International Immunology, Vol. 7, No. 2, pp. 317–330

A monovalent $C_\mu 4$-specific ligand enhances the activation of human B cells by membrane IgM cross-linking ligands

Patricia K. A. Mongini[1,2], Clare A. Blessinger[1], Nicholas Chiorazzi[3], Nirmala Rajaram[3] and **Steven M. Rudich[1,4]**

[1]Department of Rheumatology and Molecular Medicine, Hospital for Joint Diseases, New York, NY 10003, USA
[2]Department of Pathology, Kaplan Comprehensive Cancer Center, New York University School of Medicine, New York, NY 10016, USA
[3]Department of Medicine, North Shore University Hospital, Manhasset, NY 11030, USA

[4]Present address: Department of Surgery, University of California, Los Angeles, CA, USA

Abstract

The ligand–receptor binding requirements for achieving full B cell activation through the membrane immunoglobulin (mIg) signaling pathway are relatively demanding, and mIg–antigen engagements which fall below these critical thresholds cause, at most, only the partial activation of B cells. In an effort to resolve new means of enhancing the efficacy of mIgM-mediated signal transduction, as well as to further understand the process by which mIgM-mediated signals are initiated, we have explored the mechanism for a previously reported synergy between certain mixtures of murine anti-IgM mAbs in eliciting human B cell DNA synthesis. We here report that striking synergy occurs when any of several relatively high affinity mAbs specific for diverse domains of mIgM are combined in culture with the relatively low affinity $C_\mu 4$-specific ligand, mAb IG6. Although B cell activation was dependent upon the bivalency, and hence mIgM cross-linking potential, of the high affinity ligand, low affinity mAb IG6 could enhance the activation process when present as a monovalent Fab′ fragment. This did not appear due to $F(ab')_2$ contamination or Fab′ aggregation, since IG6 Fab′ preparations were notably compromised in several other functions requiring ligand bivalency. Pulsing studies revealed that the $C_\mu 4$-specific ligand exhibits its functional effects only when stimulatory mIgM receptor cross-links are being formed by bivalent ligands, and that IG6 Fab′ enhancement is most notable during the later interval of the prolonged mIgM signaling process that leads to S phase entry. A unique region of the membrane-proximal IgM domain may be important for Fab′-mediated enhancement, since Fab′ fragments that bind with higher affinities to distinct sites on $C_\mu 4$ were not as effective at mediating this phenomenon. Several possibilities for the adjuvant effects of this $C_\mu 4$-specific Fab′ on B cell responses triggered by mIgM cross-linking ligands are discussed, including the possibility that IG6 Fab′ influences the potential for mIgM dimer formation or interactions of mIgM with other signal-transducing molecules.

Introduction

Membrane-associated Ig molecules (mIg) on B lymphocytes not only function to internalize antigen for B cell processing and presentation to antigen-reactive T_H cells, but when cross-linked by antigen can also initiate signaling cascades that directly lead to B cell activation. Such signals can lead to the enhanced expression of cell surface molecules such as class II MHC, B7/BB1 and cytokine receptors, which help facilitate the receipt of cell cycle progression signals from T_H cells (1–4). Under certain circumstances, signal transduction through mIg receptors is sufficient to cause the full activation of B cells in the absence of accessory T cell signals (5–8). Studies with anti-IgM Abs as model cross-linking antigens have shown that this latter T cell independent activation process has ligand–receptor physicochemical binding requis-

Correspondence to: P. Mongini, Department of Rheumatology and Molecular Medicine, Hospital for Joint Diseases, 301 E. 17th St., New York, NY 10003, USA

Transmitting editor: Z. Ovary

Received 13 October 1994, *accepted* 9 November 1994

ites that are considerably greater than those for inducing early activation phenomena. Thus, whereas bivalent anti-IgM mAb with binding sites of low to intermediate affinity for human B cell mIgM can induce early increases in membrane class II MHC expression and G_1 entry, the cell cycle progression of activated B cells into S phase is achieved only with high concentrations of bivalent mAb with high intrinsic affinity, i.e. $K_a \geq 1 \times 10^8\ M^{-1}$ (8,9). The requirements for high concentration and high binding site affinity can be substantially reduced by increasing the number of binding sites on mIg-binding ligands of high mol. wt, as evidenced by B cell responses to anti-IgM–dextran conjugates used as models for polysaccharide antigens (7,8). The disparity in the physicochemical binding thresholds needed for achieving various stages in B cell activation has been linked to the fact that the full B cell activation through the mIgM signaling pathway requires the continued formation of new mIgM cross-links for a considerable period after initial exposure to ligand, i.e. to ~10 h before entry into S phase (9,10). Pulsing studies with bivalent ligands have suggested that the concentration and affinity requirements for achieving mIgM-mediated signal transduction during the later phase in activation are considerably greater than the ligand binding requirements for initiating signals in resting B cells (9,10). At least one factor responsible for the more rigorous binding requirements for late-phase signaling via the mIgM-mediated signaling pathway may be the diminished density of responsive receptors available for cross-link formation (8,9).

The greater insights obtained in recent years regarding the ligand–mIgM binding requirements for achieving B cell activation has led us to re-examine the explanation for a previously reported synergy between certain unique pairs of bivalent anti-IgM mAbs in achieving B cell DNA synthesis (11). In this report we examine how the binding site affinity and domain specificity of a group of anti-IgM mAbs affects their potential to display synergy in promoting B cell activation. In addition, we study the functional properties of bivalent and monovalent Fab' forms of the relevant mAb in order to discern whether the synergistic phenomenon might be explained either by a greater potential of certain pairs of bivalent mAbs to form and/or stabilize receptor cross-links through bridging mIgM molecules at distinct sites (11–13) or by some other phenomenon, e.g. a change in the conformation or disposition of mIgM that enhances the efficiency of signal transfer.

Methods

Murine anti-human Ig mAb

A full description of the derivation, epitope specificity and binding affinity of the mAbs used in this study, i.e. anti-IgM mAbs HB57 (DA4.4), 5D7, XG9, Mu18, 4-3, Mu53, IG6, P19, P24, IF11 and 196.6b, as well as anti-IgD mAb 4E5, can be found elsewhere (6,11,14,15). All the above mAbs, with the exception of mAb 5D7 (IgG2a), are of the murine IgG1 isotype. Purified mAb protein was prepared either by direct isolation of IgG from ascitic fluid on Affi-Gel protein A MAPS II columns (Bio-Rad Laboratories, Richmond, CA) or by selective elution of IgG1 on DEAE–Trisacryl (LKB Instruments, Rockville, MD) following Na_2SO_4 precipitation of ascitic fluid, as described earlier (11). Purity of the mAb preparations was assessed by 5–20% SDS–PAGE analyses as well as ELISA assays which measured binding of the anti-IgM mAbs to IgM-coated wells (8,11).

Preparation of F(ab')$_2$ and Fab' fragments of selected anti-IgM mAbs

Pepsin digestion was used to obtain F(ab')$_2$ fragments from mAbs IG6, HB57, Mu53, 4-3 IF11, 196.6b and MOPC-21 (6). Further reduction and alkylation steps generated the respective Fab' fragments (6). mAb IG6 was quite sensitive to pepsin digestion. For optimal recovery of F(ab')$_2$ and Fab' from this mAb, purified IgG at 2 mg/ml in PBS, pH 7.4, was warmed to 37°C and the pH of the solution lowered to 3.4 by the addition of 0.5 M citrate, pH 3.2. Pepsin (Sigma Chemical Co., St Louis, MO) at a concentration of 5 mg/ml in 0.1 M citrate, pH 6, was added to give a pepsin:mAb ratio of 1:120. After 30 min digestion at 37°C with rotation, 3 M Tris–HCl, pH 8.6, was added to stop the reaction (final concentration 0.25 M). In some instances, the digested protein was passed over Affi-Gel Protein A to remove residual undigested IgG1 protein. After dialysis and concentration, IG6 F(ab')$_2$ was purified by passage over a calibrated Sephacryl S-300 superfine column (Pharmacia Fine Chemicals, Uppsala, Sweden) in Tris-buffered saline (TBS; 0.01 Tris–HCl, 0.15 M NaCl, pH 7.3). IG6 Fab' was prepared by reduction of the F(ab')$_2$ preparation with 12 5 mM L-cysteine (Sigma) for 30 min at 37°C with rotation and further alkylation of the preparation with 30 mM iodoacetamide (Pierce, Rockford, IL) in the dark for 1.5 h at room temperature. After dialysis in TBS and concentration using a Centriprep 10 concentrator (Amicon, Beverly, MA), IG6 Fab' was purified by passage over the calibrated Sephacryl S-300 superfine column. Fab fragments of mAb 5D7 were prepared by papain digestion. Briefly, purified mAb 5D7 at 2 mg/ml in 0.1 M citrate, pH 7.0, was incubated with cysteine preactivated mercuri-papain (Sigma) (16) at a 1:20 enzyme:antibody ratio at 37°C for 1.5 h. Digestion was stopped by the addition of one part of 0.18 M iodoacetamide in TBS to five parts of antibody solution. After passage of a Protein A–Sepharose column to remove Fc and undigested IgG, 5D7 Fab fragments were purified by column chromatography on Sephacryl S-300. Purity of the above F(ab')$_2$, Fab' and Fab column fractions was assessed by 5–20% gradient SDS–PAGE under non-reducing and reducing conditions. HB57 Fab', 5D7 Fab and five of the seven separate IG6 Fab' preparations used in this study showed no evidence of F(ab')$_2$ contamination. Two of the seven IG6 Fab' preparations as well as 4-3 Fab', 196.6b Fab' and IF11 Fab' showed a weak F(ab')$_2$ band under non-reducing conditions that amounted to <1% contamination. For most IG6 Fab' isolations, column fractions containing Fab' at concentrations of >1 mg/ml were pooled and dialyzed in PBS without additional concentration to avoid the potential for aggregate formation. In some experiments Fab' fragments of the $C_\mu 4$-specific mAbs were airfuged at 100,000 *g* for 50 min and the upper half of the material used for experimentation. Although this sometimes diminished the enhancing properties of the preparation slightly, identical conclusions were reached regarding the synergy-inducing properties of IG6 Fab'.

Additional ligands for B cell stimulation

T cell cytokines were used to enhance anti-IgM triggered B cell DNA synthesis in selected experiments. Semi-purified low mol. wt B cell growth factor (BCGF) was obtained from Cellular Bioproducts (Buffalo, NY) and used at a concentration of either 12 5 or 25%. Recombinant human IL-4 (HuIL-4) was purchased from Amgen Biologicals (Thousand Oaks, CA) and used at a final concentration of 2.5–10 ng/ml. Recombinant HuIFN-γ was kindly contributed by Dr Michael Brunda of Hoffmann LaRoche Inc. (Nutley, NJ) and used at a final concentration of 1000 U/ml. All the concentrations used were found to be optimal in titration experiments. 4β-Phorbol-12β-myristate-13α-acetate was obtained from Sigma and stored at −20°C at a concentration of 1.6 mmol/l in absolute ethanol. In culture, it was used at a final concentration of 0.08 μmol/l.

