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Molecular analysis of ligand binding requisites for human B lymphocyte activation and tolerance through membrane IgM

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MOLECULAR ANALYSIS OF LIGAND BINDING REQUISITES FOR HUMAN B LYMPHOCYTE ACTIVATION AND TOLERANCE THROUGH MEMBRANE IGM

BY

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A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

MOLECULAR ANALYSIS OF LIGAND BINDING REQUISITES FOR
HUMAN B LYMPHOCYTE ACTIVATION AND TOLERANCE THROUGH MEMBRANE IGM

by

Steven M. Rudich

Advisor: Patricia K. A. Mongini, Ph. D.

The ligand binding requisites necessary to initiate membrane IgM-mediated human B lymphocyte clonal expansion and tolerance were investigated with a well-characterized set of soluble murine monoclonal anti-human antibodies. The anti-IgM monoclonal antibody requisites studied included the affinity and binding stoichiometry for membrane IgM in addition to the μ chain domain specificity. Anti-IgM antibody affinity and binding stoichiometry were measured by equilibrium-binding studies and Scatchard plot analysis with several membrane IgM-positive B cell lines. Specificity for $C\mu_1$, $C\mu_2$, and $C\mu_4$ IgM domains was indicated by competition radioimmunoassays with enzymatically-derived μ chain domain fragments as well as mutant IgM myeloma proteins which lacked heavy chain domains.

Diversity in ability to induce B lymphocyte DNA synthesis in splenic B cell populations was observed among the ligands which could not be attributed to inhibitory Fc-mediated signaling. Although mitogenic antibodies were identified with each domain specificity, $C\mu_1$-specific antibodies were as a whole, the least effective stimulators. The T cell-dependent mitogenic capability of the monoclonal antibodies was found to be largely a direct linear function of their affinities for B cell membrane IgM. The notable exception to this was in the case of the $C\mu_1$-specific monoclonal antibodies which bound monogamously to membrane IgM. Mixtures of both

mitogenic and non-mitogenic antibodies were found to induce marked synergy for DNA synthesis. T cell factor-independent B cell DNA synthesis was observed for certain mixtures of monoclonal antibodies which included a unique $C\mu_4$-directed antibody. This was interpreted as indicating that supra-optimal cross-linking of membrane IgM molecules on the B cell surface can obviate the requirement for growth factor signaling.

Ligand binding requirements for B lymphocyte tolerance were studied in several novel B cell malignant clonal populations which were found to be exquisitely sensitive to anti-IgM-mediated inhibition of DNA synthesis. The affinity threshold for inducing tolerance was found to be significantly less than that observed for inducing B cell clonal expansion. This, as well as other observations, indicated that the anti-IgM antibody binding requisites for tolerance induction in these B cell leukemias are much less stringent than the requisites for inducing proliferation.

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CHAPTER ONE

Introduction

Perhaps the most impressive characteristic of the immune system is its exquisite specificity for antigen (Ag). The elucidation that B lymphocytes have membrane-bound immunoglobulins (mIg), and that this mIg has the same specificity as the Ig secreted by activated cells of the same clone, helped lead to the acceptance of the idea that B cell mIg may play a key role in Ag-induced activation of B lymphocytes. However, the manner in which ligands in the extracellular milieu can trigger and elicit responses from B cells has been rigorously debated by immunobiologists for decades. This debate has focused around the following question: Can the interaction of B lymphocyte mIg with Ag directly result in B cell activation and clonal expansion, or alternatively, tolerance and clonal deletion, or, does mIg function in an entirely passive manner by focusing mitogenic moieties or T cell-reactive epitopes onto the B cell surface?

The "one non-specific" signal theory of B cell activation, originally proposed by Coutinho and Moller (1-3), suggests that mIg only serves to bind Ag onto the B cell and that no activation signal is delivered by ligation of the Ag receptor. The main function of mIg is viewed as focusing an intrinsic stimulant (polyclonal activator) present in all Ags onto a distinct non-Ig receptor on the surface of the B cell. According to this hypothesis, if the intrinsic stimulant is not a part of a particular Ag, than the Ag must have the capacity to recruit a signaling moiety, such as Ag-specific T lymphocytes, to the B cell surface to elicit B cell immune responses. This requires the linked, cognate interaction of B and T lymphocytes. The activation signal in such a case is generally believed to be transmitted when helper T cells bind, via their Ag receptor, to B cell-processed Ag associated with molecules of the major histocompatibility complex (MHC) on the surface of B cells. This Ag has to be recognized by the receptors of both the B and T cell (although not necessarily at the same time), hence the "linked" aspect of the signal. In addition, the T cell

receptor also has to recognize the MHC molecules of the B cell, hence the "cognate" aspect (4). One major short-coming presented by this theory for B cell activation is the inability to explain the need for two types of mIg receptors, mIgM and mIgD, in addition to the inability to account for the differential interactions of mIg receptors with ligands which can lead to proliferation, but may also, under certain circumstances, lead to the active induction of tolerance (5-10).

Most other models for B cell activation are modifications of the hypothesis originally proposed by Bretscher and Cohn (11) which proposes that two signals are required to elicit activation, one (although not necessarily the first [12]) being delivered through the mIg receptor, while the second is given by accessory cells or their cytokine products (13,14). The most conservative interpretation of the "two-signal" B lymphocyte activation models is that mIg ligation "pushes" the cell only so far along the activation program such that growth factor receptors are induced for expression. Proliferation, as observed by S phase DNA synthesis, is only obtained when these newly-expressed receptors are occupied by the appropriate factors.

Accordingly, it has been proposed that delivery of the first signal alone, via mIg ligation, in the absence of the second signal, would lead to tolerance induction. The effect of signal one and two on B cell immunity and tolerance was first demonstrated by Metcalf and Klinman (15), who used immature murine B cells in a T cell-dependent B cell cloning system, the splenic focus assay. They found that when cells were confronted with specific multivalent Ag in the absence of T cell help, the cells were rendered tolerant. However, when B cells saw Ag in the context of T cell help, they formed Ab-producing clones. Others have suggested that a differential recognition of Ag by mIgM and mIgD may determine whether interactions of Ag with a B lymphocyte result in the induction of immunity or tolerance (16,17).

Numerous experimental approaches have been devised to examine the role played by mIg in B cell activation. An indirect role for mIg ligation in B cell triggering is supported by reports of the ability of activated T cells or their products to induce changes in B lymphocyte physiology indicative of activation in the absence of any Ag (18,19). Another observation consistent with an Ag-focusing role for mIg is the report by Lanzavecchia (20) showing that both specific and non-

specific binding of Ag to B cells results in B cell processing and presentation of Ag to T cells in a MHC-restricted fashion. The interpretation of these studies, in addition to those by others, such as Tony, et al. (21), is that mIg acts to concentrate Ag onto the B cell, allowing it to prepare and process the Ag for linked, cognate interaction with the T lymphocyte.

There is also a great deal of experimental evidence indicating that the direct ligation of mIg receptors by Ags (or surrogate Ags in the form of anti-Ig Abs) in the absence of accessory cells or their products can induce biochemical events that lead to the activation of resting B cells (14,22,23). These activated B cells hence become receptive to T cell help for further activation and differentiation to Ig-secreting cells (13,22). In contrast to the positive effect of mIgM signaling on the activation and proliferation of mature resting B cells, interaction of the mIgM on immature B cells with either Ag or surrogate anti-Ig Ab causes them to become unresponsive and eventually die, reflecting an important tolerance mechanism (5,7,23). The different functional results obtained by mIgM ligation between mature and immature B lymphocytes may reflect either a) the distinct ways in which B cells of different activation or differentiation states can interpret the same membrane-initiated biochemical signals and /or b) differences in the kind of mIgM binding and cross-linking events and thus signaling which can take place on mature, resting versus immature B lymphocytes.

A major impediment to the study of the effects of Ag on B cell immune responses has been the clonal distribution, and hence low frequency, of cells specific for a given Ag in normal B cell populations. As a means of circumventing the enormous technical problems inherent in isolating truly homogenous populations of B lymphocytes to be triggered by selected Ags, an alternative approach in the study of B lymphocyte activation and tolerance has focused on using anti-mIg Abs as surrogate Ags. The underlying assumption in the use of anti-Ig reagents to engage mIg Ag receptors is that such an interaction mimics the cellular consequences of Ag-specific B cell triggering upon binding Ag. Besides finding use in the development of model systems to study B cell activation and tolerance, the use of anti-Ig Abs to study B cell physiology may also help elucidate the mechanisms of B cell regulation by autologous anti-idiotype Abs as

well as anti-isotypic Abs (rheumatoid factors). These latter Abs have been shown to occupy a large portion of the B cell immune repertiore, which presumably reflects some unappreciated physiological function (24,25). In addition, studies with anti-Ig Abs to modulate B cell physiology may help in the formulation of therapeutic strategies for the immunological control of certain B cell malignancies (26), some of which have already been reported to be receptive to in vitro anti-Ig-mediated signaling (27-29).

