM B Cell Membrane Molecules. The possibility was explored that the synergy exhibited by mAb 1G6 could also be obtained by incubation with mAb directed to non-IgM molecules on the B cell surface. The data in Table IV show that mAb specific to both IgD (CBDA-4E5) and Ia (22c6, SG171) did not induce proliferation and that, when these mAb were cultured with 1G6 mAb, stimulation was not enhanced. However, in the control, a significant degree of enhancement in B cell stimulation was achieved when mAb 1G6 was cocultured with Mu53 anti-μ mAb. When cultured separately at this concentration, both Mu53 and 1G6 induced no significant degree of proliferation over background.

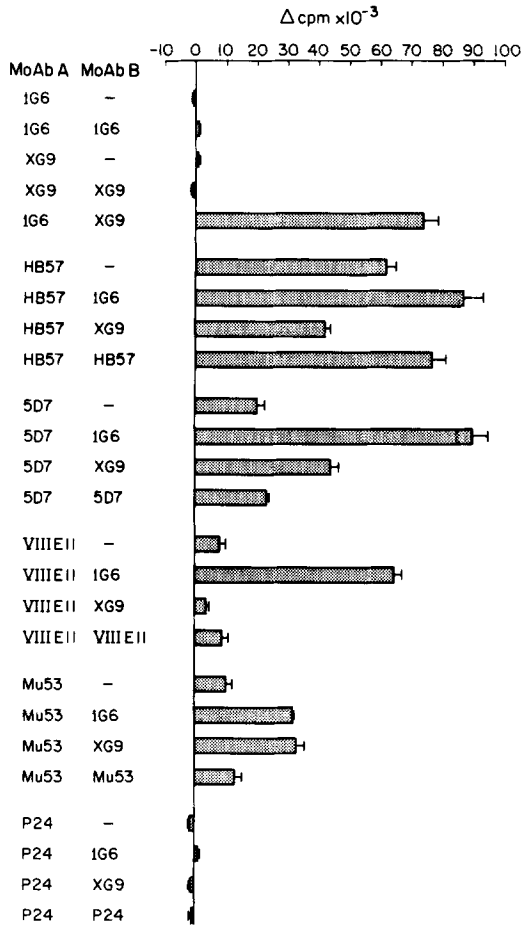

FIGURE 4. Synergy in B cell stimulation when anti-IgM mAb 1G6 or XG9 are cultured with other anti-IgM mAb. Human splenic B cells in medium containing 30% T cell supernatant were cultured with 250 μg/ml of mAb A and/or 250 μg/ml of mAb B. The data are expressed as Δcpm ± SE diff. Background stimulation was 3,293 ± 236 ($\bar{x}$ cpm ± SEM). Both 1G6 and XG9 mAb did not elicit any significant amount of [^{3}H]thymidine uptake in cultured cells when used individually; however, when mixed together, or when mixed with 5D7 or Mu53 mAb, both induced synergistic responses (see text). In addition, when 1G6 mAb was cocultured with VIIIE11, a marked synergy in the level of [^{3}H]thymidine incorporation was observed. Neither 1G6 nor XG9 mAb elicited proliferation when mixed with mAb P24. The inhibition of HB57-induced proliferation obtained when XG9 was cocultured with HB57 was reproducible in three experiments.

Comparison of the Effect of T Cell Supernatant Upon Proliferation Induced by Individual Anti-IgM mAb or mAb Mixtures. In the absence of T cell supernatant, the B cell proliferation induced by mAb HB57 was markedly reduced. Nevertheless, a Δcpm of 2,597 ± 269 was obtained with as little as 4 μg/ml mAb (Fig. 5) and plateau levels (Δcpm, 10,451 ± 2,291) of stimulation were reached with >20 μg/ml, the same concentration of HB57 that gave plateau levels in the presence of T cell supernatant (Fig. 2). The proliferation induced by all other individually mitogenic mAb was also substantially reduced in the absence of T