B cell sources and purification

Surgically excised human tonsils were obtained from 3- to 12-year-old donors at the New York Eye and Ear Infirmary, with the generous cooperation of Dr Stephen McCormick and the surgery and pathology staffs Human spleen was obtained as residual tissue following surgery for trauma and as treatment for Felty's syndrome (6,11). The tissues were processed and B cell enriched populations prepared as described previously using 2-aminoethylisothiouronium bromide-treated sheep red blood cells (SRBC) and/or neuraminidase-treated SRBC to deplete T lymphocytes (8,11). Experiments with tonsil B cells used Percoll density gradient centrifugation to enrich for B cells of high density, resting phenotype, as described previously (9). Although most of the experiments described in this report utilized fresh cell preparations, in some cases B lymphocytes were stored in the vapor phase of liquid nitrogen until use. The leukemic B cell population (LUB) was obtained from Dr Janet Cuttner of Mount Sinai School of Medicine as a peripheral blood specimen and stored in liquid nitrogen until use. The phenotype and functional properties of this clonal population have been described previously (6,15,17).

Cell culture and assessment of DNA synthesis

Unless indicated otherwise, B cell preparations were cultured in triplicate in microculture plates at 2–3 × 10^5 cells/0.2 ml. Culture medium was a 1:1 Ham's–Iscove's mixture with 15% FCS and additional supplements as described elsewhere (11). DNA synthesis was measured by quantitating the uptake of a pulse of 1 μCi [^{3}H]thymidine (72.5 Ci/mmol) (New England Nuclear, Boston, MA) as previously indicated (11,17).

Capping analysis

To evaluate the potential of mAb IG6 ligands to induce capping of mIgM, resting tonsil B cells in culture medium were incubated with mAb IG6 in Fab', F(ab')$_2$ or IgG form, or with mAb MOPC-21 control Fab', each at 100 μg/ml, for 25 min at 37°C. The cells were washed three times with cold PBS + 1% BSA + 0.1% sodium azide (assay medium) before staining with FITC-conjugated sheep F(ab')$_2$ anti-mouse Ig, with specificity for light and heavy chains, in assay medium. After 30 min on ice, cells were washed four times in cold assay medium and suspended in 50% glycerol/50% assay medium before transfer to a slide for subsequent fluorescence analysis with a Leitz Ortholux II microscope, generally after being stored at 4°C for 12 h. In assays designed to evaluate the potential of IG6 Fab' and IG6 F(ab')$_2$ to enhance cap formation by bivalent mAb HB57, mAb 5D7 and mAb XG9, resting tonsil B cells in culture medium were incubated with IG6 Fab', IG6 F(ab')$_2$, MOPC-21 F(ab')$_2$ or medium, in the presence or absence of each of the bivalent mAb as IgG. Each of the ligands were added at a final concentration of 100 μg/ml. In the seven assays performed at 37 or 20°C, cap formation was allowed to proceed for 3–4 or 4–5 min respectively. Cells were washed in the presence of azide as indicated above and the degree of cap formation was assessed after staining with FITC-conjugated goat F(ab')$_2$, anti-mouse IgG (Fc-specific). All assays were performed with coded samples, and at least 100 cells were counted for each determination.

Radiolabeling of surface membrane proteins with ^{125}I

B cells from the RAMOS cell line (4 × 10^7) were washed three times with PBS and resuspended in 1 ml of PBS containing lactoperoxidase (1 mg/ml; Sigma) and ^{125}I (4–6 mCi; DuPont NEN, Bloomington, DE). Four 50 μl aliquots of 0.03% H_2O_2 were added to the above reaction mixture at 5 min intervals at room temperature. Radiolabeled cells were then washed three times and resuspended in ice-cold PBS.

Immunoprecipitation of radiolabeled surface membrane proteins

Prior to lysis, 1 × 10^6 radiolabeled RAMOS B cells were incubated on ice with 100 μg of mAb IG6 Fab' at concentrations of either 100 or 800 μg/ml. After 1 h, cells were washed with PBS and lysed in 100 μl of PBS containing digitonin (2%; Sigma), EDTA (5 mM; Sigma), phenylmethylsulfonyl fluoride (2 mM; Sigma), aprotinin (0.15 TIU/ml; Sigma) and leupeptin (10 μg/ml) (PBS–digitonin). Lysed suspensions were then microfuged at 16,000 *g* for 30 min at 4°C and frozen in aliquots at −80°C.

For immunoprecipitation, protein lysates from 2 × 10^6 cell equivalents were shaken gently overnight at room temperature with 50 μl goat anti-rabbit IgG-conjugated agarose beads (Accurate Chemical Co., Westbury, NJ) that had been pre-incubated for 2 h at room temperature with 20 μl of either normal rabbit serum (NRS) or affinity-purified rabbit anti-human IgM antibodies. Beads were then washed three times with PBS–digitonin, resuspended in 100 μl of Laemmli's buffer and boiled for 5 min prior to electrophoresis through a 12% SDS–polyacrylamide vertical slab gel under reducing conditions with mol. wt markers. Radiolabeled proteins were identified and quantified both by standard autoradiography using X-ray film (Kodak, Rochester, NY) and by a PhosphorImager SF (Molecular Dynamics, Sunnyvale, CA).

Results

Unique pairs of anti-human IgM mAbs show pronounced synergy in the induction of human B cell DNA synthesis

Consistent with our previous findings (11), the data in Table 1 show that certain unique combinations of murine mAbs

Table 1. Mixtures of anti-human IgM mAb display synergy in inducing human B cell DNA synthesis

mAb A (50 μg/ml)	c.p.m. [^{3}H]thymidine uptake						
	None	mAb B (50 μg/ml)					
		HB57	5D7	XG9	Mu53	IG6	P24
None	2177	12,694	2811	854	1640	1100	763
HB57	12,694	10,984	5029	9756	10,579	30,325	11,060
5D7	2811		3072	8232	4100	19,296	4860
XG9	854			1305	4141	10,075	1009
Mu53	1640				1953	2284	1241
IG6	1100					1513	1281
P24	763						1141

T cell-depleted spleen cells (2×10^5/well) were cultured with each mAb alone or with 1:1 mixtures of mAb A and mAb B for 72 h. DNA synthesis was assessed by uptake of a 1 μCi pulse of [^{3}H]thymidine during the last 18 h of culture. The values shown represent the mean of [^{3}H]thymidine uptake of triplicate cultures. The underlined values represent the c.p.m. from mixtures showing the greatest synergy.

Table 2. Influence of anti-IgM domain specificity and binding affinity on mAb potential to display synergy in inducing B cell DNA synthesis

mAb A		K_a (M^{-1})	mAb B									
			$C_\mu 1$-specific		$C_\mu 2$-specific				$C_\mu 4$-specific			
			XG9	P19	HB57	Mu18	Mu53	P24	5D7	4-3	IG6	IF11
$C_\mu 1$ specific	XG9	7×10^7	±[a]	±	0	+	±	±	±	±	+++	0
	P19	4×10^6		0	−	0	0	0	+	0	±	0
$C_\mu 2$ specific	HB57	5×10^8			0	±	0	0	0	±	++	0
	Mu18	5×10^7				0	0	±	+	±	+	±
	Mu53	2×10^7					0	0	±	0	±	0
	P24	2×10^6						±	±	±	±	±
$C_\mu 4$ specific	5D7	1×10^8							0	++	+++	±
	4-3	2×10^7								0	0	±
	IG6	7×10^6									±	+
	IF11	2×10^5										0

The data represent the mean of three experiments in which each of the above anti-IgM mAbs was placed in culture at 150 μg/ml with T cell-depleted spleen cells. Domain specificity and intrinsic affinity of each mAb for mIgM was determined as described elsewhere (6,14).

[a]The synergy index for DNA synthesis ([^{3}H]thymidine uptake) was calculated as [(c.p.m. with mAb A + mAb B) + (background c.p.m.)]/[(c.p.m. with mAb A) + (c.p.m. with mAb B)]. Synergy indices were scored as follows: −, <0.49; 0, 0.5–1.249; ±, 1.25–2.49; +, 2.5–4.9; ++, 5.0–9.9; +++, >10.

specific for human B cell mIgM can elicit levels of B cell [^{3}H]thymidine uptake which are significantly greater than that induced by either antibody alone. Analysis of the responses obtained when B cells were cultured with 1:1 combinations of a group of ten highly characterized anti-IgM mAbs provided insights into how the domain specificity and the intrinsic affinity of the anti-IgM mAbs influences the potential for synergy (Table 2). Three notable observations were made. Firstly, the most profound synergy is consistently noted when relatively low affinity $C_\mu 4$-specific mAb IG6 ($K_a = 7 \times 10^6$ M^{-1}) is one component of the mAb mixture. Secondly, mAb IG6 can exhibit synergy with mAbs specific for diverse domains, e.g. $C_\mu 1$ in the case of mAb XG9, $C_\mu 2$ in the case of mAb HB57 and $C_\mu 4$ in the case of mAb 5D7. Thirdly, the ability of mAb IG6 to exhibit synergy with another mAb appears to be dependent upon the relatively high binding site affinity of the non-IG6 mAb. The three mAbs which showed the best synergy with mAb IG6 have binding affinities for mIgM of $K_a = 5 \times 10^8$ M^{-1} (mAb HB57), 1×10^8 M^{-1} (mAb 5D7) and 7×10^7 M^{-1} (mAb XG9) (6).

Fab' fragments of mAb IG6 enhance B cell responses to mIgM cross-linking ligands

It has been well documented that pairs of mAbs directed to distinct epitopes on soluble or cell-bound antigens can create circular complexes that have a higher binding avidity than that measured when either of the individual mAbs engages with antigen (12,13)—a phenomenon due to the complex-stabilizing effect of bridging antigenic molecules at two distinct sites per molecule. Since signaling of B cell S phase entry by mIgM-binding ligands requires cross-linking of receptors and, furthermore, has exceedingly high affinity requirements

for induction, it was considered possible that the ability to link pairs of mIgM molecules at two distinct sites might be responsible for all of the above synergy.

To discern between the above and other possible mechanisms for enhancement, we have evaluated whether bivalency of each anti-IgM antibody in a synergistic pair was required. Table 3 shows data from representative experiment which tested the ability of mAb HB57 + mAb IG6 or mAb 5D7 + mAb IG6 combinations to exhibit synergy when one or both of the mAbs was present as a monovalent Fab' fragment. Surprisingly, we found that although bivalency of the high affinity ligand (mAb HB57 or mAb 5D7) was important for a functional response, the low affinity, $C_\mu 4$-specific ligand (mAb IG6) induced notable synergy when added as monovalent Fab'. $F(ab')_2$ and Fab' fragments of mAb HB57 were compared for ability to manifest synergy with $F(ab')_2$ and Fab' fragments of mAb IG6 in two experiments (one shown in Table 3). While the enhanced [^{3}H]thymidine uptake in cultures containing HB57 $F(ab')_2$ and low affinity IG6 Fab' was 50–80% of that noted in cultures containing both mAbs in bivalent form, the enhanced [^{3}H]thymidine uptake induced in cultures containing high affinity HB57 Fab' and bivalent IG6 was only 4–12% of that induced in cultures containing both bivalent mAbs. $F(ab')_2$ and Fab' fragments of mAb 5D7 could not be compared because of the difficulty in obtaining 5D7 $F(ab')_2$ fragments. The enhancing effect of IG6 Fab' was consistently observed with several different Fab' preparations derived from diverse subclones of IG6 hybridoma (data not shown), and was not due to a non-antibody contaminant since activity was retained when IG6 Fab' was further affinity-purified on IgM–Sepharose columns (data not shown).