It has been more than twenty years since Sell and Gell (30) first showed that rabbit lymphocytes can become activated to proliferate by treatment with anti-allotype sera, confirming that the mIg on B lymphocytes can play a role in the elicitation of the B cell immune response. The first report documenting stimulation of human lymphocytes using anti-Ig sera appeared shortly thereafter (31). Unrefutable molecular evidence obtained with these ligands has indicated that mIg molecules are signal transducers, most analogous to åa-1 adrenergic receptors in their mode of signal transmission. Specifically, recent evidence reported by Cambier and co-workers (14,22), Bijsterbosch et al. (32), amongst others (33) indicates that activation of mIg receptors by Ag (or surrogate anti-Ig Ab) prompts the hydrolysis of polyphosphoinositides by membrane-bound phospholipase C yielding inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 stimulates the release of Ca^{++} from the endoplasmic reticulum and a later Ca^{++} influx; this Ca^{++} acts in concert with DAG to cause translocation of protein kinase C (PKC) to the plasma membrane. Here, activated, membrane-associated PKC phosphorylates its substrate(s), altering the activity of structures responsible for ion transport. This leads via unknown intermediary events and /or third messengers to membrane depolarization (34), increased expression of class II MHC gene products (35,36), as well as the proto-oncogenes c-myc and c-fos (37,38). Cells which have undergone these events reach an activation state defined as Go* (39). Progression from Go* to the G1 stage of the cell cycle, which is characterized by increases in cell size and significant increases in mRNA content, may require even more rigorous signaling conditions than those for the Go to Go* transition. Thus while most T-cell dependent Ags can induce this later transition, they cannot drive the cell past Go* (40,41). Entry into S phase and DNA synthesis appears to be

another control point in the cell cycle for B cell activation. B cells driven to G1 will not go any further in the cycle unless they receive T cell signals (such as those delivered by T cell-derived growth factors) or instead have been cultured from the resting state with certain T cell-independent Ags or certain anti-Ig Abs in high concentrations (42-44).

Recently, Fahey and DeFranco et al. (45), studying tolerance induction in the immature B lymphoma cell line WEHI-231, have determined that mIgM ligation on these cells can trigger the polyphosphoinositide signaling pathway as well. The very early biochemical changes in B cell physiology produced as a result of mIgM binding appear to be the same between WEHI-231 and mature, resting B lymphocytes, despite the fact that anti-IgM Abs cause opposite proliferative responses in these two cell types.

The demonstration that Fab' fragments of anti-Ig Abs do not stimulate B lymphocyte activation (34,46), but that the addition of a second bivalent Ab against the bound Fab' moieties induced mitogenesis (47), argued strongly in support of the view that for anti-Ig Abs to mediate signal transduction, cross-linkage of mIg molecules is mandatory. If ligand multivalency is indeed an essential requirement for B lymphocyte activation, then redistribution of surface Ig probably plays an important role in the initiation of the activation cascade. In the redistribution of mIgM, patching of small aggregates appears to preceed extensive capping of ligand-receptor complexes (48). The work of Klinman, et al. (7) with specific Ags and the work of others (49) with anti-Ig ligands has also suggested that cross-linkage of mIg receptors is mandatory for inducing tolerance.

Dintzis, et al. (50,51), using well-characterized co-polymers of acrylamide and hapten with known haptenic densities, concluded that in order for T cell-independent Ags to activate B cell immune responses, a rather large mIg-ligand cluster, formed from approximately 10-20 bound mIg molecules, was necessary. Each of these clusters has been called an immunon, and the idea that multiple clusters of such cross-linked mIg molecules must be simultaneously ligated on the B cell surface in order to actuate stimulation of B cell responses, has been designated the "immunon concept". In addition, they observed that clusters formed from co-polymers of less than the

haptenic density to create an immunon, were able to induce tolerance (51). Recently, however, Perelson (52) has argued that such large, cross-linked latticed clusters of mIg receptors may not be obligatory for stimulatory signal transduction but rather that two or three cross-linked membrane receptor molecules may be adequate for maximal B cell immune responses. In this regard, it should be noted that degranulation of mast cell IgE Fc receptors, which like the mIg Ag receptor, are thought to signal in an alpha-1 adrenergic-type manner, requires simple bivalent bridging of bound IgE molecules (53).

In their formulation of the immunon concept for B cell immune triggering, Dintzis and co-workers did not determine whether smaller cross-linked aggregates of mIgM, which could not be classified as immunons, could nevertheless initiate some of the biochemical events associated with activation. One might argue that in order to obtain S phase DNA synthesis, perhaps a critical signal threshold must be exceeded, and this can only be accomplished by the immunon, but that all mIg-signaling is not just mediated through the formation of such structures.

One enigma in regards to anti-Ig-mediated B cell signaling has been the requirement for a relatively high concentration of soluble anti-Ig Ab being needed to induce B cells to synthesize DNA. Most anti-Ig ligands do not stimulate B cell proliferation at concentrations < 5-10 μg/ml, optimal stimulation is often observed with doses ten times greater (42-44). In contrast, membrane depolarization and mIa hyper-expression are clearly demonstrated at anti-Ig Ab concentrations of < 0.5 μg/ml (54). These findings, plus the finding that 24-36 hours of exposure to anti-Ig ligands is necessary for signaling B cell proliferation (42), require that something in addition to initial modulation of mIg from the B cell surface is needed for entry into the G1 stage of the cell cycle. This has suggested that signaling through mIgM cross-linking must exceed some threshold level for a critical period of time to stimulate B cells to synthesize DNA (42,55). Once mIg has initially been modulated from the B cell surface, its rate of replacement is sufficiently slow such that higher concentrations of anti-Ig may be required later on in culture to keep the rate of mIg cross-linkage above the critical level required to induce triggering.

Regardless of whether or not the immunon is the only signaling unit derived from mIg cross-linkage, a question of fundamental importance is just how are mIgM cross-linking events transduced into intracellular signals which can alter B lymphocyte homeostasis? One of the most likely mechanisms by which mIg cross-linking leads to intracellular changes in B cell physiology involves the association of mIg with other membrane-bound molecules. This has been supported by co-precipitation and chemical cross-linking studies (56-60). Petrini et al (60) has evidence indicating that a membrane molecule analogous to Gc protein (a vitamin D binding protein) associates with mIgM upon activation. They suggest that since Gc protein has a high affinity for actin (61), this protein may play a key role in promoting the interaction of mIg ligation with actin and hence in initiating the membrane modulation events that result in B cell clonal expansion. While mIgM ligation which results in triggering of DNA synthesis may or may not involve mIgM association with actin, it would appear that mIgM ligation that results in triggering of active tolerance does not. Teale and Klinman (62) have found that pharmacological inhibitors of patching and capping do not interfere with the tolerance process.

Since it is abundantly clear that anti-Ig Abs modulate B cell physiology via cross-linkage of mIg receptors, it has been puzzling that some anti-Ig Abs can actuate B cell stimulation while other anti-Ig Abs cannot (63-71). This is an especially intriguing question in the face of the paradoxical observations that all anti-Ig Abs appear capable of inhibiting the lectin-driven B lymphocyte DNA synthesis (49, 72-74). The qualitative differences between mitogenic and non-mitogenic anti-Ig ligands is as yet largely unresolved. In addition, those ligand-binding factors responsible for the intiation of a tolerogenic or proliferative signal remain to be investigated. These qualitiative differences between anti-Ig ligands, ie. the binding requisites for mIg that characterize individual Abs, and which may be very important in the differentiation between mitogenic and non-mitogenic anti-Ig Abs, as well as in the elicitation of an activating or tolerogenic signal, will form the basis of this thesis work.

Although some of the diversity in mitogenic effects of anti-Ig ligands was shown to be attributable to inhibitory signals delivered by the Fc portion of the anti-Ig Ab upon ligation of the

IgG Fc receptor (64, 75,76), Fc-mediated inhibitory effects are clearly not the sole explanation for the limited signaling properties of some ligands since $F(ab')_2$ fragments of anti-Ig Ab preparations are sometimes found to be also non-stimulatory (71,73). Rather, it has been suggested that the differences in the ability of anti-Ig preparations to deliver signals to B lymphocytes may involve inherent differences in the V-region-derived binding properties of the anti-Ig ligands. That is, differences in mIg binding requisites, such as site specificity, affinity or total avidity, as well as binding stoichiometry for mIgM determinants, may all lead to the initiation of different kinds and /or degrees of signals which can directly regulate B cell responsiveness. Because most observations regarding anti-Ig-mediated signaling were obtained using polyclonal anti-Ig sera, a critical assessment of how these factors influence the ability of anti-Ig ligands to modulate B cell physiology, was not feasible. Recently, with the widespread usage of monoclonal Abs, such an analysis is possible.

Evidence that affinity/avidity differences between anti-Ig preparations can lead to different kinds of B cell signaling is provided by the repeated demonstration, originally made by Parker (77), that non-mitogenic anti-Ig Abs can be made mitogenic by presenting them to the B cell as an insoluble matrix - such as through coupling to acrylamide beads (78). The process of insolubilization creates a matrix of ligands at high fixed density on the surface of the bead, the result of which is a much greater avidity of interaction with mIgM molecules. In addition, in one of the earliest studies which used MoAbs to probe B cell signaling, Hamano and Asofsky (79) observed that the relative ability of two MoAb ligands to affect B cell proliferation and differentiation was correlated with their relative affinity for mIgM. Similarly, Zitron and Clevinger (80), using two murine anti-allo-IgD MoAbs, also noted that differences in the ability to induce murine B cell proliferation may reside in affinity differences between the two reagents.