TABLE IV

Inability of 1G6 Anti-IgM mAb to Synergize with mAb Directed to Other B Cell Membrane Molecules

mAb A	Δcpm (SE diff)					
	mAb B					
	None	1G6	Mu53	4E5 Anti-IgD	22c6 Anti-DR	SG157 Anti-DR
None	—	−1,507 (552)	−67 (696)	−446 (620)	−2,287 (610)	−801 (582)
1G6	−1,507 (552)	−748 (591)	8,302 (929)	−1,064 (549)	183 (597)	−1,200 (566)

Purified B cells were cultured with 50 μg/ml of purified mAb A and/or mAb B in medium containing 30% T cell supernatant. Δcpm represents the cpm in mAb-containing cultures above that in cultures with medium alone (3,864 ± 539 cpm).

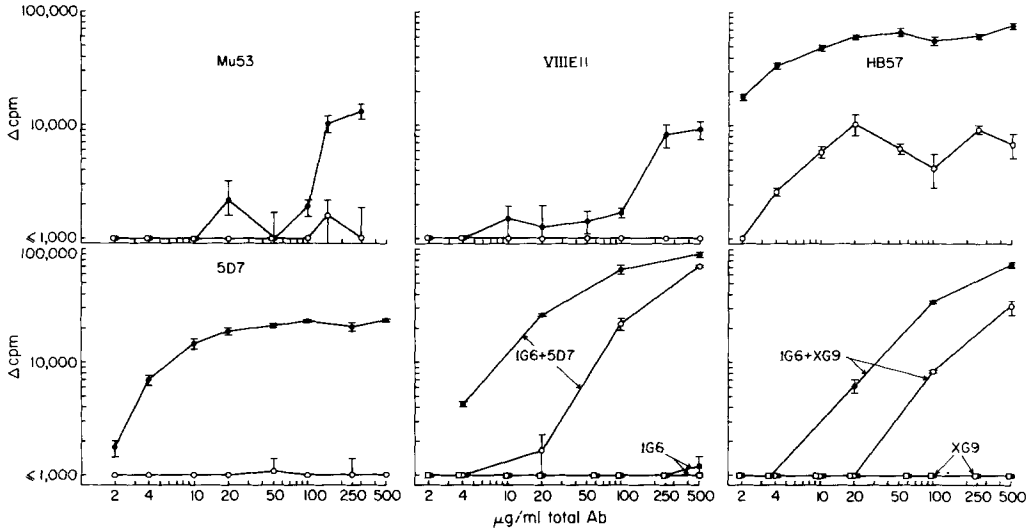

FIGURE 5. Comparison of B cell proliferation induced by single anti-IgM mAb or mAb mixtures in the presence and absence of T cell supernatant. T cell-depleted splenic lymphocytes were cultured with the indicated concentrations of individual anti-IgM mAb or 1:1 mAb mixtures in the presence (closed symbols) or absence (open symbols) of 30% T cell supernatant. The data are expressed as Δcpm ± SE diff. Background proliferation with T cell supernatant was 3,293 ± 236 ($\bar{x}$ cpm ± SEM). In the absence of T cell supernatant, the proliferation induced by each individual mAb was either markedly reduced or not detectable. However, when mAb mixtures were tested, a highly significant degree of proliferation was obtained in the absence of an exogenous T cell factor source. In some instances (mAb 1G6 + 5D7 and 1G6 + XG9), the level of stimulation observed in the absence of T cell supernatant approached that obtained for the same cultures in the presence of supernatant.

cell supernatant; in fact, low levels of stimulation (Δcpm, 1,000–2,500) were observed only in three of seven experiments in which B cells were cultured with maximal concentrations of 5D7 or Mu53 mAb.

Fig. 5 also illustrates that, in contrast to individual mAb, certain mixtures of anti-IgM mAb induced a significant degree of B cell proliferation in the absence of exogenous T cell supernatant. Moreover, the level of proliferation approached that for the same cultures containing the added T cell factor source.