To rule out the possibility that the enhancement noted with IG6 Fab' (at 100 μg/ml) was due to contamination with non-reduced $F(ab')_2$ at levels <1% (see Methods), bivalent IG6 $F(ab')_2$ and monovalent IG6 Fab' were compared, on a concentration basis, for ability to enhance B cell DNA synthesis stimulated by high affinity bivalent mAbs HB57, 5D7 and XG9. The data in Fig. 1 show that monovalent IG6 Fab' was only 2- to 3-fold less effective than IG6 $F(ab')_2$ at

Table 3. Optimal synergy between high affinity anti-IgM mAb and low affinity mAb IG6 requires bivalency of the high affinity mAb but not mAb IG6

	c.p.m. [^{3}H]thymidine uptake (48–70 h)		
	Medium	+ bivalent mAb IG6	+ mAb IG6 Fab'
Medium	114 ± 30	76 ± 28	106 ± 12
mAb HB57 IgG	1401 ± 159	5686 ± 355	5802 ± 354
mAb HB57 Fab'	74 ± 19	239 ± 42	134 ± 49
mAb 5D7 IgG	480 ± 18	5197 ± 170	5253 ± 159
mAb 5D7 Fab'	63 ± 22	263 ± 6	69 ± 14
Medium	500 ± 128	731 ± 167	600 ± 101
mAb HB57 $F(ab')_2$	1793 ± 125	20,590 ± 1074	10,442 ± 265
mAb HB57 Fab'	551 ± 186	2972 ± 103	346 ± 34

Intrinsic binding affinities of mAbs HB57, 5D7 and IG6 for mIgM have been determined to be $5\ 3 \times 10^8$, 1.2×10^8 and $7.0 \times 10^6\ M^{-1}$ respectively (6). mAb proteins were each added at a final concentration of 100 μg/ml to cultures of resting tonsil B cells. Bivalent mAb IG6 was in the form of IgG (first experiment) or $F(ab')_2$ (second experiment). mAb 5D7 could not be tested as $F(ab')_2$ because of the difficulty in generating such fragments. In these experiments, the binding activity of Fab' fragments of mAbs HB57, 5D7 and IG6 was compared with that of the respective intact antibodies in RIA on IgM-coated wells using ^{3}H-labeled anti-murine kappa l.c antibody as a probe. Fab' and IgG forms of the higher affinity ligands (mAbs 5D7 and HB57) bound IgM with comparable efficiency. Consistent with its lower intrinsic affinity, monovalent IG6 Fab' bound IgM with less efficiency than bivalent mAb IG6 IgG (data not shown).

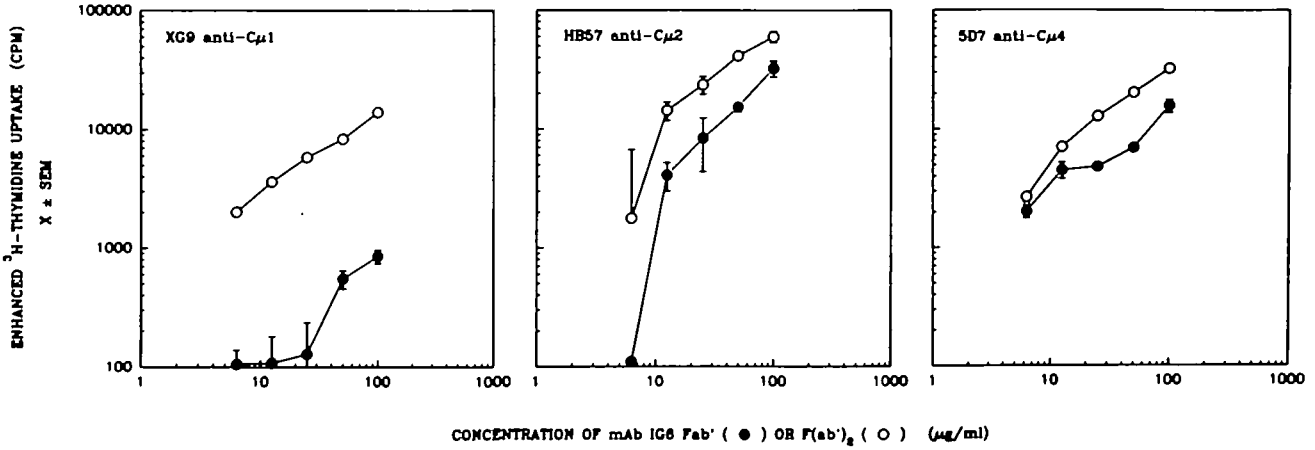


Fig. 1. Effect of various concentrations of mAb IG6 Fab' or $F(ab')_2$ at enhancing B cell DNA synthesis in cultures containing intact mAb XG9 anti-$C_\mu 1$, HB57 anti-$C_\mu 2$ or 5D7 anti-$C_\mu 4$. mAbs XG9, HB57 and 5D7 were added to cultures of resting B cells at a concentration of 100 μg/ml. [^{3}H]Thymidine uptake was measured during the last 24 h of a 72 h culture period. Data are expressed as enhanced c.p.m. uptake, calculated as follows: (Δc.p.m. in cultures with mAb IG6 Fab' or $F(ab')_2$ + intact mAb) − [(Δc.p.m. in cultures with IG6 Fab' or $F(ab')_2$ alone) + (Δc.p.m. in cultures with intact mAb alone)]. Values for Δc.p.m. in cultures with 100 μg/ml of mAb IG6 Fab' or $F(ab')_2$ alone were 157±42 and 777±194 respectively in the experiment in which mAb XG9 and mAb 5D7 were tested and −106±73 and 1133±376 respectively in the experiment in which mAb HB57 was tested. Values for Δc.p.m. in cultures with 100 μg/ml of mAbs XG9, HB57 or 5D7 present alone were 58±107, 34,265±3385 and 1994±59 respectively.

Table 4. IG6 Fab' can enhance response to bivalent $C_\mu 1$-specific mAb XG9 in the presence of additional mIgM cross-linking ligand

Experiment	mAbs	c.p.m [^{3}H]thymidine uptake		
		Medium	+ IG6 Fab'	+ bivalent IG6
A	None	191 ± 47	192 ± 68	276 ± 56
	XG9 anti-$C_\mu 1$	400 ± 52	3071 ± 196	17,796 ± 2138
	Mu53 anti-$C_\mu 2$	1947 ± 334	2995 ± 71	nd
	XG9 + Mu53	7477 ± 737	24,185 ± 873	nd
B	None	428 ± 74	477 ± 28	1461 ± 151
	XG9 anti-$C_\mu 1$	2559 ± 281	10,837 ± 489	51,794 ± 2189
	Mu53 anti-$C_\mu 2$	641 ± 110	947 ± 58	18,977 ± 681
	XG9 + Mu53	8249 ± 82	31,170 ± 978	46,688 ± 2239

Bivalent anti-IgM mAbs XG9, Mu53 and IG6, and mAb IG6 Fab' were each added to resting tonsil B cell cultures at 100 μg/ml. In experiment A bivalent mAb IG6 was added as IgG, in experiment B as $F(ab')_2$.

exhibiting synergy with the two bivalent ligands of highest affinity, i.e. $C_\mu 2$-specific mAb HB57 and $C_\mu 4$-specific mAb 5D7. This clearly indicates that contamination with $F(ab')_2$ cannot explain the activity of the IG6 Fab' preparations. The slightly greater effectiveness of IG6 $F(ab')_2$ over IG6 Fab' at enhancing responses to bivalent mAb HB57 or mAb 5D7 may at least in part reflect the low affinity of the mAb IG6 binding site ($K_a = 7 \times 10^6\ M^{-1}$). Thus, although the functional effects of the mAb IG6 ligand may not be dependent upon the capacity of this ligand to cross-link mIgM molecules, engagement by two binding sites may diminish the ligand's rate of dissociation from each membrane IgM molecule and hence increase its functional effect when bound to $C_\mu 4$ Furthermore, the enhanced functional activity of IG6 $F(ab')_2$ may reflect the potential of this latter ligand to enhance the signaling process not only through direct engagement with a unique site on the $C_\mu 4$ domain (as observed with IG6 Fab'), but also through creating an additional bridge through which mIgM molecules are cross-linked.

Consistent with this latter explanation is the substantially greater potential of IG6 $F(ab')_2$ versus IG6 Fab' at inducing synergy with bivalent mAb XG9 (Fig. 1). The latter $C_\mu 1$-specific ligand had been found to be somewhat impaired in its ability to cross-link mIgM and to activate B cells because it often binds in a monogamous fashion to monomeric IgM, i.e. both mAb binding sites engaged with the dual $C_\mu 1$ epitopes on a single IgM molecule (6). We consider it quite likely that the diminished effectiveness of IG6 Fab' at exhibiting synergy with mAb XG9 could reflect an insufficient amount of receptor cross-linking in these cultures. To more effectively monitor the activation-promoting function of monovalent IG6 Fab' in mAb XG9-containing cultures, we attempted to replace the bridging role of mAb IG6 with another bivalent ligand. $C_\mu 2$-specific mAb Mu53 was selected as a replacement because it binds to human IgM with an affinity near that of mAb IG6, and does not interfere with the binding of mAb XG9 to $C_\mu 1$ (6,14). Table 4 shows results from two representative experiments in which the enhancing properties of IG6 Fab' were assessed in B cell cultures containing both $C_\mu 1$-specific mAb XG9 and $C_\mu 2$-specific mAb Mu53. The results clearly indicate that in such cultures Fab' preparations of mAb IG6 can cause a substantial

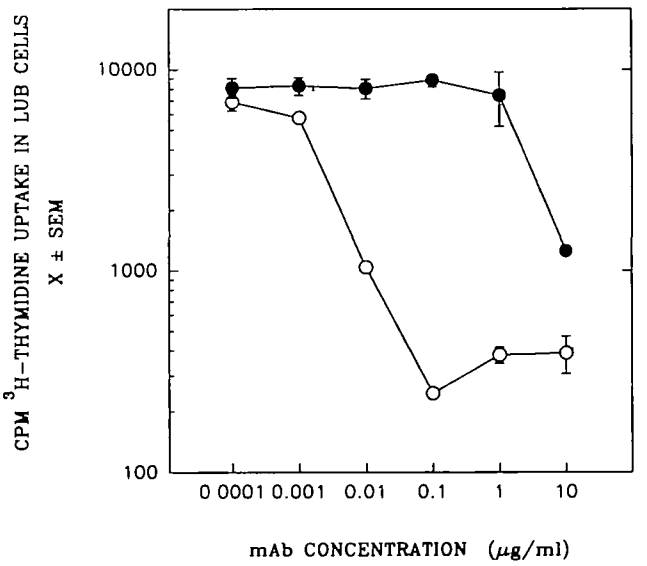


Fig. 2. Fab' fragments of mAb IG6 are ineffective at signaling inhibition of DNA synthesis in human leukemic B cells. Various concentrations of IG6 Fab' (●) or IG6 $F(ab')_2$ (O) were tested for ability to inhibit the spontaneous *in vitro* DNA synthesis of LUB hairy cell leukemia cells. LUB cells were cultured at 1×10^5 cells/200 μl for a total of 4 days. A 1 μCi pulse of [^{3}H]thymidine was added during the final 24 h of culture.

increase in the DNA synthesis observed. Taken together, the available data suggest that, under circumstances where ample cross-linking of mIgM molecules occurs, Fab' preparations of mAb IG6 can have a significant effect at enhancing the signaling process.