Convincing evidence that site specificity of anti-Ig ligands may play a major role in the diversity observed in the ability of anti-Ig ligands to directly trigger B lymphocytes is lacking, although it has been proposed by others to explain heterogeneous effects in the ability of different anti-Ig Ab preparations to elicit B cell responses (80,81). Some precedent for site-

specific regulatory activity of Abs to other cellular membrane receptors does exist. Valente, et al. (82,83) have suggested that the growth-promoting or cAMP-inducing properties of Abs to the thyroid thyrotropin receptor may depend upon their ligation to alternative domains of this cell-surface receptor.

There are several possible explanations as to why the binding of an anti-Ig ligand to distinct sites may vary in the efficiency of signal transduction. Site-specific regulation may determine the ease with which conformational changes are initiated and propagated along the mIg molecule when distinct molecules are cross-linked by ligand. Some allosteric changes due to specific ligand binding may be necessary to induce association of mIg with other extra-membraneous constituents which may play an instrumental role in conveying activation signals to the cell interior. Since cross-linking has been shown to be obligatory for B cell signaling (34,46), it is possible that MoAbs which are more effective cross-linking agents, by virtue of either the location or number of epitopes to which they bind per mIgM molecule, may be more effective at B cell signal transduction.

Through the use of a large panel of anti-IgM MoAbs with well-defined affinities, binding stoichiometries, as well as mIgM site specificites, we have attempted to determine the contribution that each of these factors play in the ability of anti-IgM MoAbs to transduce signals which result in the alteration of B lymphocyte physiology. In particular, our attention will focus on the ligand binding requisites necessary to induce human B cell activation, as measured by S phase DNA synthesis, as well as the anti-IgM ligand binding requisites needed to inhibit the S phase DNA synthesis of several novel human B cell malignant clones.

Using these model systems to study signaling requisites, we intend to address the following questions:

1. Do MoAbs specific for human IgM differ in their ability to stimulate human B cell clonal expansion or alternatively, clonal tolerance? If there are differences in the abilities of these MoAbs to activate and inhibit B cell DNA synthesis, is there any correlation?

2. Do anti-IgM MoAbs differ in their ability to signal via a high-dose, T cell independent or a low-dose, T cell dependent mechanism?

3. Is there any indication for stimulatory, inhibitory, or null (neutral) sites on the membrane-bound IgM molecule, which, upon being bound by anti-IgM ligand, deliver different signals to human B lymphocytes?

4. What role does the affinity of the anti-IgM ligand for mIgM play in B cell activation, as well as for inhibition of leukemic B cell DNA synthesis?

5. To what extent is cross-linkage of mIgM molecules by anti-IgM ligand necessary to elicit B cell activation? inhibition? Is a large mIgM receptor-anti-IgM ligand cross-linked cluster (immunon) always mandatory to transduce signals, or are clusters of smaller size capable of signal transduction?

This thesis will present four manuscripts, three of which were previously published (and used in entirety with the permission of the copyright holder), which have addressed these questions.

CHAPTER TWO

Human B Cell Activation: Evidence for Diverse Signals Provided by Various Monoclonal Anti-IgM Antibodies*

INTRODUCTION

Anti-immunoglobulin antibodies have been frequently used to stimulate B lymphocytes (6,63,71,84,85) since Sell and Gell's initial observation that anti-allotype Abs induce the proliferation of rabbit lymphocytes (30). However, certain investigators have been unable to induce B cell proliferation with anti-Ig Abs (1,72) while others have observed that these reagents inhibit the activation of B lymphocytes (49,64,65).

In some instances, these divergent findings could be attributed to possible differences in lymphocyte population subset (66), maturational stage (66-68), cell cycle (69,70,86), or to the presence of accessory cells or factors (70,84,85,87). In contrast, other evidence suggests that the nature of the polyclonal Ab itself is important in determining whether of 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 (64,75,76), the fact that inhibition has also been observed with Ab preparations in which the Fc region has been digested (71,73), 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 reponse of the B lymphocyte to anti-Ig Abs is influenced by the affinity and/or epitope specificity of the Ab. Since polyclonal antisera consist of indeterminant mixtures of multiple Abs with distinct affinities and differing specificities, this hypothesis can be examined more directly by the use of MoAbs. A number of distinct anti-IgM MoAbs were assayed for the ability to stimulate human B cell proliferation in the

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presence and absence of T cell supernatant. In addition, defined combinations of these MoAbs were tested to determine whether the signals delivered by combinations of MoAbs differ from the signals provided by individual MoAbs.

The data obtained suggest that the ligand-binding characteristics of individual anti-IgM MoAbs influence the presence or absence of B cell proliferation, and the degree of the response obtained. Furthermore, the data show that combinations of certain individually non-mitogenic MoAbs induce pronounced B lymphocyte proliferation. Moreover, at high ligand concentration, certain MoAb mixtures, but not individual MoAbs, deliver signals for B cell activation which obviate the need for ancillary signals provided by T cell supernatants.

MATERIALS AND METHODS

Preparation of Murine Anti-Human Hybridomas. MoAbs VIIIE11, 1G6 and XG9 were obtained in two fusions of the non-secreting murine myeloma Sp 2/0 with spleen cells from BALB/c mice following standard immunization and fusion protocols (88,89). The same two human IgM myeloma proteins were immunogens for both fusions. MoAbs Mu53 and P24 were generously donated by Drs. David Posnett and Henry Kunkel and were derived similarly from BALB/c mice immunized with an aggregated human μ chain and intact human IgM monoclonal antibody, respectively. Anti-IgM MoAb 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 MoAb HB57 was purchased from the American Type Culture Collection (J. Kearney, donor).

CBDA-4E5 anti-human IgD MoAb and PMG3A-4C6 anti-human lambda light chain MoAb were prepared as described for anti-IgM MoAbs except that a IgDλ myeloma protein and a IgG3λ myeloma protein, respectively, were used as immunogens. HB45 anti-human kappa light chain MoAb was obtained from the American Type Culture Collection (J. Kearney, donor). 22c6 and SG171 MoAbs bind to monomorphic determinants on human Ia molecules (90,91).

Isolation and Purification of Monoclonal Antibodies. Anti-IgM-producing hybridomas were detected in a solid-phase enzyme linked immunosorbent assay (ELISA) (horse-radish peroxidase-goat anti-mouse Ig assay kit; New England Nuclear, Boston, MA) and cloned at limiting dilution. Clones were screened for binding to nine human IgM and fourteen human myeloma proteins of other Ig classes. Selected hybrids were propagated in irradiated (350R), pristane-primed BALB/c mice. Ascitic fluid was cleared by centrifugation at 17,300 x g for 1/2 hour, made 0.1% w/v with sodium azide and stored at 4°C.

Anti-IgM MoAbs 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% w/v Na_2S0_4. The resolubilized precipitates were dialyzed against 0.2M PO_4 buffer, pH 8, before being loaded onto protein-A Sepharose. The differential pH elution technique of Ey et al. (92) was used for purification in which contaminating IgG1 was removed by washing with 0.1M citrate buffer, pH 6, and the bound IgG2a or IgG3 hybridoma protein was eluted with 0.1M citrate, pH 4.5. The remaining anti-IgM MoAbs of IgG1 mouse isotype (HB57, Mu53, 1G6, XG9, and P24) were purified by Na_2S0_4 precipitation and ion-exchange chromatography over DEAE-Trisacryl (LKB Instruments, Rockville, MD) as described by Parham et al. (93), with slight modificatons. Protein was loaded onto columns in 0.005M PO_4 buffer, pH 8, and eluted by a linear salt gradient from 0.005M to 0.1M PO_4 buffer, pH 8. All anti-IgM MoAbs eluted at a conductivity of between 2.21-4.46 x 10^3 μMHOs.

The purified anti-IgM MoAbs were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (94) under both native and denaturing conditions in a 5 to 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 non-reduced and reduced gels. Calculations based upon densitometric tracings revealed levels of purity in all preparations of between 90-95%. In addition, immuno-electrophoresis using goat anti-mouse serum and goat anti-mouse Ig as preciptating reagents showed only one precipitation line.

CBDA-4E5 anti-human IgD MoAb (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 MoAbs (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 Waldenstrom's macroglobulinemia or from patients with mixed cryoglobulinemia who exhibited a monoclonal IgM spike. IgM proteins were puified by euglobulin fractionation (0.005M phosphate buffer, pH 8.6) and either Sephacryl S-300 gel filtration or protein-A Sepharose affinity chromotography 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 (1) absorption and selective elution from protein-A Sepharose, (2) affinity chromatography over Sepharose columns conjugated with purified murine hybridoma Abs specific for human Ig isotypes or (3) Na_2S0_4 precipitation followed by Pevikon block electrophoresis. IgA myeloma proteins were purified from serum by a sequence of steps which involved euglobulin precipitation to remove IgM, Na_2S0_4 precipitation to enrich for IgG and IgA, $ZnSO_4$ precipitation to separate IgG from IgA (95), 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 (96) but included 1% phytohemagglutinin (PHA). After incubating for 3 days at 37°C, 6% CO_2 and 90% relative humidity, cells were pelleted at 2000 rpm x 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 five hours followed by sterile filtration. Preliminary experiments using an affinity-purified goat anti-human IgM Ab established that a 30% v/v 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 MoAbs 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 MoAbs (data not shown).