The ability of T cell supernatants to enhance the proliferation of B lymphocytes cultured with various concentrations of individual or 1:1 mixtures of anti-IgM mAb is evaluated in Table V. The data demonstrate that: (*a*) the proliferation

TABLE V

Comparison of the Degree of T Cell Supernatant Dependence Exhibited in B Cell Cultures Stimulated by High and Low Concentrations of Mitogenic Anti-IgM mAb

Anti-IgM mAb	Index of enhancement by T cell supernatant* with total Ab concentration at:					
	500 μg/ml			20 μg/ml		
	Exp. A	Exp. B	Exp. C	Exp. A	Exp. B	Exp. C
HB57	11.12	5.32	11.09	7.25	4.77	10.96
5D7	—‡	27.10	29.13	—	—	16.63
VIIIE11	—	—	4.67	—	—	—
Mu53	13.11	—	11.64	—	—	2.65
1G6 + XG9	2.39	2.04		—	—	
+ HB57	1.78	1.40		7.50	5.53	
+ 5D7	1.27	1.62		15.95	15.11	
+ VIIIE11	3.29	2.83		10.45	5.90	
+ Mu53	7.19	3.03		—	—	
XG9 + HB57	5.47	4.73		8.10	6.93	
+ 5D7	7.73	8.35		17.29	11.23	
+ Mu53	7.79	5.42		—	—	
HB57 + 5D7	8.74	5.55		9.92	6.26	
+ VIIIE11	12.23	5.50		9.21	7.17	
+ Mu53	4.32	4.24		16.68	8.41	
5D7 + VIIIE11	13.23	8.32		24.80	8.78	
+ Mu53	11.35	8.45		—	24.37	
VIIIE11 + Mu53	10.99	4.15		—	6.17	

* Index of T cell supernatant (T-supt) enhancement was calculated by dividing the Δcpm in anti-IgM–stimulated cultures containing T-supt by the Δcpm in anti-IgM–stimulated cultures without T-supt.

‡ Index was not calculated because the denominator (Δcpm in cultures without T-supt) was too low to give a reliable index. Index values were only calculated when both the Δcpm observed in anti-IgM–stimulated cultures containing T-supt and the Δcpm observed in stimulated cultures without T-supt were each >2.5 standard deviations of the $\bar{x}$ cpm in the respective control cultures. Control cultures without T-supt exhibited $\bar{x} \pm$ SD values of 2,049 ± 370, 2,177 ± 355, and 759 ± 122 cpm in Exps. A, B, and C, respectively. Control cultures containing 30% T-supt exhibited 3,293 ± 471, 3,124 ± 225, and 1,950 ± 170 cpm in Exp. A, B, and C, respectively.

induced by each individual anti-IgM mAb was markedly enhanced in the presence of T cell supernatant, as indicated by the large index values (see Materials and Methods). (*b*) Cultures stimulated with the low-dose stimulatory mAb (HB57 or 5D7) showed no significant difference in T cell supernatant dependence at high or low mAb concentrations. Even when mAb doses suboptimal for maximal proliferation were tested (data not shown), T cell supernatants did not enhance stimulation to any greater degree than when optimal concentrations of mAb were used. (*c*) In contrast, cultures stimulated with mAb mixtures containing 1G6 generally exhibited a high degree of T cell supernatant independence (i.e., low index values), manifested at high mAb doses but diminishing at low doses. (*d*) Cultures stimulated by mAb mixtures without 1G6 showed a much less

pronounced, in many cases negligible, difference in T cell supernatant dependence at high and low mAb concentrations.

Comparison of T Cell Supernatant-replacing Ability of mAb 1G6 and XG9. Fig. 6 illustrates that the synergy obtained by coculturing mAb 1G6 with each of the anti-μ mAb in the absence of T cell supernatant was at least equivalent to the synergy obtained when the non-1G6 mAb were cocultured with T cell supernatant alone. Only with mAb Mu53 did the T cell factor source function better as a costimulator than 1G6. Compared with 1G6, XG9 was considerably less effective as a costimulator than the T cell supernatant.