As an independent ligand, IG6 Fab' functions poorly in phenomena which require cross-linking of mIgM

It was important to rule out the possibility that the functional effect of IG6 Fab' was due to the non-covalent aggregation of Fab' fragments during storage, or in culture, to generate complexes with a potential to cross-link mIgM molecules.

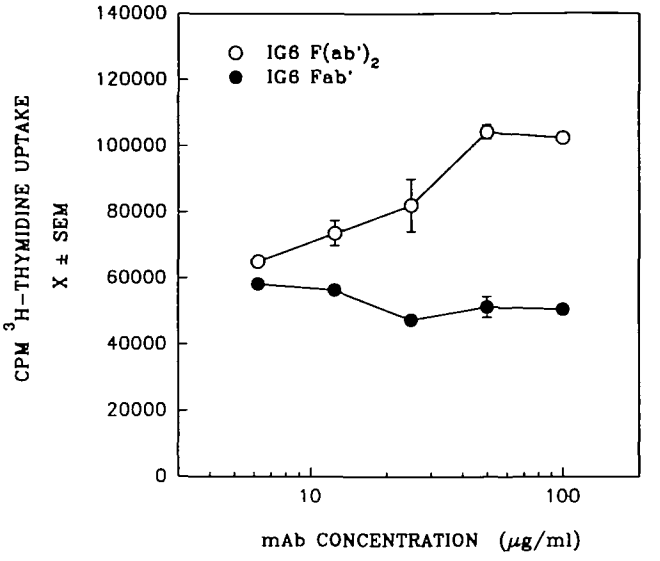


Fig. 3. Fab' and F(ab')$_2$ fragments of mAb IG6 differ in potential to synergize with PMA in inducing the DNA synthesis of normal B cells. Various concentrations of IG6 Fab' or F(ab')$_2$ were placed in culture with 0.08 μM of PMA. Uptake of an 18 h pulse of [^{3}H]thymidine was assessed at the end of a 72 h culture period. Cultures with PMA alone exhibited 41,888±1194 c.p.m

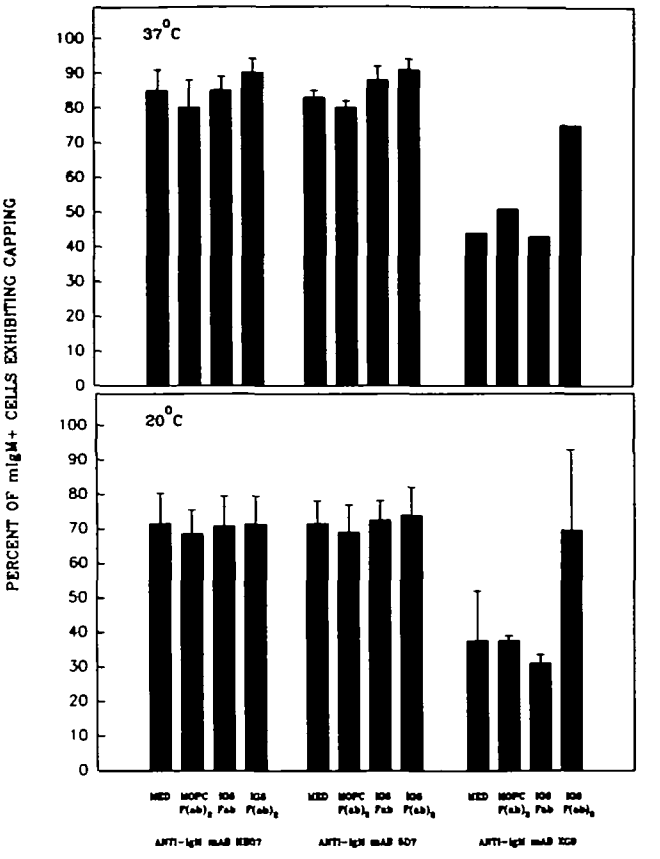
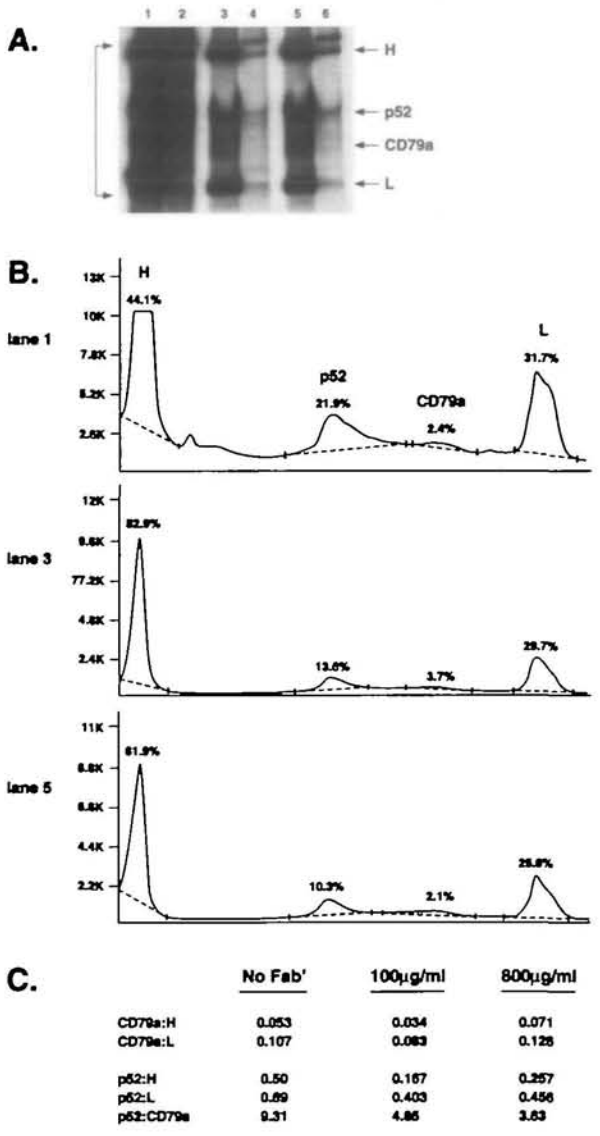


Fig. 4. Monovalent IG6 Fab' does not enhance the potential of bivalent anti-IgM mAb to cap mIgM Capping studies were performed by incubating resting tonsil B cells for 3–4 min at 37°C or 4–5 min at 20°C with bivalent IgG forms of mAbs HB57, 5D7 or XG9 in the presence or absence of IG6 Fab', IG6 F(ab')$_2$ or control MOPC-21 F(ab')$_2$, as indicated in Methods. The data shown for mAbs HB57- and 5D7-induced capping represent the mean±SEM of four capping experiments performed at 37°C and three separate capping experiments performed at 20°C. mAb XG9-induced capping was evaluated in one of the experiments performed at 37°C and in two of the experiments performed at 20°C. In all experiments, cells incubated with MOPC-21 F(ab')$_2$, IG6 Fab' or IG6 F(ab')$_2$, in the absence of intact IgG forms of anti-IgM mAb, showed no staining with the Fc-specific FITC-conjugated goat F(ab')$_2$ anti-mouse IgG probe.

This issue has been addressed through three experimental approaches. Firstly, Fab' and F(ab')$_2$ preparations of mAb IG6 were compared for potential to initiate inhibitory signals in a mIgM-positive leukemic B cell population previously shown to be sensitive to low concentrations of all tested bivalent anti-IgM mAb, including mAb IG6 (6). In contrast to the 2- to 3-fold lesser dose effectiveness of IG6 Fab' versus IG6 F(ab')$_2$ at enhancing normal B cell DNA synthesis induced by mAb HB57 or 5D7 (Fig. 1), IG6 Fab' was 1000-fold less effective than IG6 F(ab')$_2$ at inducing the suppression of LUB cell DNA synthesis (Fig. 2). The inhibition noted at the highest concentration of IG6 Fab' could be explained by a 0.1% contamination of the IG6 Fab' preparation with F(ab')$_2$, or comparable aggregates. A similar experiment with a different IG6 Fab' preparation showed no inhibition at a Fab' concentration of 100 μg/ml, while maximal inhibition was achieved by 0.1 μg/ml of IG6 F(ab')$_2$ (data not shown). In a second type of assay, IG6 F(ab')$_2$ and IG6 Fab' were compared for their potential to enhance DNA synthesis in PMA-containing B cell cultures (Fig. 3). Consistent with reports in which PMA and anti-IgM antibodies show synergy in eliciting human B cell activation (18,19), PMA-stimulated cultures containing IG6 F(ab')$_2$ generally exhibited a notable increase in DNA synthesis. In the experiment in Fig. 3, cultures with the least amount of IG6 F(ab')$_2$ present, i.e. 3 μg/ml, showed higher levels of DNA synthesis than those observed with a 33-fold greater concentration of IG6 Fab' (100 μg/ml). As a final approach to testing whether IG6 Fab' preparations contained aggregates capable of cross-linking mIgM, resting B cells were preincubated for 25 min at 37°C with 100 μg/ml of mAb IG6 [as Fab', F(ab')$_2$ or IgG] in culture medium, washed and stained in the presence of sodium azide with a FITC-conjugated anti-mouse Ig probe at 4°C. While capping of mIgM was noted on 73 and 57% of the mIgM-positive cells incubated with IG6 F(ab')$_2$ and IG6 IgG respectively, only 14% of the mIgM-positive cells incubated with IG6 Fab' showed a weak fluorescence profile that might be consistent with capping. The great majority (79%) of the positive cells exposed to IG6 Fab' exhibited a weak peripheral stain. The above findings from three different experimental approaches strongly suggest that the notable ability of IG6 Fab' preparations to synergize with bivalent anti-IgM mAbs in the induction of B cell DNA synthesis is not due to an appreciable re-aggregation of the Fab' fragments in culture. Hence, we conclude that a monovalent interaction of the IG6 $C_\mu 4$-specific ligand with the membrane-proximal domain of mIgM can induce changes which enhance the potential of B cells to



C.