Solid-phase Radioimmunoassays. The determination of MoAb in hybridoma culture supernatants was performed by solid phase radioimmunoassay (RIA) using a modification of techniques reported previously (97). Briefly, 10 μg/ml of purified human myeloma protein was incubated on wells of a polyvinylchloride plate (PVC) for 1 1/2 hours at 37°C. After 3 washes, diluted hybridoma Ab was added to myeloma-coated wells and incubated for an additional 1 1/2 hours. Bound MoAb was detected by incubating wells with approximately 25,000 cpm of tritium (^{3}H)- labeled, affinity-purified Ab specific for different murine Ig isotypes (97).

Competitive-Binding Radioimmunoassay. Analogously to the direct-binding RIA, each purified anti-IgM MoAb was coated onto PVC wells (in duplicate) by incubating 50 μl of a 10 μg/ml solution of MoAb in each well at 37°C for 90 min. One-half hour before the end of this initial coating, 1:1 mixtures composed of 1 μg/ml of a ^{3}H-labeled human IgM myeloma protein (specific activity = 667,000 cpm/μg) and each of the soluble anti-μ MoAbs, at concentrations ranging from 0.08 to 50 μg/ml in 5-fold dilutions, were prepared. These mixtures were incubated in a 24-well Linbro plate at 37°C until the end of the coating incubation, at which time all MoAb-coated wells were washed and 50 μl of each inhibition mixture was added to wells coated with each anti-μ MoAb. After a final 3 hour 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 MoAb were used as an inhibitor. Percent inhibition values were calculated as follows:

$$100 \times [1 - (\text{cpm } ^3\text{H-IgM bound in presence of soluble MoAb inhibitor}) / (\text{cpm } ^3\text{H-IgM bound in absence of soluble MoAb inhibitor})].$$

Immunofluorescence Studies. For all immunofluorescence studies, 2 x 10^5 cells in PBS-BSA-azide (assay buffer) were incubated with 100 μl of 1000 μg/ml or 10 μg/ml of purified MoAb

in a 96-well V-bottomed microtiter plate (Linbro, Flow Laboratories) for 1 hour at 4°C. MoAbs were tested at a concentration of 1000 μg/ml to insure saturation binding of potentially low affinity Abs (93). All tested MoAbs had previously been ultracentrifuged at 108,000 x g for 80 min at 4°C. After washing four times with assay buffer (200 μl/wash), tetramethylrhodamine-labeled $F(ab')_2$ goat anti-mouse Ig was incubated with the cells for 20 mins on ice before washing. Cells used for staining included: (a) Daudi, a lymphoblastoid B cell line (μ, k); (b) two mIgM-positive chronic lymphocytic leukemias (μ, k 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, Sunnyvale, CA), cells were analyzed by both forward narrow angle light scatter and fluorescence emission upon excitation at 514nm (98). After gating for viable cells on the basis of the scatter signal, the fluorescence of between 10,000 to 15,000 cells was compared with that obtained when cells were stained with 100 μl of 1000 μg/ml or 10 μg/ml of irrelevant control Abs: MOPC-245 for γ1 MoAbs, LPC-1 for γ2a and FLOPC-21 for γ3 MoAbs. Data were collected using logarithmic amplification and plotted as number of cells (ordinate) vs. log relative fluorescence intensity (abscissa). All gain and offset parameters were kept constant between different MoAbs.

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 x g) to eliminate debris, the resulting single-cell suspension was layered on Ficoll-Hypaque and centrifuged (2000 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-k and anti-λ MoAbs, anti-Leu 1, and P9 and R17 anti-monocyte MoAbs (99), respectively, cells from both spleen sources were found to be between 31-38% Ig+, 35-40% Leu 1+, and <2%+ for monocytic markers.

Immediately before an experiment, T cells were removed by rosetting twice with neuraminidase-treated sheep red blood cells (100). 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 MoAb 4F2 (101) was expressed weakly on <1% of these T cell-depleted splenocytes.

Cell Culture Conditions for Inducting B Cell Stimulation. T cell-depleted splenic lymphocytes were cultured in flat-bottomed wells of a 96-well plate (Linbro) at 2 x 10^5/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 (102) and 15% fetal calf serum (Rehatuin, Armour Pharmaceutical, Phoenix, AZ, lot. No. W60006). After 56 hours of incubation, proliferation was assessed by an additional 16 hour 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 ± standard error of the mean (SEM) from three identical cultures are reported; otherwise, when Δcpm values are shown, standard error of the difference (SE diff) between test triplicates 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 Monoclonal Anti-Human IgM Abs. Table I is a compilation of five experiments demonstrating the specificity of MoAbs HB57, 5D7, VIIIE11, Mu53, 1G6, 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 MoAbs are 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 non-myeloma Ig. Similar results obtained with seven other IgM, five additional IgG, and two more IgA myeloma proteins, not illustrated, confirmed the specificity assignment of the MoAbs for human IgM.

Selective Binding of Anti-IgM MoAbs to Epitopes Expressed on IgM-bearing B Cells. The fluorescence distribution histograms shown in Fig. 1 demonstrate that the anti-IgM MoAbs bound to determinants exposed on the surface of a B cell lymphoblastoid line expressing mIgM. The MoAbs do 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 MoAbs were tested on three additional leukemias that express mIgM (data not shown). The histograms illustrated were obtained using 1000 μg/ml of MoAb; similar results were also obtained when 10 μg/ml of each MoAb was used for staining (data not shown).

Recognition of Distiinct Epitopes on Human IgM by Anti-IgM MoAbs. A cross-inhibition assay was performed to evaluate which anti-μ MoAbs are directed to distinct determinants on human IgM. MoAbs which 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) MoAb 1G6 bound to a unique epitope on human IgM as shown by the fact that no other MoAb was capable of competing with it in a reciprocal fashion. (b) MoAbs 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 MoAbs since no other MoAb competed reciprocally. (c) MoAb 5D7

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 (97) 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.

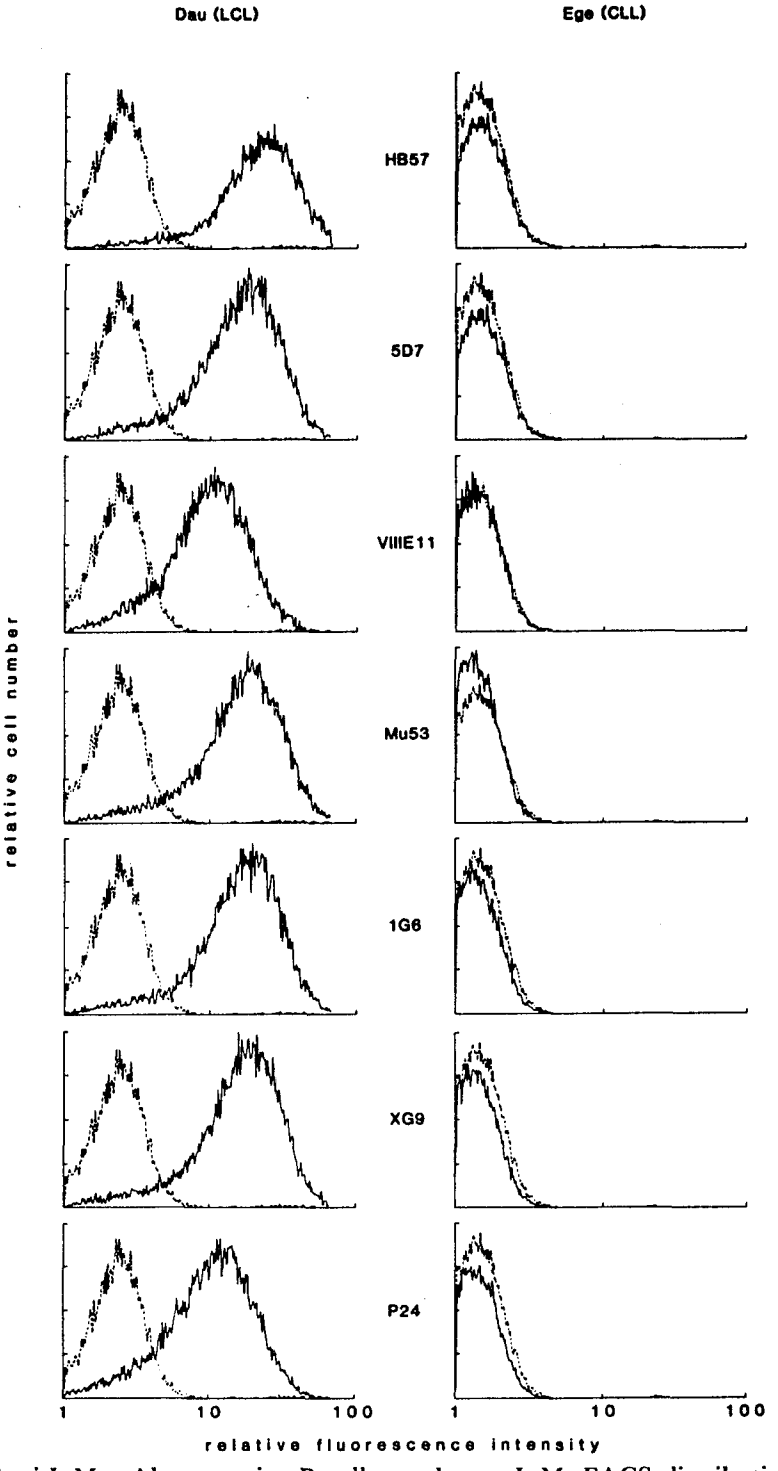

FIGURE 1. Anti-IgM mAb recognize B cell membrane IgM. FACS distribution histograms with logarithmic amplification for both an mIgM-positive lymphoblastoid cell line (Dau) and an mIgM-negative chronic lymphocytic leukemia (Ege) are shown. Cells were incubated with 1,000 μg/ml of ultracentrifuged mAb followed 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), VIIIE11 (9,463), Mu53 (8,253), 1G6 (9,611), XG9 (9,058), and P24 (867).

presumably binds to a unique epitope since it competed only minimally with HB57 and was inhibited only by IG6, in a non-reciprocal fashion. (d) MoAbs HB57 and Mu53 could have specificity for the same or very neighboring site, as they are able to reciprocally compete at low concentrations. However, unlike MoAb Mu53, the binding of HB57 to ^{3}H-IgM could be inhibited to some degree by all the MoAbs. (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 MoAbs.