We considered the possibility that DEAE-purified 1G6 was contaminated with

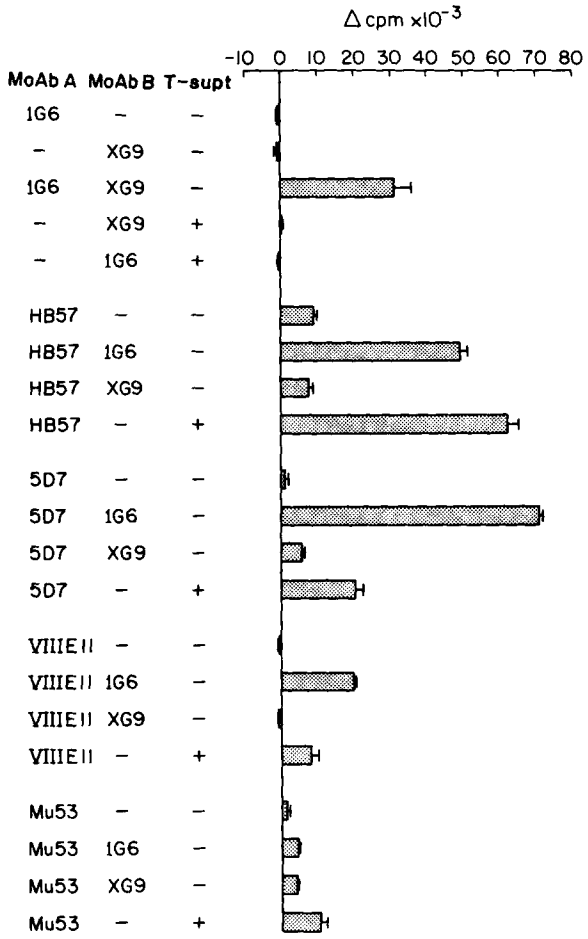

FIGURE 6. Comparison of T cell supernatant-replacing ability of mAbs 1G6 and XG9. Cultures of T cell-depleted splenic lymphocytes containing 250 μg/ml of mAb A were supplemented with 250 μg/ml of mAb B (1G6 or XG9) or 30% T cell supernatant (*T-supt*). The data are expressed as Δcpm ± SE diff of triplicate cultures. Background cpm in control cultures was 3,124 ± 225 ($\bar{x}$ cpm ± SEM). In nearly all cultures, mAb 1G6 functions as well, if not better, than T cell supernatant in augmenting B cell stimulation; the only exception being mAb Mu53-stimulated cultures. In contrast, mAb XG9 was unable to augment B cell proliferation when used as a costimulator in the absence of T cell supernatant.

a B cell growth factor (BCGF)-like factor. Further purification of 1G6 on protein A Sepharose or on Sepharose coupled with purified goat anti–mouse Ig antibody, revealed that the synergistic properties were associated with the bound mouse IgG antibody (data not shown).

Discussion

One principal finding in this study was that various anti-IgM mAb differed significantly in their ability to induce proliferation of human splenic B lymphocytes. HB57 and 5D7 produced high plateau levels of stimulation at very low concentrations. These two mAb induced significant proliferation above background with concentrations as low as 2 μg/ml. VIIIE11 and Mu53, in contrast, induced a lesser degree of proliferation and only at high concentrations. Finally, anti-IgM mAb 1G6, P24, and XG9 did not produce significant proliferation, but usually caused an inhibition of background [^{3}H]thymidine incorporation over a broad dose range.

These findings, together with the results of recent reports (17, 21, 38–40), bear importantly on past studies (1–16, 18–20, 22) of the effect of polyclonal anti-IgM Ab on B cells. The Ab in anti-Ig sera are a mixture of distinct species that are likely drawn from the different prototype mAb described above. As a result, the degree of proliferation elicited and the dose required for optimal stimulation by polyclonal Ab will to some degree reflect the nature of the individual Ab comprising such polyclonal preparations.