	No Fab'	100μg/ml	800μg/ml
CD79a:H	0.053	0.034	0.071
CD79a:L	0.107	0.083	0.126
p52:H	0.50	0.157	0.257
p52:L	0.89	0.403	0.456
p52:CD79a	9.31	4.86	3.63

Fig. 5. Analysis of CD79a (Igα) co-precipitation with IgM in lysates of B cells preincubated with $C_\mu 4$-specific IG6 Fab'. RAMOS B cells labeled with ^{125}I were incubated with medium (lanes 1 and 2) or with *IG6 Fab' at 100 μg/ml (lanes 3 and 4) and 800 μg/ml (lanes 5 and* 6) for 1 h prior to lysis in digitonin (Methods). IgM and associated proteins were immunoprecipitated from the lysates by exposure to goat anti-rabbit IgG-conjugated beads preincubated with rabbit anti-human IgM antibody (lanes 1, 3 and 5) or control Ig from normal rabbit serum (lanes 2, 4 and 6) (Methods). (A) Autoradiographic detection of IgM and associated proteins Four major proteins were immunoprecipitated by anti-IgM· μ chain (H), light chain (L), CD79a (Igα) and an unidentified protein of 52 kDa mol. wt (p52) The identity of the band designated CD79a is supported by other immunoprecipitation studies with anti-CD79a and anti-CD79b peptide antibody (N. Rajaram and N. Chiorazzi, data not shown). (B) PhosphorImager scan of labeled proteins in SDS–PAGE gels of anti-IgM immunoprecipitates. A region of the gel corresponding to that shown in the bracketed region in (A) was scanned with a PhosphorImager SF for lanes 1, 3 and 5. The proportional recovery of radioactivity was determined by integrating the area of each peak above the dashed baseline and by then expressing the area in each peak as a percentage of the total area of the four pooled peaks within each lane. (C) Ratio of the relative recovery of various anti-IgM immunoprecipitated proteins in RAMOS cells exposed or not exposed to $C_\mu 4$-specific IG6 Fab'. Ratios were computed using the original c.p.m. values obtained by computer analyses from the designated areas under the various peaks revealed by the PhosphorImager The data from this and other replicate experiments show no significant increase in the association of CD79a with IgM in B cells preincubated with IG6 Fab'.

become activated upon cross-linking of mIgM by other ligands. The remainder of this study attempts to examine in greater detail some of the functional properties of this monomeric Fab' ligand.

Monovalent IG6 Fab' does not enhance the potential of bivalent mAb to cap mIgM in resting B cells

The failure of IG6 Fab' to induce the cross-linking of mIgM molecules when added as an isolated ligand does not preclude the possibility that IG6 Fab' might, through a means independent of its own cross-linking potential, enhance the ability of bivalent ligands to do so. Of interest in this regard are the recent observations of Mecheri *et al.* that binding of immunogenic peptides by class II MHC Ia molecules reduces the membrane mobility of Ia, as well as significantly increases the potential of bivalent anti-Ia antibodies to aggregate Ia (20). In an attempt to evaluate whether the IG6 Fab' enhancement of B cell activation reflected an IG6 Fab'-mediated enhancement of mIgM cross-linking by bivalent mAb HB57 or mAb 5D7, we have assessed the extent of cap formation in resting B cells incubated with the bivalent mAbs in the presence or absence of monovalent IG6 Fab'.

The pooled data from seven experiments show no evidence for an enhanced degree of cap formation when B cells were exposed to high affinity bivalent mIgM cross-linking ligands in the presence of IG6 Fab' for 3–4 min at 37°C or 4–5 min at room temperature (20°C) (Fig. 4). Under the same conditions, the presence of bivalent IG6 $F(ab')_2$ only slightly, if at all, enhanced the degree of capping observed with the high affinity mAb HB57 or 5D7, but did appear to enhance the amount of cap formation noted in B cells exposed to the $C_\mu 1$-specific mAb XG9 (which has a propensity to bind mIgM in a monogamous, i.e. non-cross-linking, fashion). This provides additional support for our earlier stated hypothesis that mAb IG6-assisted bridging, or cross-linking, of mIgM may be important for the enhanced B cell activation in mAb XG9-containing cultures.

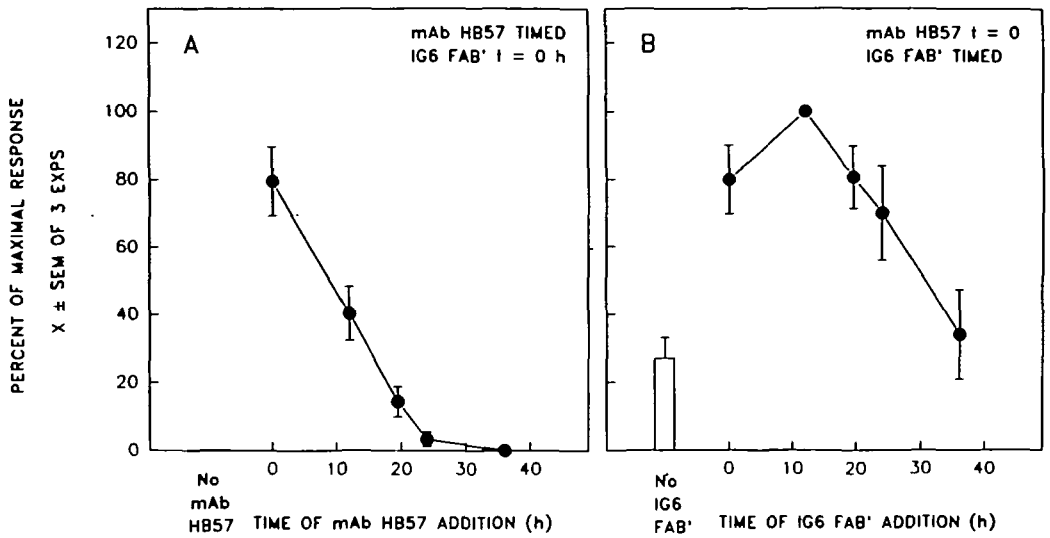


Fig. 6. Enhanced B cell DNA synthesis can be achieved with a 24 h delay in the addition of IG6 Fab' but not a similar delay in the addition of bivalent anti-IgM. In each of the three experiments, 100 μg/ml of bivalent mAb HB57 was added at differing time points to cultures containing 100 μg/ml of IG6 Fab' at $t = 0$ h (A) or 100 μg/ml IG6 Fab' was added at differing time points to cultures containing 100 μg/ml mAb HB57 at $t = 0$ h (B). Each point represents the mean ± SEM of the percent of maximal response calculated for each of the three experiments [^{3}H]Thymidine uptake was measured at 48–61(67) h of culture. Cultures with IG6 Fab' alone from $t = 0$ exhibited only 0.11±0.5% of the maximal response (bar not evident). The responses noted when mAb HB57 was added at varying time intervals to cultures with IG6 Fab' present from $t = 0$ h did not differ from the responses noted when mAb HB57 was added at the same time intervals to cultures containing medium alone (data not shown).

Monovalent IG6 Fab' does not appear to enhance mIgM associations with Igα (CD79a)

Signal transduction upon mIgM cross-linking is dependent upon non-covalent association of mIgM with other integral membrane proteins (21–25). As such, the adjuvant function of IG6 Fab' might be attributed to IG6 Fab'-induced conformational changes which affect these intermolecular engagements. In an effort to explore the possibility that IG6 Fab' might enhance mIgM association with mb-1-encoded Igα (CD79a) (21–23), we have evaluated whether a prior incubation of monovalent IG6 Fab' with iodinated mIgM + Raji cells (30 min at 4°C) can increase the extent to which Igα is co-precipitated with mIgM, once these cells are lysed in digitonin. The results from one out of three executed experiments in which rabbit anti-human IgM antibody was used to immunoprecipitate the complexes is shown in Fig. 5. Immunoprecipitated, gel-separated proteins were revealed by autoradiography (Fig. 5A) and by PhosphorImager analysis of labeled bands (Fig. 5B). These studies provided no evidence for an enhanced association of Igα (CD79a) with mIgM in B cells exposed to IG6 Fab'. Thus, the band corresponding to Igα (CD79a) represented 2.35% of the total radiolabeled protein (within the scanned region spanning light chain to μ heavy chain) immunoprecipitated from lysates of RAMOS cells not exposed to IG6 Fab', and 2.13 and 3.74% of that from lysates of cells exposed to 100 and 800 μg/ml of mAb IG6 Fab' respectively. In an additional experiment, in which adequate levels of Igα were available for analysis, Igα (CD79a) represented 5.83% of the total immunoprecipitated protein from cells not exposed to IG6 Fab' and 4.77% of the immunoprecipitated protein from cells exposed to IG6 Fab' (800 μg/ml). The CD79a:μ chain ratios in immunoprecipitates of cells exposed and not exposed to IG6 Fab' were not significantly different (Fig. 5C).

An ^{125}I-labeled protein of ~52 kDa was consistently recovered in the anti-IgM immunoprecipitates of Raji cell lysates. In the experiment shown in Fig. 5, B cell pre-exposure to IG6 Fab' appeared to reduce the proportional recovery of p52, as indicated by the lower p52:μ, p52:L chain and p52:CD79a ratios in cells exposed to IG6 Fab' versus cells not exposed to the $C_\mu 4$-specific ligand (Fig. 5C). These reduced ratios were not observed in all experiments, however (data not shown). The identity of the p52 protein is unknown, but it may be equivalent to a protein(s) of 52–56 kDa which co-isolates with mIgM in certain other B cell studies (26–31).

An additional observation in most of the immunoprecipitation experiments was that the amount of immunoprecipitated protein recovered from lysates of cells incubated with IG6 Fab' was lower than that from lysates of an identical number of cells not exposed to the $C_\mu 4$-specific ligand. This might reflect a diminished ability of the immunoprecipitating antibody to bind mIgM due to steric hindrance from the bound IG6 Fab'. Alternatively, it might reflect the ability of IG6 Fab' to reduce the detergent solubility of mIgM through promoting mIgM–cytoskeleton interactions (32). We cannot currently distinguish between these possibilities.

IG6 Fab' enhances B cell DNA synthesis despite its delayed addition to anti-IgM stimulated cultures

A notable aspect of the mIg-mediated activation process in normal B cells is that B cell entry into S phase appears to require the continued formation of mIg receptor cross-links for a prolonged (24–36 h) period after initial B cell exposure

to the mIg cross-linking ligand (9,10). In order to determine when during this extended triggering period IG6 Fab' functions to enhance B cell S phase entry, the monovalent ligand was added after various time intervals to cultures that had received 100 μg/ml of bivalent mAb HB57 at $t = 0$ h (Fig. 6B) DNA synthesis was assessed by uptake of [^{3}H]thymidine during a 48–61(67) h pulse period. Quite interestingly, maximal enhancement in DNA synthesis was observed when IG6 Fab' was added 12 h after culture initiation and notable enhancement could still be observed despite a 24 h delay. (In fact, experiments with occasional tonsil B cell preparations exhibiting a strong reactivity to bivalent anti-IgM antibody showed maximal responses with the 24 h delay in addition of IG6 Fab'.) In striking contrast to the late-acting effects of IG6 Fab', any delay in the addition of bivalent anti-IgM resulted in a significantly diminished level of B cell DNA synthesis at 48–61(67) h (Fig. 6A).