Results obtained from inhibition assays in which P24 MoAb 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 MoAb for IgM or a diminution in its binding capacity when the MoAb is immobilized onto PVC wells. It should be noted that soluble P24 MoAb was very effective at binding to IgM-coated wells at MoAb concentrations as low as 2.5 μg/ml (data not shown).

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

The concentration response profiles of MoAbs Mu53 and VIIIE11 were similar to one another but distinct from those of HB57 and 5D7. A much greater concentration (>100 μg/ml) of these MoAbs was needed to induce a substantial degree of ^{3}H-thymidine uptake. Furthermore, the maximal level of stimulation obtained was always much lower with HB57 or 5D7 MoAbs. Concentrations of Mu53 and VIIIE11 MoAbs ≤1000 μg/ml did not produce a definable plateau in stimulation.

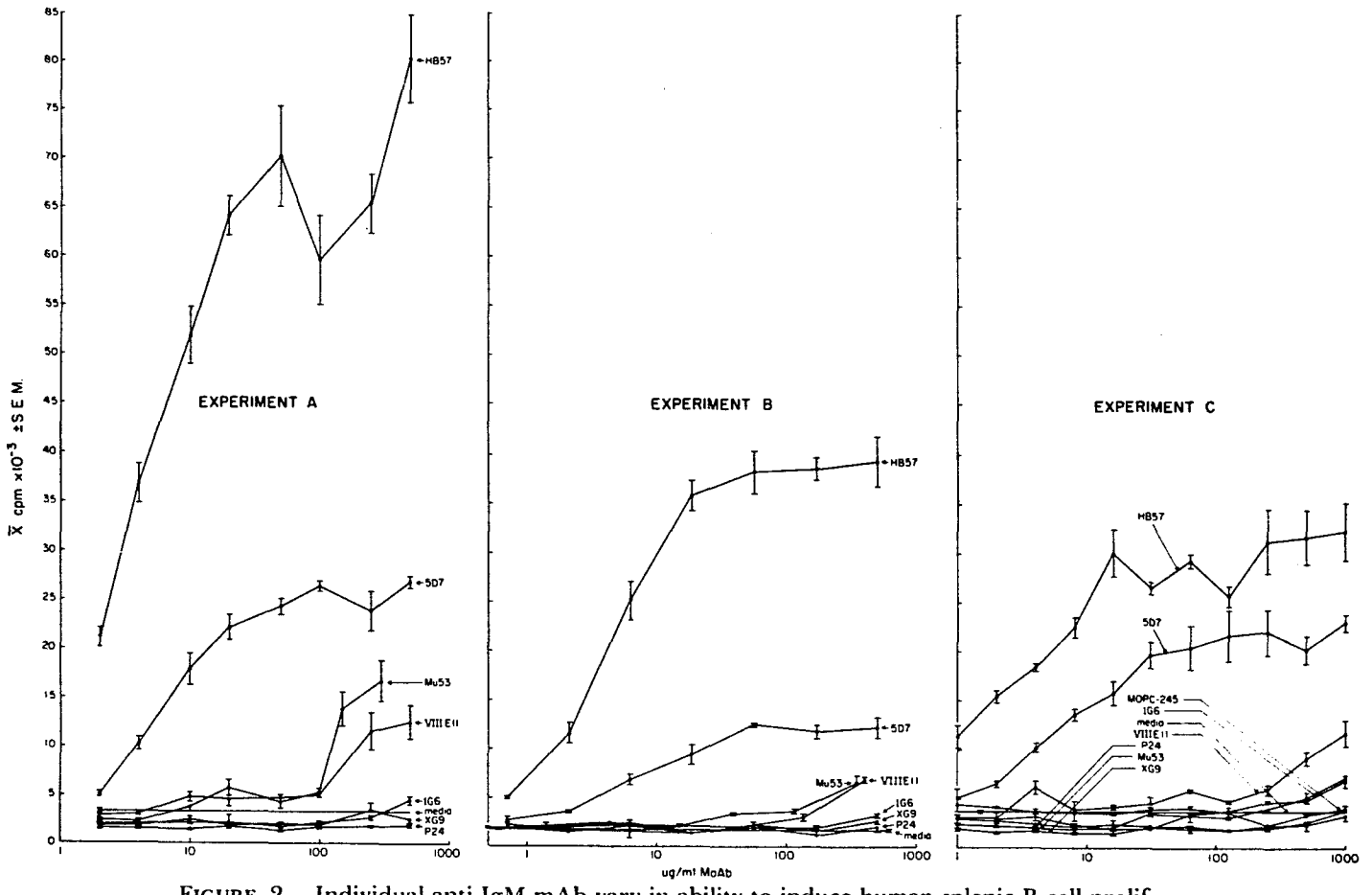

FIGURE 2. Individual anti-IgM mAb vary in ability to induce human splenic B cell proliferation. T cell–depleted human splenocytes from two separate donors (experiments A and B, donor 1; experiment C, donor 2) were cultured with various concentrations of purified anti-μ mAb in the presence of 30% T cell 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 i ıduced an inhibition of background proliferation, indicated by the solid horizontal line.

In contrast, MoAbs 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. MoAbs 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 ≤1000 μg/ml.

When experiments were performed using ultracentrifuged MoAb 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 MoAbs 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 MoAb-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 MoAbs. Fig. 3 illustrates that a 1:1 mixture of the two individually non-stimulatory MoAbs 1G6 and XG9 resulted in a marked enhancement of B cell activation. While the individual MoAbs did not induce >1,209 ± 462 cpm of ^{3}H-thymidine incorporation (Δcpm ± SE diff) at any dose tested, the combined MoAbs (250 μg/ml of each) induced 74,391 ± 4,399 cpm. This level of stimulation was comparable to that elicited by the most stimulatory individual MoAb, HB57. However, while maximal stimulation induced by MoAb HB57 was achieved at 20 μg/ml, maximal stimulation induced by the 1:1 1G6 - XG9 MoAb mixture required >500 μg/ml of total Ab.

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

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.

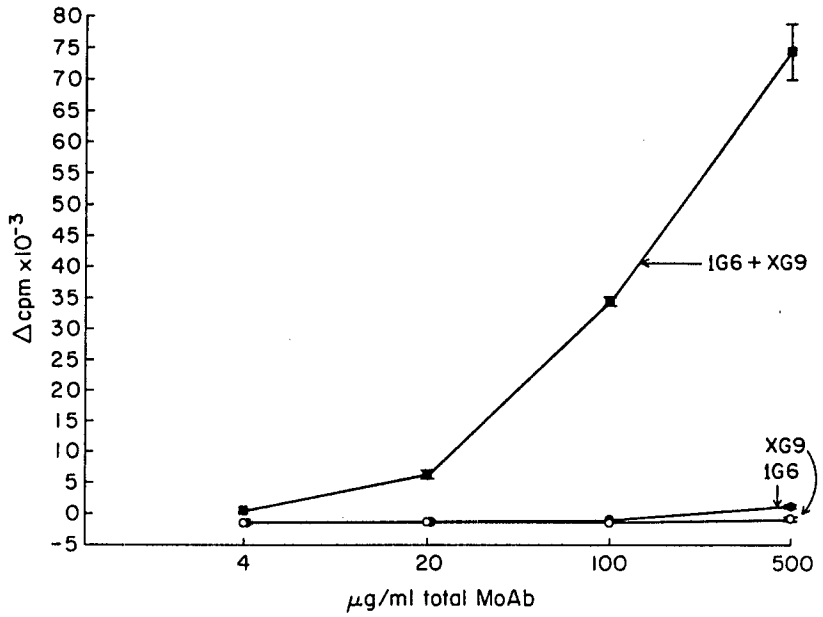

FIGURE 3. Synergy in B cell proliferation when anti-IgM mAb 1G6 and XG9 were cocultured. T cell–depleted splenic lymphocytes were cultured with 1G6 mAb (●) or XG9 mAb (○) or 1:1 mixtures of the two (■) at various mAb concentrations, in the presence of 30% T cell supernatant. The mAb concentrations indicated 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.