How is it that different mAb, each with specificity for the same molecule, are able to elicit such different degrees of stimulation? A number of factors may influence the ability of distinct anti-IgM mAb to induce proliferation of B lymphocytes. The avidity of an mAb for epitopes on membrane Ig is a likely factor. In this regard, Hamano and Asofsky have recently described an association between the affinity of two rat mAb to mouse IgM and their ability to induce differentiation of a murine B cell line (41).

The ability to induce B cell proliferation could also be influenced by the epitope specificity of the mAb. This association could reflect the mechanism by which B cells receive stimulatory signals. There are at least two explanations of how anti-Ig Ab trigger B cell proliferation, which are not necessarily mutually exclusive. Binding to certain epitopes may cause a conformational change in the mIgM molecule that aids in the initiation of intracellular signals required for proliferation. Alternatively, signals may arise from the aggregation of mIgM molecules (43). With regard to this latter mechanism, it is likely that an mAb which recognizes an epitope multiply expressed on an mIgM molecule will be more efficient at crosslinking mIgM molecules than an mAb that recognizes a singly expressed epitope.

Many of the anti-IgM mAb found to exhibit distinct patterns of stimulation in this study also recognize unique epitopes on IgM. These results are compatible with the possibility that epitope specificity influences mitogenic potential. In addition, Julius et al. (39), as well as Zitron and Clevinger (38), have reported that two mAb pairs, directed to epitopes on different domains of the murine IgM and IgD molecule, respectively, vary in their ability to induce B cell proliferation. The binding to a particularly sensitive, domain-associated epitope

may be important in signaling since the recent data of Leptin (40) indicate that two soluble rat anti–murine IgM mAb which share domain specificity and isotype with the mitogenic mAb described by Julius et al. (39) both fail to induce murine B cell proliferation.

The effects of Ab binding to Fc receptors has been shown to influence B cell activation by anti-Ig Ab (10, 19–21). However, it is unlikely that an Fc region–mediated mechanism is responsible for all differences in the mitogenic character of the individual anti-μ mAb in this study, since five of those that displayed functional differences were of the same γ1 isotype. The possibility cannot be excluded that the difference in stimulatory capacity between mAb XG9 (γ1 murine isotype) and VIIIE11 (γ3 murine isotype) could be due to Fc differences, since the available evidence indicates that they both bind to the same epitope.

Of particular interest is the finding that the individually nonstimulatory mAb 1G6 and XG9 give a very strong mitogenic response when incubated together. Moreover, synergy was observed when either 1G6 or XG9 was cocultured with all but two of the anti-IgM mAb. This was apparent not only at high concentrations (250 μg/ml) of each mAb, but also at concentrations as low as 10 μg/ml. Indeed, the only instance in which 1G6 did not markedly augment existing stimulation was in co-incubation with HB57 in the presence of T cell supernatant. This could reflect the fact that in such cultures HB57 mAb alone elicits a near-maximal B cell response. This possibility is strengthened by the observation that, in the absence of T cell supernatant, 1G6 strikingly enhances the maximal stimulation achieved with HB57. It is important to consider that mAb 1G6 induced synergy in B cell proliferation when combined with other anti-IgM mAb but not when combined with mAb to other surface molecules, such as anti-Ia or anti-δ. However, since a limited number of anti-Ia and anti-δ mAb were tested, the possibility remains that others might display synergy with mAb 1G6.

At present, there is no satisfactory interpretation for the absence of synergy between mAb XG9 and HB57 even in the absence of T cell supernatant. One reasonable explanation may be that, when combined, they have a reduced ability to induce positive signals for B cell proliferation. This could also explain the inability of mAb 1G6 and XG9 to induce B cell proliferation when either was used in combination with P24.