The possibility that IG6 Fab' might substitute for the presence of cross-linking ligand during the late phase of the prolonged mIgM-mediated signaling process was evaluated. Resting tonsil B cells were pulsed with the bivalent ligand and/or IG6 Fab' for 24 h, washed extensively and recultured with HB57 mAb and/or IG6 mAb Fab' for the remainder of a 65 h culture period. The data from a representative experiment in Table 5 shows that the addition of IG6 Fab' during the late phase of B cell activation in the absence of the bivalent anti-IgM fails to enhance B cell DNA synthesis and, furthermore, that synergy in inducing DNA synthesis is substantially more apparent when IG6 Fab' and the mIgM cross-linking ligand are co-incubated during the later phase in activation than when the ligands are co-incubated solely during the first 24 h period. Taken together, the above results indicate that IG6 Fab' exhibits its functional effects only when stimulatory mIgM receptor cross-links are being formed and that IG6 Fab'-

Table 5. Enhancing effect of IG6 Fab' during the later phase of B cell activation requires the concomitant presence of a mIgM cross-linking ligand

mAb present at 0–24 h of culture	mAb present at 24–65 h of culture			
	None	IG6 Fab'	HB57 mAb	HB57 mAb + IG6 Fab'
None	873 ± 93[a]	1033 ± 131	1357 ± 145	4390 ± 337
IG6 Fab'	841 ± 123	917 ± 109	1011 ± 128	4189 ± 461
HB57 mAb	3641 ± 203	3769 ± 167	13,290 ± 264	58,181 ± 1795
HB57 mAb + IG6 Fab'	6494 ± 335	7589 ± 63	16,864 ± 671	63,556 ± 2331

Resting tonsil B cells were incubated in round-bottomed 17 × 100 mm culture tubes at 10^7/2 ml with mAb HB57 ± IG6 Fab', each at 200 μg/ml, or medium alone. After 24 h, cells were washed three times and recultured in microtiter wells at 3×10^5/200 μl with mAb HB57 ± IG6 Fab', each at 100 μg/ml, or medium alone.

[a]Values are c.p.m. [^{3}H]thymidine uptake.

Table 6. Comparison of IG6 Fab' and T cell cytokines for capacity to enhance anti-IgM and anti-IgD-triggered B cell DNA synthesis

Experiment	Bivalent mAb	Concentration (μg/ml)	Δ c.p.m. [^{3}H]thymidine uptake				
			Medium	IG6 Fab' (100 μg/ml)	low mol. wt BCGF (12.5%)	rIL-4 (10 ng/ml)	rIFN-γ (1000 U/ml)
A	HB57 anti-IgM	100	1395 ± 33	5438 ± 152	7537 ± 694	19,663 ± 757	4361 ± 215
		10	814 ± 19	3005 ± 302	5615 ± 329	16,419 ± 721	2673 ± 133
		1	85 ± 34	420 ± 45	1682 ± 122	8978 ± 278	419 ± 45
	5D7 anti-IgM	100	527 ± 35	3505 ± 177	1797 ± 207	6102 ± 270	1275 ± 122
		10	418 ± 138	3518 ± 77	1995 ± 273	6266 ± 373	742 ± 76
		1	6 ± 21	744 ± 88	126 ± 124	3274 ± 98	−124 ± 14
B	HB57 anti-IgM	100	12,724 ± 523	41,458 ± 5169	28,732 ± 1186	41,666 ± 2644	79,446 ± 2183
		10	4980 ± 464	21,885 ± 825	26,191 ± 904	31,498 ± 1983	47,340 ± 2929
		1	346 ± 123	1478 ± 65	4297 ± 307	8930 ± 192	2447 ± 193
	5D7 anti-IgM	100	2384 ± 116	31,633 ± 1377	6983 ± 398	16,130 ± 102	12,838 ± 348
		10	953 ± 82	20,637 ± 1755	6238 ± 376	12,339 ± 1081	4723 ± 224
		1	−84 ± 9	1704 ± 41	506 ± 132	2551 ± 130	63 ± 47
C	HB57 anti-IgM	100	1199 ± 51	5212 ± 338	7662 ± 382	12,046 ± 878	ND
	4E5 anti-IgD[a]	100	25 ± 23	46 ± 50	3677 ± 350	8635 ± 306	ND

Δ c.p.m. [^{3}H]thymidine uptake represents the c.p.m. uptake above that observed in cultures with medium, IG6 F(ab') or T cell cytokine only. Background c.p.m. in cultures containing medium, IG6 Fab', low mol. wt BCGF, rIL-4 and rIFN-γ was 167 ± 77, 176 ± 33, 1279 ± 79, 347 ± 51 and 275 ± 105 respectively for experiment A; 176 ± 38, 105 ± 13, 294 ± 52, 350 ± 44 and 204 ± 57 respectively for experiment B; and 290 ± 17, 395 ± 30, 512 ± 51 and 520 ± 51 for experiment C.

[a]*Intrinsic binding affinity of mAb 4E5 for mIgD has been previously established to be* $K_a = 7.6 \times 10^7\ M^{-1}$ (15).

mediated enhancement is most pronounced during the later interval of the prolonged mIgM signaling process that leads to S phase entry.

Comparison of IG6 Fab' and T cell cytokines for capacity to enhance anti-IgM and anti-IgD triggered DNA synthesis

Numerous studies have indicated that soluble cytokines from activated T cells can enhance the clonal proliferation of mIg-triggered B cells (4,33,34). Cytokines such as rIL-4 and low mol. wt BCGF appear to function, at least in part, through contributing signals which further the cell cycle progression of B cells which have reached the G_1 phase of the cell cycle via the mIg signaling pathway (33). The apparent efficacy of IG6 Fab' at enhancing the mIgM-signaled $G_1 \rightarrow S$ phase transition suggests that this $C_\mu 4$-specific ligand might be an effective substitute for T cell cytokines in promoting a full B cell activation response. The relative effectiveness of IG6 Fab' and several cytokines (rIL-4, low mol. wt BCGF, and rIFN-γ) at enhancing the S phase entry of resting human B cells triggered by bivalent anti-IgM ligand was compared (Table 6). Data from the representative experiments shows that IG6 Fab' was generally less effective than rIL-4 at enhancing B cell S phase entry in cultures containing high affinity mAb HB57 or mAb 5D7. However, the $C_\mu 4$-binding Fab' was as effective or more effective than optimal concentrations of rIFN-γ or low mol. wt BCGF. In this and multiple other experiments, IG6 Fab' not only enhanced the level of DNA synthesis elicited by high optimal concentrations of these ligands but also permitted mAbs HB57 and 5D7 to trigger B cell S phase entry at concentrations that were non-mitogenic in the absence of the monovalent ligand Although IG6 Fab' could enhance anti-IgM triggered DNA synthesis to a level comparable to that observed in the presence of optimal concentrations of certain T cell cytokines, the Fab' ligand did not substitute for the signaling effects of cytokines. Thus, the level of [^{3}H]thymidine uptake observed in mAbs HB57- and 5D7-stimulated cultures containing both IG6 Fab' and optimal concentrations of rIL-4, rIFN-γ or low mol. wt BCGF was consistently greater than that observed in cultures supplemented with either IG6 Fab' or the T cell cytokine (data not shown).

An important additional finding shown in Table 6 is that IG6 Fab' did not enhance the level of B cell [^{3}H]thymidine uptake in B cells triggered by the IgD-specific mAb 4E5, despite the fact that this mAb could induce significant B cell S phase entry in the presence of rIL-4 or low mol. wt BCGF. These latter results suggest that the enhancing effect of IG6 Fab' is restricted to B cells activated through the mIgM receptor, and that the similar functional effect of IG6 Fab' and cytokines at facilitating the S phase entry of mIgM-triggered B cells is not due to IG6 Fab' cross-reactivity with a cytokine receptor.

Enhancing potential of other Fab' fragments specific for distinct sites on the $C_\mu 4$ domain

Fab' fragments of mAbs specific for five distinct sites on $C_\mu 4$ (14) were evaluated for potential to facilitate B cell activation by the mIgM cross-linking ligands mAbs HB57 and 5D7 (Fig. 7). Consistent with the results obtained with intact mAbs (Table 2), Fab' from mAb IG6 was the most effective monovalent ligand at promoting enhanced B cell DNA synthesis. While Fab' fragments from mAbs 5D7, 4-3, 196.6b and IG6 could facilitate [^{3}H]thymidine uptake in mAb HB57-stimulated cultures, enhanced DNA synthesis in mAb 5D7-stimulated cultures was observed only upon the addition of IG6 Fab' or

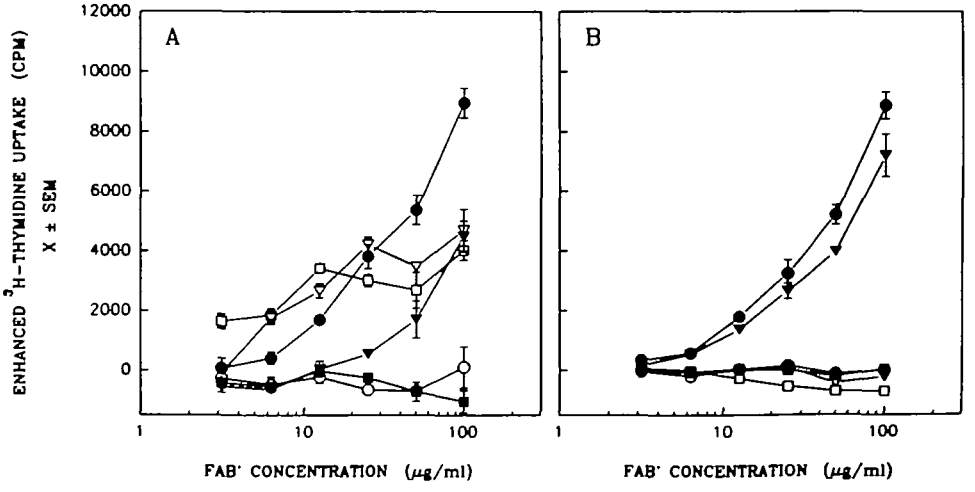


Fig. 7. Enhancement of anti-IgM triggered DNA synthesis by Fab' fragments specific for diverse sites on $C_\mu 4$ Resting tonsil B cells were stimulated with bivalent anti-IgM mAb HB57 (A) or mAb 5D7 (B), each at 100 μg/ml, in the presence of various concentrations of airfuged IG6 Fab' (●), 4–3 Fab' (▼), 196.6b Fab' (▽), 5D7 Fab (□), IF11 Fab' (■) or control MOPC-21 Fab' (○). Data are expressed as enhanced [^{3}H]thymidine uptake (48–70 h) as calculated in Fig. 1 and Table 5. Background c.p.m. in cultures with medium alone was 312±32; c.p.m. in cultures containing bivalent mAb HB57 alone and mAb 5D7 alone was 5109±349 and 1250±58 respectively. None of the Fab' ligands induced significant [^{3}H]thymidine uptake above background when cultured in the absence of the bivalent mAb. The data shown are representative of two such titration experiments. In addition, five other experiments with Fab' ligands tested at 100 μg/ml showed the following hierarchy in terms of enhancement potential for mAb HB57-triggered DNA synthesis: IG6 > 196.6b ≥ 4.3 ≥ 5D7 >> IF11, and for mAb 5D7-triggered DNA synthesis: IG6 > 4.3 >> 196.6b = IF11. The affinities of most of the anti-IgM Fab' for B cell mIgM are indicated in Table 2. (The affinity of 196.6b Fab' has not been determined.)