The ability of 1G6 and XG9 MoAbs to induce synergy in B cell stimulation is illustrated in Fig. 4, which shows that: (a) MoAb 1G6 markedly enhanced the proliferation induced by MoAbs XG9, 5D7, and VIIIE11 but induced a minimal degree of enhancement with HB57 and Mu53 and none at all with P24. (b) MoAb XG9 exhibited synergy with 1G6, 5D7, and Mu53, but did not manifest this phenomenon with HB57, VIIIE11, or P24. (c) When cultured with MoAb Mu53, 1G6 and XG9 induced equivalent levels of ^{3}H-thymidine incorporation. In all other cases, the combination of a given MoAb with 1G6 always induced a greater degree of proliferation than the combination of the given MoAb with XG9. (d) The inability of XG9 to induce synergy when co-cultured with VIIIE11 is consistent with their apparent recognition of the same epitope on the IgM molecule (see Table II). However, these two MoAbs consistently induced different levels of proliferation when cultured with other anti-μ MoAbs. For example, the co-culture 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 MoAbs 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 MoAb P24. The proliferation obtained when P24 was co-cultured with individually-mitogenic anti-IgM MoAbs was not any lower than that obtained when these latter MoAbs were used singly (data not shown).

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

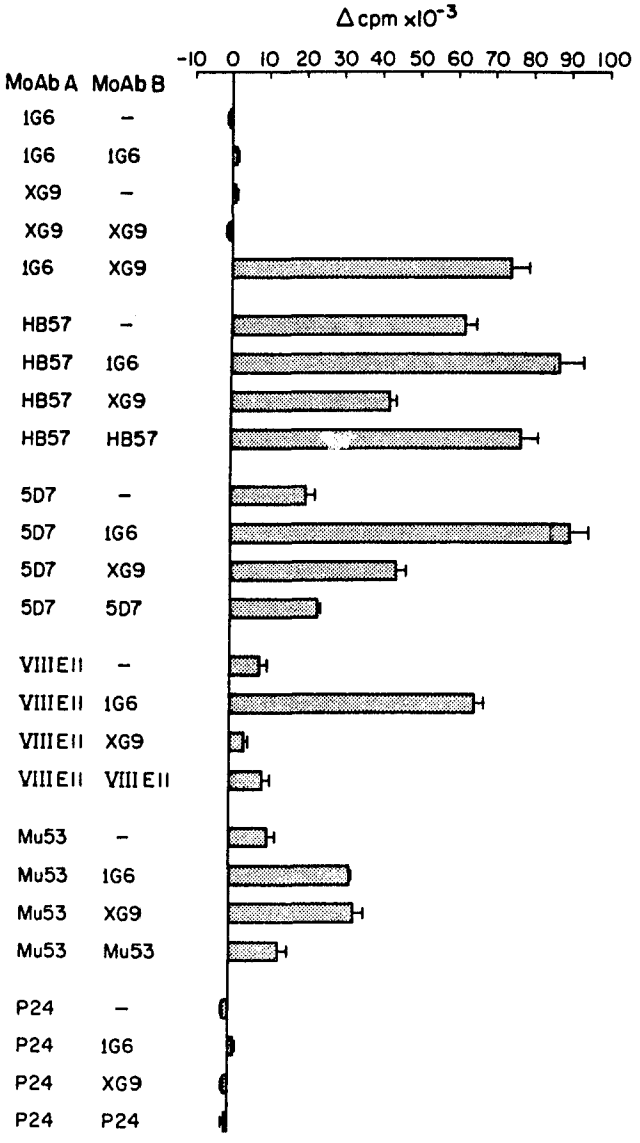

FIGURE 4. Synergy in B cell stimulation when anti-IgM mAb 1G6 or XG9 are cultured with other anti-IgM mAb. Human splenic B cells in medium containing 30% T cell supernatant were cultured with 250 μg/ml of mAb A and/or 250 μg/ml of mAb B. The data are expressed as Δcpm ± SE diff. Background stimulation 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.

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).

Comparison of the Effect of T Cell Supernatant Upon Proliferation Induced by Individual Anti-IgM MoAbs or MoAb Mixtures. In the absence of T cell supernatant, the B cell proliferation induced by MoAb HB57 was markedly reduced. Nevertheless, a Δcpm of 2,597 ± 269 was obtained with as little as 4 μg/ml of MoAb (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 MoAbs was also substantially reduced in the absence of T 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 MoAbs.

Fig. 5 also illustrates that, in contrast to individual MoAbs, certain mixtures of anti-IgM MoAbs 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 MoAbs is evaluated in Table V. The data demonstrated that: (a) the proliferation induced by each individual anti-IgM MoAb 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 MoAbs (HB57 or 5D7) showed no significant difference in T cell supernatant dependence at high of low MoAb concentrations. Even when MoAb 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 MoAb were used. (c) In contrast, cultures stimulated with MoAb mixtures containing 1G6 generally exhibited a high degree of T cell supernatant independence (i.e., low index values), manifested at high MoAb doses but diminishing at low doses. (d) Cultures stimulated by MoAb mixtures without 1G6 showed a much less pronounced, in many cases negligible, difference in T cell supernatant dependence at high and low MoAb concentrations.

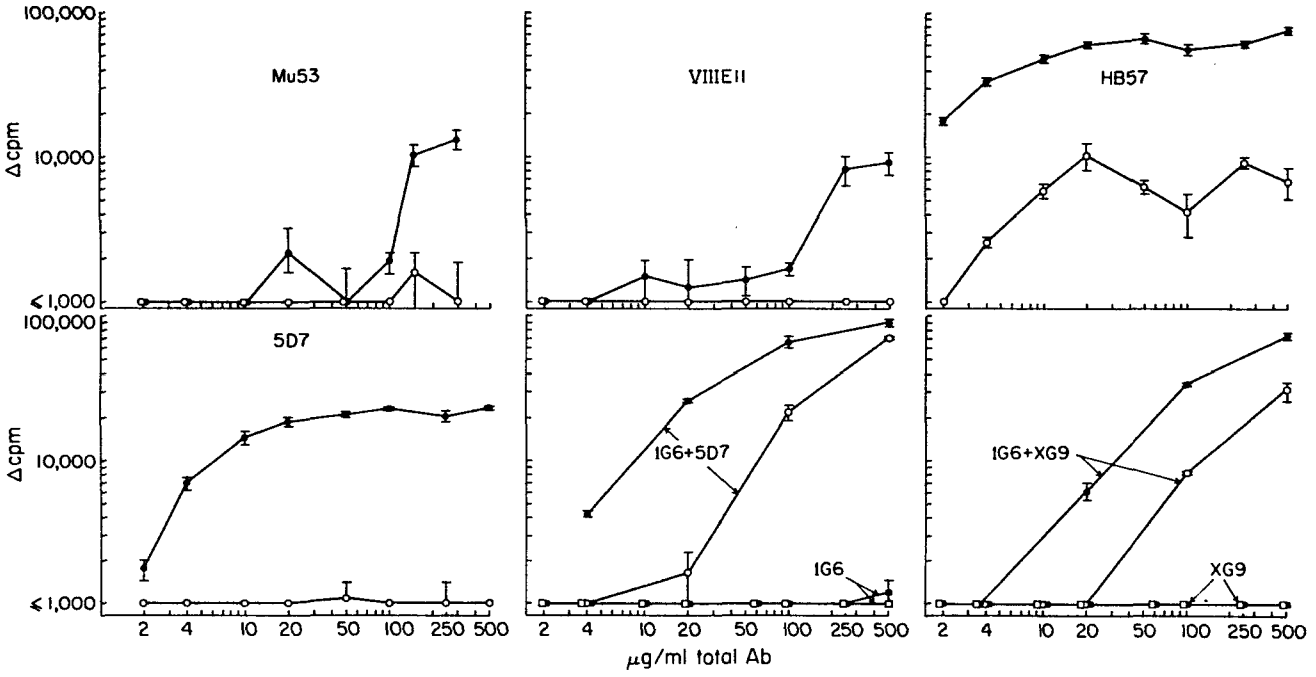

FIGURE 5. Comparison of B cell proliferation induced by single anti-IgM mAb or mAb mixtures in the presence and absence of T cell supernatant. T cell–depleted splenic lymphocytes were cultured with the indicated concentrations of individual anti-IgM mAb or 1:1 mAb mixtures in the presence (closed symbols) 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.

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.