At least three possible mechanisms for the synergy demonstrated in cocultures of anti-μ mAb with mAb 1G6 (or XG9) can be considered: (*a*) cyclic complexes may form between mAb 1G6, mIgM, and other anti-μ mAb, which lead to a greater overall avidity of the ligands for mIgM. Ehrlich et al. (43) have recently described cyclic complexes of mAb that display a higher affinity for antigen than the individual mAb. In addition, Parham (44) has shown that the binding of certain mAb to a major histocompatibility complex molecule induces conformational changes that affect the affinity with which other mAb bind to alternative epitopes on the molecule. (*b*) The ability of mAb 1G6 to induce synergy may simply reflect its unique ability to collaborate with other mAb in inducing efficient crosslinkage of mIgM molecules and consequent stimulatory micropatch formation (42) on the B cell membrane. It is unlikely that this mechanism is solely responsible for the observed synergy since other pairs of mAb that bind to unique epitopes and thus should be capable of increasing crosslinkage (e.g.,

5D7 and VIIIE11 or Mu53 and VIIIE11) do not display synergy when combined. (*c*) The exceptional ability of 1G6 mAb to synergize may reflect its unique capability to induce conformational changes that enhance the efficiency of signal transfer from mIgM to the B cell interior after binding to mIgM with another anti-IgM Ab.

Studies with anti-IgM Ab from polyclonal antisera (45, 46) have shown that B cell proliferation elicited by low Ab concentrations is dependent upon T cell factors, while proliferation elicited by high concentrations of Ab is relatively T cell independent. In contrast, our experiments with individual anti-IgM mAb indicate that, irrespective of the dose of Ab, the proliferation induced by each individual mAb is significantly enhanced by the presence of a T cell factor source.

Of particular interest is our finding that the cocultivation of mAb 1G6 with other anti-IgM mAb circumvents the need for a second signal to be delivered to B cells by T cell supernatant. We did not examine whether these mAb mixtures, at high concentrations, recruit the proliferation of a B cell subpopulation that does not require second signals from exogenous factors, or whether signals from these mixtures abrogate the need for additional signaling to one responsive B cell subpopulation. In addition, although the data clearly show that mAb 1G6 has specificity for human IgM, they do not rule out the possibility that 1G6 may crossreact with the BCGF receptor on human B lymphocytes and thereby mimic the effects of BCGF. Given that B cells are capable of secreting B cell growth factor when infected with Epstein-Barr virus (47), it is also conceivable that the signals delivered by mAb 1G6, in combination with another anti-IgM mAb, induce B cells to secrete their own endogenous growth factor that eliminates the need for exogenous factor. These possible mechanisms for the unique properties of mAb 1G6 remain to be investigated.

Summary

Seven murine monoclonal antibodies (mAb) with different binding characteristics for human IgM varied markedly in their ability to induce proliferation of T cell–depleted human splenocytes. Two mAb (HB57 and 5D7) that bound to distinct epitopes on IgM were highly effective initiators of B cell proliferation at very low concentrations, in the presence of a T cell factor source. In the absence of T cell supernatant, both HB57 and 5D7 mAbs produced a markedly reduced degree of stimulation at all concentrations. Two additional anti-IgM mAb (VIIIE11 and Mu53) were distinctive in that, even at high concentrations, only limited proliferation was observed compared with the first group of mAb. This proliferation depended on the presence of T cell supernatant. Competitive-binding studies revealed that the epitope recognized by mAb Mu53 may be identical or very proximate to that recognized by HB57. Three other mAb (1G6, XG9, and P24) induced little or no proliferation. 1G6 bound to a unique epitope on the IgM molecule, whereas XG9 shared a determinant with VIIIE11 mAb. Regulatory influences of Fc receptor binding cannot account for all the diversity in proliferation observed with the individual anti-IgM mAb.

Markedly augmented proliferation was obtained when B cells were cultured with certain combinations of anti-IgM mAb in the presence of exogenous T cell supernatant. The proliferation induced in the absence of T cell supernatant by

high concentrations of mAb mixtures that included 1G6 approached that observed for the same mixtures in the presence of T cell supernatant. The data suggest that certain signals delivered through membrane IgM can bypass the need for T cell supernatant in the activation of human B lymphocytes.

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