4-3 Fab'. MOPC-21 Fab' (control) and low affinity anti-IgM mAb IF11 Fab' ($K_a \approx 2 \times 10^5\ M^{-1}$) did not enhance B cell responses to either cross-linking ligand. The finding that 5D7 Fab' blocks the triggering of B cell DNA synthesis in mAb 5D7-stimulated cultures was expected given that the monovalent ligand can compete with bivalent mAb 5D7 for epitopes and prevent the requisite cross-linking of mIgM necessary for triggering B cell activation. The further inability of 196.6b Fab' to enhance mAb 5D7-triggered DNA synthesis is consistent with the fact that mAb 196.6b interferes sterically with mAb 5D7 binding to IgM in RIA cross-competition analyses with pentameric IgM (14). Interestingly, at high Fab' concentrations the relative potential to facilitate DNA synthesis triggered by $C_\mu 2$-specific mAb HB57 did not correlate directly with Fab' binding affinity: 4-3 Fab' and 5D7 Fab bind $C_\mu 4$ with significantly higher affinity than does IG6 Fab' (Table 2). At low concentrations, however, high affinity 5D7 Fab could function more effectively than low affinity IG6 Fab'.

Three of the $C_\mu 4$-specific Fab' fragments which can enhance B cell responses to $C_\mu 2$-specific mAb HB57—Fab' from mAbs IG6 and 4-3, and mAb 196.6b—have been shown by cross-competition RIA analyses to bind to proximal epitopes on $C_\mu 4$ (14) These observations, taken together with the fact that enhancing potential does not correlate directly with binding affinity for $C_\mu 4$, suggest that the region of the $C_\mu 4$ domain bound might be a relevant factor in Fab'-mediated enhancement. The site bound by mAb IG6 Fab' may be a conformational and not a linear epitope since attachment of this ligand to pentameric IgM was not inhibited by three CNBr cleavage fragments which together span the entire $C_\mu 4$ domain (14). Furthermore, the $C_\mu 4$ domain obtained from trypic digests of IgM, isolated in 5 M guanidine hydrochloride, a denaturing solvent, and then dialyzed in PBS, was much less effective than isolated μ chain at inhibiting mAb IG6 binding to radiolabeled IgM (14).

Discussion

The above studies describe the novel finding that a monomeric ligand specific for the membrane-proximal $C_\mu 4$ domain of mIgM can enhance human B cell activation achieved through the mIgM-signaling pathway. The activation-promoting function of this ligand (mAb IG6 Fab') was only noted when mIgM molecules on the responsive B cells were cross-linked by bivalent mIgM-specific ligands and not when B cells were triggered by a bivalent antibody specific for mIgD co-expressed on the resting B lymphocytes. IG6 Fab' engagement with $C_\mu 4$ appears to lower somewhat the very high physicochemical binding requirements for triggering B cell S phase entry by cross-linking ligands. Thus, IG6 Fab' increased the S phase entry of B cell populations cultured with bivalent ligands near the minimal ligand–receptor affinity threshold for signaling S phase; facilitated a minor degree of [^{3}H]thymidine uptake in cells cultured with anti-IgM mAbs of affinities below the threshold (P. Mongini, unpublished results); and lowered the minimal concentration threshold for triggering B cell DNA synthesis by ligands of high binding affinity. Because the available evidence suggests that the enhancing effects of the $C_\mu 4$-specific Fab' are independent of the site bound by an effective cross-linking ligand, it is highly possible that this monomeric $C_\mu 4$-specific molecule may also enhance B cell responses to cross-linking antigens specific for IgM variable region domains.

A particularly interesting observation in the above studies was that IG6 Fab' could effectively function at enhancing anti-IgM triggered DNA synthesis when added during the late phase of the prolonged period of mIgM-mediated signaling necessary for T cell-independent B cell S phase entry (9,10). The presence of IG6 Fab' could not substitute for mIgM cross-linking ligands during the late phase in activation. Rather, the functional effects of IG6 Fab' required the concomitant presence of the cross-linking ligand. Since previous studies from this and other laboratories have shown that the affinity and concentration requirements for ligand-initiated signal transduction during the late phase are much more demanding than the binding requirements for signaling through mIgM during the early phase (9,10), the current observations suggest that Fab' engagement with $C_\mu 4$ is functionally most relevant under conditions where it is difficult to achieve effective mIgM receptor cross-linking and subsequent signal transduction. Although IG6 Fab' appears to be most active during the later phase in mIgM-triggered activation that leads to S phase entry, the $C_\mu 4$-specific ligand is not without effect during earlier stages in activation. Preliminary studies indicate that IG6 Fab' can enhance the potential of anti-IgM bivalent ligands to trigger enhanced class II MCH expression in resting B cells (P. Mongini, unpublished results).

The relatively high concentration of IG6 Fab' required for optimal function is consistent with the relatively low affinity of this monomeric ligand. Thus, the EC_{50} of IG6 Fab', i.e. the concentration which should bind 50% of total available mIgM epitopes under conditions of ligand excess, has been calculated to be 7 μg/ml at 4°C (6). Because Fab' dissociation rates generally rise with increasing temperature (35), it is expected that at a culture temperature of 37°C, the EC_{50} of IG6 Fab' should be even greater. This consideration, taken together with the fact that IG6 Fab' should bind a high percentage of mIgM molecules in order to assure simultaneous engagement of mIgM molecules by both IG6 Fab' and the cross-linking anti-IgM ligand, are quite consistent with the high concentration requirements for IG6 Fab' function.

Several possible explanations for the adjuvant effects of this $C_\mu 4$-specific monomeric ligand have been considered. The possibility that IG6 Fab' functions through inducing allosteric changes in mIgM which enhance the affinity of the bivalent ligands for their respective epitopes appears unlikely. Firstly, IG6 Fab' can exhibit synergy with high affinity ligands specific for different domains, including domains that are separated by one to two additional domains from that bound by IG6 Fab'. Secondly, past competition RIA analyses failed to suggest that binding of mAb IG6 to pentameric IgM enhances the binding of mAb HB57, Mu53, XG9 or 5D7 (14) (S. Rudich and P. Mongini, unpublished results). Finally, immunocytofluorimetric analyses of B cells, incubated with limiting concentrations of mAbs HB57 and 5D7 in the presence or absence of 100 μg/ml of IG6 Fab' and stained with a fluorescein-conjugated probe specific for mouse IgG Fc, did not reveal significant changes in staining intensity in the presence of monovalent Fab' (P. Mongini, unpublished results).

The possibility that IG6 Fab' enhances the cross-linking of mIgM by bivalent ligands through some other means cannot be fully excluded. Although IG6 Fab' did not appear to enhance IgM cap formation in B cells exposed to bivalent anti-IgM mAbs, subtle changes in the kinetics of cross-link formation and/or dissociation may have been missed in these experiments, and such changes could have a major effect on signal transduction, particularly in preactivated B cells. This might occur through Ig domain interactions between bound IG6 Fab' molecules in a cross-linked mIgM complex. Alternatively, allosteric changes in the mIgM molecule induced by IG6 Fab' binding might enhance the potential for mIgM–mIgM associations. Both these phenomena could help stabilize mIgM aggregates initially created by the bivalent high affinity ligands. This issue will have to be more thoroughly investigated by measurements of the association and dissociation rates for bivalent anti-IgM in the presence and absence of IG6 Fab'.

An interesting possibility is that the functional effects of IG6 Fab' involve changes in the association of B cell mIgM with other integral membrane proteins of signal-transducing potential, i.e. Igα, Igβ, CD19 or CD22 (21–25,36,37). All of these latter proteins belong to the Ig superfamily (23,25,36) and exhibit extracellular domain-like structures known to have a high potential for homophilic or heterophilic binding (38). IG6 Fab'-induced changes in the conformation or orientation of $C_\mu 4$ may alter the potential of mIgM to interact with certain of these membrane molecules. That the membrane-proximal $C_\mu 4$ domain might have some particular functional significance can be inferred from the high degree of homology between murine and human $C_\mu 4$, i.e. 81 versus 47, 63 and 56% homology for $C_\mu 1$, $C_\mu 2$ and $C_\mu 3$ respectively (39). Because studies by Reth and colleagues had suggested that the function of Igα in the mIgM signal-transducing complex might involve interactions of the single extracellular Ig-like domain of Igα (CD79a) with $C_\mu 3$ or $C_\mu 4$ (23,37,40), it was a particularly intriguing possibility that this latter association might be altered by IG6 Fab'. Our current studies to test this have provided no evidence that IG6 Fab' binding to $C_\mu 4$ increases the affinity of mIgM–CD79a interactions, however. Although the known weak association between mIgM and Igα (CD79a) (22,23) suggested that an IG6 Fab'-induced increase in the binding affinity of the two molecules should have been revealed in these experiments, we cannot fully exclude the possibility that IG6 Fab' may modulate mIgM–CD79a interactions The negative findings could reflect a limited ability of the low affinity IG6 Fab' to remain bound to the mIgM–CD79a complexes (and hence maintain the proper $C_\mu 4$ conformation for increased mIgM–CD79a association) once the complexes have been solublized by detergent.

In the above studies, IG6 Fab' did not appear effective at independently initiating signals in B cells. Thus, IG6 Fab' preparations did not trigger DNA synthesis; were inefficient at exhibiting synergy with PMA, a direct activator of protein kinase C; and were ineffective at signaling inhibition of DNA synthesis in leukemic B cells previously found to be exceedingly sensitive to inhibition by low affinity mIgM cross-linking ligands (6). In more recent experiments, we have begun to explore whether IG6 Fab' may induce very early biochemical events following receptor engagement. This has involved evaluating the potential of bivalent mAb HB57, bivalent mAb IG6 and monovalent IG6 Fab' to induce early tyrosine phosphorylation in both resting tonsil B cells and the $mIgM^+$ human B cell line, Daudi. In several experiments with resting B cells, IG6 Fab' has not induced reproducible increases in the phosphorylation of cellular proteins under conditions in which bivalent mAbs IG6 and HB57 do so. In an experiment with Daudi cells, the Fab' ligand did, however, induce significant tyrosine phosphorylation above background, although the intensities of most of the phosphorylated bands were lower than those induced by IG6 $F(ab)_2$ (P. Mongini and C. Blessinger, unpublished observations). Further studies are needed to clarify whether the binding of the IG6 Fab' ligand to the $C_\mu 4$ domain may induce some early pertubation of mIg receptor associated tyrosine kinases.