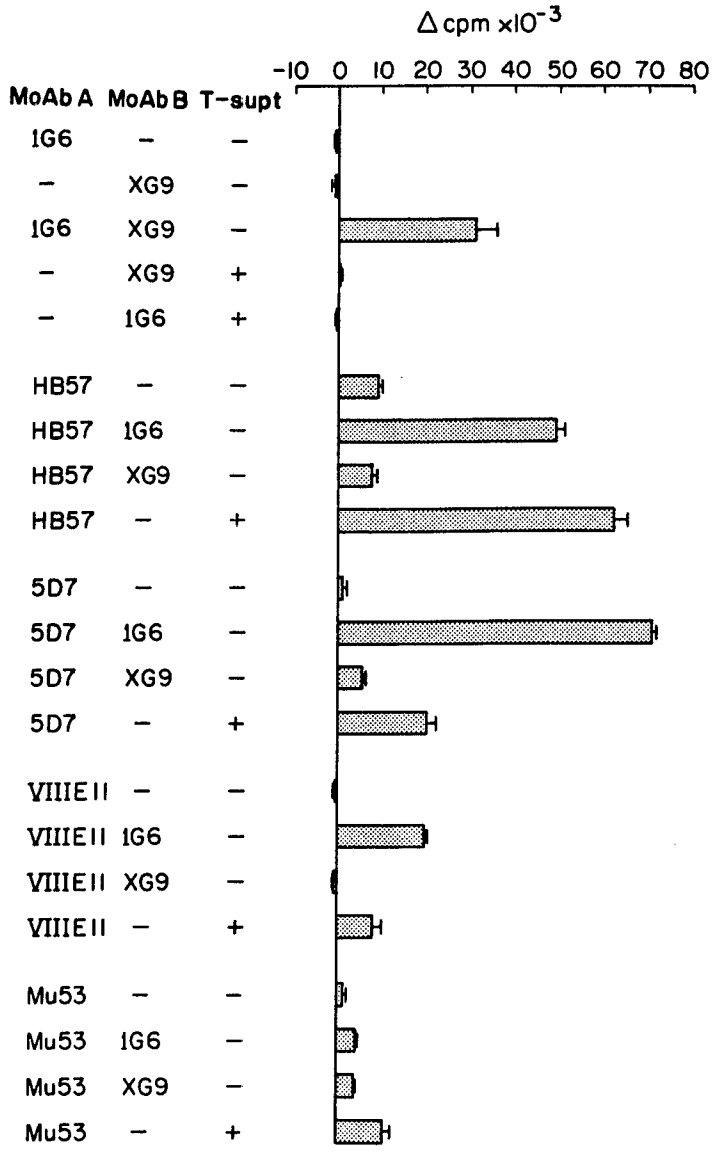

FIGURE 6. Comparison of T cell supernatant-replacing ability of mAbs 1G6 and XG9. Cultures of T cell-depleted splenic lymphocytes containing 250 μg/ml of mAb A were supplemented with 250 μg/ml of mAb B (1G6 or XG9) or 30% T cell supernatant (*T-supt*). The data are expressed as Δcpm ± SE diff of triplicate 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.

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

We considered the possibility that DEAE-purified 1G6 was contaminated with 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 MoAbs 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 MoAbs induced significant proliferation above background with concentrations as low as 2 μg/ml. MoAbs VIIIE11 and Mu53, in contrast, induced a lesser degree of proliferation and only at high concentrations. Finally, anti-IgM MoAbs 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 (73,78,80,86,103), bear importantly on past studies (1,6,30,49,63-72,75-77,84,85,87) of the effect of polyclonal anti-IgM Abs on B cells. The Abs in anti-Ig sera are a mixture of distinct species that are likely drawn from the different prototype MoAbs described above. As a result, the degree of proliferation elicited and dose required for optimal stimulation by polyclonal Abs will to some degree reflect the nature of the individual Abs comprising such polyclonal preparations.

How is it that different MoAbs, 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 MoAbs to induce proliferation of B lymphocytes. The avidity of an MoAb 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 MoAbs to mouse IgM and their ability to induce differentiation of a murine B cell line (79).

The ability to induce B cell proliferation could also be influenced by the epitope specificity of the MoAb. This association could reflect the mechanism by which B cells receive stimulatory signals. There are at least two explanations of how anti-Ig Abs trigger B cell proliferation, which are not necessarily mutually exclusive. Binding to certain epitopes may cause a conformational change in the mIgM molecule which aids in the initiation of intracellular signals required for proliferation. Alternatively, signals may arise from the aggregation of mIgM molecules (104). With regard to this latter mechanism, it is likely that an MoAb which recognizes an epitope multiply - expressed on a mIgM molecule will be more efficient at cross-linking mIgM molecules than an MoAb that recognizes a singly expressed epitope.

Many of the anti-IgM MoAbs 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. (103), as well as Zitron and Clevinger (80), have reported that two MoAb 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 (78) indicate that two soluble rat anti-murine IgM MoAbs which share domain specificity and isotype with the mitogenic MoAb described by Julius, et al. (103) 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 Abs (64,73,75,76). However, it is unlikely that an Fc region-mediated mechanism is responsible for all differences in the mitogenic character of the individual anti-μ MoAbs in this study, since five of those that displayed functional differences were of the same $\gamma 1$ isotype. The possibility cannot be excluded that the difference in stimulatory capacity between MoAbs 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 non-stimulatory MoAbs 1G6 and MoAbs XG9 give a very strong mitogenic response when incubated together. Moreover, synergy was observed when either MoAbs 1G6 or XG9 was co-cultured with all but two of the anti-IgM MoAbs. This was apparent not only at high concentrations (250 µg/ml) of each MoAb, 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 MoAb 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 MoAb 1G6 induced synergy in B cell proliferation when combined with other anti-IgM MoAbs but not when combined with MoAbs to other surface molecules, such as anti-Ia or anti-δ. However, since a limited number of anti-Ia and anti-δ MoAbs were tested, the possibility remains that others might display synergy with MoAb 1G6.

At present, there is no satisfactory interpretation for the absence of synergy between MoAbs 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 MoAbs 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 co-cultures of anti-µ MoAbs with MoAbs 1G6 (or XG9) can be considered: (a) cyclic complexes may form between MoAb 1G6, mIgM and other anti-µ MoAbs, which lead to a greater overall avidity of the ligands for mIgM. Ehrlich et al. (104) have recently described cyclic complexes of MoAbs that display a higher affinity for antigen than the individual MoAbs. In addition, Parham (105) has shown that the binding of certain MoAbs to a MHC molecule induces conformational changes that affect the affinity with which other MoAbs bind to alternative epitopes on the molecule. (b) The ability of MoAb 1G6

to induce synergy may simply reflect its unique ability to collaborate with other MoAbs in inducing efficient cross-linkage of mIgM molecules and consequent stimulatory micro-patch formation (50) on the B cell membrane. It is unlikely that this mechanism is solely responsible for the observed synergy since other pairs of MoAbs which bind to unique epitopes and thus should be capable of increasing cross-linkage (e.g. MoAbs 5D7 and VIIIE11 or MoAbs Mu53 and VIIIE11) do not display synergy when combined. (c) The exceptional ability of 1G6 MoAb 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 Abs from polyclonal antisera (44,106) 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 MoAbs indicate that, irrespective of the dose of Ab, the proliferation induced by each individual MoAb is significantly enhanced by the presence of a T cell factor source.

Of particular interest in this study is our finding that the co-cultivation of MoAb 1G6 with other anti-IgM MoAbs circumvents the need for a second signal to be delivered to B cells by T cell supernatant. We did not examine whether these MoAb 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 MoAb 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 (107), it is also conceivable that the signals delivered by MoAb 1G6, in combination with another anti-IgM MoAb, 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 MoAb 1G6 remain to be investigated.

SUMMARY

Seven murine monoclonal antibodies (MoAbs) with different binding characteristics for human IgM varied markedly in their ability to induce proliferation of T cell-depleted human splenocytes. Two MoAbs (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 MoAbs produced a markedly reduced degree of stimulation at all concentrations. Two additional anti-IgM MoAbs (VIIIE11 and Mu53) were distinctive in that, even at high concentrations, only limited proliferation was observed compared with the first group of MoAbs. This proliferation depended on the presence of T cell supernatant. Competitive-binding studies revealed that the epitope recognized by MoAb Mu53 may be identical or very proximate to that recognized by HB57. Three other MoAbs (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 MoAb. Regulatory influences of Fc receptor binding cannot account for all the diversity in proliferation observed with the individual anti-IgM MoAbs.

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

CHAPTER THREE

Analysis of the Domain Specificity of Various Murine Anti-Human IgM Monoclonal Antibodies Differing in Human B Lymphocyte Signaling Activity*

INTRODUCTION

For some years polyclonal antibodies that bind to membrane immunoglobulin on B cells have been recognized as transducers of signals that modulate both B lymphocyte expansion (6,63,108) and Ig secretion (85). Recently, work in this (109), as well as other laboratories (78,80,81,110) has demonstrated that considerable heterogeneity exists in the capacity of individual anti-immunoglobulin monoclonal antibodies to transduce signals for both the induction of DNA synthesis in resting B lymphocytes (103) as well as for the inhibition of DNA synthesis in certain activated B cell populations (111). Because anti-IgM antibodies of the same murine isotype exhibited different functions, the basis for this heterogeneity was shown not to be related to Fc region differences in the MoAb (109). Accordingly, characteristics of the variable regions of the anti-Ig MoAbs were analyzed to determine the nature of the signal delivered to the B lymphocyte.

As part of an effort to more precisely understand the parameters involved in the surface-IgM - ligand interaction that might be responsible for divergent MoAb B cell signaling capabilites, we have localized the site of binding of these MoAbs to particular domains on the IgM molecule. In addition, through the use of MoAb cross-inhibition radioimmunoassays, the question of whether Abs that bind to the same domain are directed to the same or different epitopes was

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* Reprinted with permission from Molecular Immunology, vol. 24, Steven M. Rudich, Edith Mihaesco, Robert Winchester and Patricia K. A. Mongini, Analysis of the Domain Specificity of Various Murine Anti-Human IgM Monoclonal Antibodies Differing in Human B Lymphocyte Signaling Activity,

examined. Information on MoAb site specificity, combined with a critical appraisal of anti-IgM MoAb affinity and stoichiometry to B cell membrane IgM (Rudich and Mongini, in preparation), may give us better insights into the nature of the binding events required for signaling by anti-IgM MoAbs.

MATERIALS AND METHODS

Human IgM proteins. Human IgM myeloma macroglobulin "Pan" was of kappa light chain type and did not possess rheumatoid factor activity. This euglobulin was purified by dialysis against cold distilled water and passage over a 90 x 2.6 cm Sephacryl S-300 gel filtration column (Pharmacia) in PBS-A (phosphate-buffered saline + 0.02% azide). IgM "Pan", from the middle portion of the excluded peak, was used for all enzymatic digestions and was also the probe used in either tritiated or iodinated form for radioimmunoassays (RIAs).