Although the above studies have not fully delineated the mechanism of action of this $C_\mu 4$-specific Fab', they indicate that engagement of a monovalent ligand with the membrane-proximal portion of the mIgM molecule can enhance signal transduction achieved through the mIgM–receptor complex. This suggests the development of a novel type of B cell adjuvant, i.e. a mIg–receptor complex-targeted ligand, which, although not fully stimulatory by itself, can significantly enhance the activation of B cells challenged with mIg cross-linking antigens. Although the IG6 ligand itself would not be a suitable *in vivo* adjuvant due to its specificity for secreted as well as membrane forms of IgM, it is conceivable that other monovalent ligands specific for epitopes on the unique membrane-proximal portion of mIgM (39,41,42), might show comparable effects.

Acknowledgements

This work was supported by US Public Health Service grants R01 GM35174 (PKAM) and ROI AI35984 (NC).

Abbreviations

BCGF	B cell growth factor
Hu	human
LUB	leukemic B cell population
mIg	membrane immunoglobulin
NRS	normal rabbit serum
PMA	4β-phorbol-12β-myristate-13α-acetate
SRBC	sheep red blood cells
TBS	Tris-buffered saline

References

1 Janeway, C. A., Bottomly, K. B., Babich, J., Conrad, P., Conzan, S., Jones, B., Kaye, J., McVay, L., Murphy, D. and Tite, J. 1984. Quantitative variation in Ia antigen expression plays a central role in immune regulation. *Immunol. Today* 5:99.

2 Noelle, R. J. and Snow, E. C. 1991. T helper cell-dependent B cell activation. *FASEB J.* 5:2770.

3 Clark, E. A. and Lane, P. J. L. 1991. Regulation of human B-cell activation and adhesion. *Annu. Rev Immunol.* 9:97.

4 Jelinek, D. F. and Lipsky, P. E. 1987. Regulation of human B lymphocyte activation, proliferation, and differentiation. *Adv. Immunol.* 40:1.

5 Goroff, D. K., Stall, A., Mond, J. J. and Finkelman, F. D. 1986. *In vitro* and *in vivo* B lymphocyte activating properties of anti-delta antibodies. *J. Immunol.* 136:2382

6 Rudich, S M., Roux, K. H., Winchester, R J. and Mongini, P. K A 1988. Anti-IgM-mediated B cell signaling. Molecular analysis of ligand binding requisites for human B cell clonal expansion and tolerance. *J. Exp. Med.* 168:247

7 Rehe, G. T., Katona, I. M., Brunswick, M , Wahl, L. M., June, C. H and Mond, J. J. 1990. Activation of human B lymphocytes by nanogram concentrations of anti-IgM–dextran conjugate. *Eur. J. Immunol* 20:1837.

8 Mongini, P. K. A., Blessinger, C. A., Highet, P F. and Inman, J. K 1992 Membrane IgM-mediated signaling of human B cells. Effect of increased ligand binding site valency on the affinity and concentration requirements for inducing diverse stages of activation. *J Immunol.* 148:3892.

9 Mongini, P. K A , Blessinger, C A. and Dalton, J. P. 1991. Affinity requirements for induction of sequential phases of human B cell activation by membrane IgM-cross-linking ligands. *J. Immunol.* 146.1791

10 DeFranco, A. L., Raveche, E. S. and Paul, W. E. 1985. Separate control of B lymphocyte early activation and proliferation in response to anti-IgM antibodies. *J. Immunol.* 135.87.

11 Rudich, S. M., Winchester, R. J. and Mongini, P K. A. 1985. Human B cell activation: evidence for diverse signals provided by various monoclonal anti-IgM antibodies *J Exp Med.* 162.1236.

12 Holmes, N. J. and Parham, P. 1983. Enhancement of monoclonal antibodies against HLA-A2 is due to antibody bivalency. *J. Biol. Chem.* 258:1580.

13 Ehrlich, P. H , Moyle, W. R , Moustafa, Z A and Canfield, R. E. 1982 Mixing two monoclonal antibodies yields enhanced affinity for antigen. *J. Immunol* 128:2709.

14 Rudich, S. M , Mihaesco, E., Winchester, R and Mongini, P. K. A. 1987. Analysis of the domain specificity of various murine anti-human IgM monoclonal antibodies differing in human B lymphocyte signaling activity. *Mol. Immunol* 24:809.

15 Mongini, P. K. A., Blessinger, C , Posnett, D. N. and Rudich, S. M 1989. Membrane IgD and membrane IgM differ in capacity to transduce inhibitory signals within the same human B cell clonal populations. *J. Immunol.* 143:1565.

16 Parham, P., Androlewicz, M. J., Brodsky, F. M., Holmes, N. J. and Ways, J. P. 1982 Monoclonal antibodies: purification, fragmentation, and application to structure and functional studies of class I MHC antigens *J Immunol. Methods* 53:R101

17 Mongini, P , Blessinger, C., Seremetis, S., Winchester, R. and Rudich, S. 1987. Human leukemic B cell activation: functional consequences of membrane IgM interaction with anti-IgM ligand is an alterable cell characteristic. *Blood* 70:1193.

18 Francois, D. T., Katona, I. M., June, C. H., Wahl, L. M , Feuerstein, N., Huang, K.-P. and Mond, J. J. 1988. Anti-Ig-mediated proliferation of human B cells in the absence of protein kinase C. *J. Immunol.* 140:3338.

19 Suzuki, T., Butler, J. L. and Cooper, M. D. 1985. Human B cell responsiveness to B cell growth factor after activation by phorbol ester and monoclonal anti-μ antibody. *J. Immunol.* 134:2470.

20 Mecheri, S., Edidin, M., Dannecker, G., Mittler, R. S. and Hoffmann, M. K. 1990. Immunogenic Ia-binding peptides immobilize the Ia molecule and facilitate its aggregation on the B cell membrane. Control by the Mls-1 gene. *J. Immunol.* 144:1361.

21 Reth, M., Hombach, J., Wienands, J., Campbell, K. S., Chien, N , Justement, L. B. and Cambier, J. C. 1991. The B-cell antigen receptor complex. *Immunol. Today* 12:196.

22 van Noesel, C. J. M., Brouns, G. S., van Schijndel, G. M. W., Bende, R. J., Mason, D. Y., Borst, J. and van Lier, R. A. W. 1992. Comparison of human B cell antigen receptor complexes: membrane-expressed forms of immunoglobulin (Ig)M, IgD, and IgG are associated with structurally related heterodimers. *J Exp. Med.* 175:1511.

23 Reth, M. 1992. Antigen receptors on B lymphocytes. *Annu. Rev. Immunol.* 10:97.

24 Pesando, J. M., Bouchard, L. S. and McMaster, B. E 1989. CD19 is functionally and physically associated with surface immunoglobulin. *J. Exp Med* 170:2159

25 Clark, E. A. 1993. CD22, a B cell-specific receptor, mediates adhesion and signal transduction. *J. Immunol.* 150:4715.

26 Yellen-Shaw, A. J. and Monroe, J G. 1992. Developmentally regulated association of a 56-kD member of the surface immunoglobulin M receptor complex. *J. Exp Med.* 176:129.

27 Gold, M R., Matsuuchi, L., Kelly, R. B. and Defranco, A. L 1991. Tyrosine phosphorylation of components of the B-cell antigen receptors following receptor crosslinking. *Proc. Natl Acad Sci. USA* 88:3436.

28 Kuwahara, K., Matsuo, T., Nomura, J., Igarashi, H., Kimoto, M , Inui, S and Sakaguchi, N 1994. Identification of a 52-kDa molecule (p52) coprecipitated with the Ig receptor-related mb-1 protein that is inducibly phosphorylated by the stimulation with phorbol myristate acetate *J Immunol* 152:2742

29 Petrini, M , Emerson, D L and Galbraith, R M 1983. Linkage between surface immunoglobulin and cytoskeleton of B lymphocytes may involve Gc protein. *Nature* 306:73.

30 Koch, N. and Haustein, D. 1983. Association of surface IgM with two membrane proteins on murine B lymphocytes detected by chemical crosslinking *Mol Immunol* 20:33.

31 Rosenspire, A. J. and Choi, Y. S. 1982. Relation between actin-associated proteins and membrane immunoglobulin in B cells. *Mol Immunol* 19:1515.

32 Albrecht, D. L. and Noelle, R. J. 1988. Membrane Ig-cytoskeletal interactions. I. Flow cytofluorometric and biochemical analysis of membrane IgM-cytoskeletal interactions *J. Immunol* 141:3915

33 Erikstein, B. K., Schwarze, P., Kiil Blomhoff, H., Funderud, S., Stokke, T and Smeland, E B 1991. Use of size fractionation of in vitro-activated human B lymphocytes for studies of cell cycle-dependent growth regulation. *Scand. J. Immunol.* 33:51.

34 DeFrance,T , Vanbervliet, B., Aubry, J -P , Takebe, Y , Arai, N., Miyajima, A., Yokota, T., Lee, F., Arai, K.-I., DeVries, J. E. and Banchereau, J. 1987. B cell growth-promoting activity of recombinant human IL4. *J. Immunol.* 139.1135.

35 Johnstone, R. W., Andrew, S M , Hogarth, M P., Pietersz, G A. and McKenzie, I. F. C. 1990. The effect of temperature on the binding kinetics and equilibrium constants of monoclonal antibodies to cell surface antigens. *Mol. Immunol.* 27:327.

36 Stamenkovic, I. and Seed, B. 1988. CD19, the earliest differentiation antigen of the B cell lineage bears three extracellular immunoglobulin-like domains and an Epstein–Barr virus-related cytoplasmic tail. *J. Exp Med* 168:1205

37 Hombach, J , Tsubata, T., Leclercq, L., Stappert, H. and Reth, M. 1990. Molecular components of the B cell antigen receptor complex of the IgM class *Nature* 343:760.

38 Williams, A. F. and Barclay, A. N. 1988. The immunoglobulin superfamily—domains for cell surface recognition. *Ann. Rev. Immunol.* 6:381.

39 Kabat, E. A., Wu, T. T., Reid-Miller, M., Perry, H. M. and Gottesman, K.S., eds. 1987. *Sequences of Proteins of Immunologic Interest,* 4th edn. US Department of Health and Human Services, Public Health Service, NIH, Bethesda, MD.

40 Hornback, J., Lottspeich, F. and Reth, M 1990. Identification of the genes encoding the IgM- and IgM components of the IgM antigen receptor complex by amino-terminal sequencing. *Eur. J. Immunol.* 20:2795.

41 Oi, V. T., Bryan, V. M , Herzenberg, L A. and Herzenberg, L. A. 1980. Lymphocyte membrane IgG and secreted IgG are structurally and allotypically distinct. *J. Exp. Med.* 151:1260

42 Davis, F. M., Gossett, L. A. and Chang, T. W. 1991. An epitope on membrane-bound but not secreted IgE: implications in isotype-specific regulation. *Biotechnology* 9:53.