Mu heavy chain disease (μHCD) proteins BOT and DAG, described previously (112,113), were also used in some experiments. They are mutant proteins that completely lack V_H and $C\mu_1$ heavy chain domains as well as light chains. However, they are pentameric and do possess normal IgM $C\mu_2$, $C\mu_3$, and $C\mu_4$ domains.

Preparation of enzymatic fragments from Human IgM "Pan".

Fc5 μ. IgM was treated with TPCK-treated trypsin (Sigma Chemical Co., St. Louis, MO, No. T-8642) in a 20-to-1 substrate-to-enzyme (S:E) weight ratio at 65°C for 10 min, following the method of Plaut and Tomasi (114). Digestion was terminated by the addition of soybean trypsin inhibitor (Sigma, No. T-9003). After cooling, the digestion mixture was placed over a calibrated Sephacryl S-300 gel filtration column (90 x 2.6 cm) equilibrated with 0.1 M Tris-HCl, 0.15 M NaCl, pH 7.3 (TBS). $Fc_5\mu$ eluted in the second of five peaks.

Cμ4. Non-reducing 5-20% gradient SDS-PAGE analysis of the protein in the first peak from the 65°C trypsin digest showed only two bands - one at approximately 380 kiloDaltons (kDa) and another at 14 kDa. Upon reduction, the smaller band remained intact. Because of its size, the fact that it was non-reducible, and the reported tendency of enzymatically cleaved $C\mu_4$ to aggregate with other IgM moieties (115), we considered that this smaller fragment may have been $C\mu_4$.

To separate the apparent $C\mu_4$ fragment from the larger molecular weight (mol. wt) moieties, the protein in peak 1 was vacuum concentrated and then dialyzed at 4°C against 5 M guanidine hydrochloride (GuHCl). The protein was then applied to a 64 x 1.3 cm column of AcA-54 equilibrated with 5 M GuHCl. The second peak obtained, containing the low mol. wt $C\mu_4$ domain, exhibited a single band at 14 kDa and was consequently dialyzed against PBS-A.

Cμ3. 5-20% gradient SDS-PAGE analysis of the fourth peak from the 65°C trypsin digest showed two bands - one at approx. 63 kDa, corresponding to contaminating Fabμ from the neighboring higher mol. wt peak, and another, much more prominent non-reducible band of 20 kDa, which was considered to be possibly $C\mu_3$ (116). To isolate this low mol. wt fragment, the protein in this fourth peak was vacuum concentrated and applied to an AcA-54 gel filtration column (93 x 1.3 cm) equilibrated in PBS-A. The elution profile showed an initial small peak of Fabμ, followed by a large second peak of $C\mu_3$ and subsequent peaks of lower mol. wt peptides.

F(ab')2 μ. IgM was treated with TPCK-treated trypsin in a 100-to-1 S:E weight ratio at 37°C following the procedure of Miller and Metzger (117). The digestion was terminated after 24 hrs by the addition of soybean trypsin inhibitor. After cooling, the mixture was applied to a 146 x 3.1 cm column of Sephacryl S-200 equilibrated in PBS-A. Three peaks were obtained, the second of which contained the $F(ab')_2\mu$ fragment.

Cμ2. The cold pepsin cleavage method of Lin and Putnam (118) was followed to produce the $C\mu_2$ fragment. Upon gel filtration through Sephacryl S-200 (146 x 3.1 cm), two well-defined peaks were obtained - the first of which contained $C\mu_2$.

Preparation of reduced products from IgM "Pan".

H-L. Half molecules of IgM monomer (H-L) were produced as essentially detailed by Vidal and Conde for the production of IgM "subunits" (119). IgM was made 0.05 M with l-cysteine (Sigma, No. C-7755) and rotated for 8 min at room temperature. After cooling the mixture on ice, a 10% molar excess of iodoacetamide (Sigma, No. I-6125) was added. After an additional 1 hr incubation at 4°C, the mixture was run over a Sephacryl S-300 gel filtration column (90 x 2.6 cm). The single peak obtained was analyzed by non-reducing SDS-PAGE which revealed that

approximately 80% of the protein was in the form of H-L (mol. wt 96 kDa). The remaining components were divided almost equally into μ and light chains (mol. wts 78 and 25.5 kDa, respectively), and IgM monomer (mol. wt 210 kDa).

Other IgM fragments.

μ chain. Mu chain was purchased from Behring Diagnostics (Calbiochem-Behring Corp., La Jolla, CA, cat. no. 401850); it gave a single band of approximately 78 kDa on both non-reduced and reduced SDS-PAGE.

Tryptic cleavage peptide T27 (Gln272 - Lys287). This fragment was produced from trypsin cleavage of μHCD protein BOT as detailed previously (120) and is part of the amino acid sequence inside the disulphide bond loop of the IgM $C\mu_2$ domain (see Fig. 7B).

CNBr cleavage fragments H5 (Phe313 -Met331), H6 (Cys332 -Met484), H7 (Gln485 - Met501), H8 (Pro502 -Met563). These fragments were produced from CNBr cleavage of the $Fc_5\mu$ fragment of BOT as described by Barnikol-Watanabe et al. (121). Together, they constitute the COOH-terminal IgM segment comprising the last 22 residues of $C\mu_2$, continued with the entire $C\mu_3$ and $C\mu_4$ domains, excluding the COOH-terminal octapeptide of the μ chain. The numbering scheme for the CNBr cleavage fragments as well as for peptide T27 is based upon IgM macroglobulin Gal (122).

Radiolabeling of IgM Pan.

Preparation of 3H-IgM. IgM was tritiated with ^{3}H-sodium borohydride (New England Nuclear) by the reductive methylation procedure (123). The specific activity of the preparation used was 667,000 cpm/μg.

Preparation of 125I-IgM. IgM was iodinated with carrier-free ^{125}Iodine (New England Nuclear) using the chloramine-T method (124). Typical specific activities of ^{125}I-IgM prepared were greater than 1.5×10^6 cpm/μg.

Radioimmunoassays.

IgM fragment inhibition radioimmunoassay. Polyvinyl chloride (PVC) wells were coated with 25 μl of a 25 μg/ml solution of anti-IgM MoAb diluted in PBS-A for 1 1/2 to 2 hrs at 37°C. After

washing with PBS, non-specific binding sites were blocked by a 1/2 hr incubation with 125 µl of 1%BSA-PBS-A. Upon an additional PBS wash, 25 µl of the indicated inhibitors, at concentrations usually of 0.15, 1.5, 15, 150, and 300 µg/ml in 1%BSA-PBS-A, were added to the appropriate wells for 2-2 1/2 hrs. Finally, 25 µl of ^{125}I-IgM (70-90,000 cpm) was added to each well for 1 1/2 hrs. Plates were washed and individual wells evaluated for bound ^{125}I-IgM by use of a gamma counter. Inhibition curves were plotted by determining the percent inhibition obtained when various concentrations of IgM or IgM fragment were used as an inhibitor of ^{125}I-IgM binding to the well-bound anti-IgM MoAb. Percent inhibition values were calculated as follows: 100 x [1- (cpm ^{125}I-IgM bound in the presence of inhibitor ÷ cpm ^{125}I-IgM bound in the absence of inhibitor)]. The picomole (pmole) amount of inhibitor necessary to diminish the binding of ^{125}I-IgM to immobilized anti-IgM MoAb by 50% was used as a means of evaluating the presence of epitopes on different IgM fragments.

Cross-inhibition radioimmunoassay. The ability of various soluble anti-IgM MoAbs to inhibit the binding of ^{3}H-IgM to PVC well-bound anti-IgM MoAb was evaluated by methods previously described (109). The only modification consisted of a change in the MoAb coating concentration from 10 µg/ml to 25 µg/ml. Inhibition curves were determined in a manner analogous to that used in the IgM fragment inhibition RIA.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to the method of Laemmli (94). Samples run under reducing conditions contained 5% 2-mercaptoethanol in the sample buffer. Mol. wt determination was by comparison to standard curves prepared from the electrophoresis on the same gel of proteins of known mol. wt (Sigma, Nos. MW-SDS-7 and MW-SDS-6H). Mol. wts could not be determined for intact IgM as well as for µHCD proteins BOT and DAG under non-reducing conditions. All proteins were analyzed with either 5-15% or 5-20% gradient acrylamide gels.

Preparation, characterization, and purification of murine monoclonal anti-human IgM antibodies (anti-IgM MoAbs). The preparation, isolation, and purification of seven of the nineteen

murine anti-human IgM MoAbs used in this study, i.e. XG9, VIIIE11, HB57, Mu53, P24, 5D7, and 1G6 has been previously described (109).

Generation of additional murine anti-human IgM MoAbs described in this report. MoAbs VIIIF10, IIF5, 2B11, VIIIB5, IX11, XG4, and IF11 were obtained in the same two fusions in which MoAbs XG9, VIIIE11, and 1G6 were generated (109). MoAb B∝1 was obtained in a separate fusion using a different immunogen - a μk macroglobulin having rheumatoid factor activity. MoAbs Mu18 and P19 were derived from the same fusions as MoAbs Mu53 and P24, respectively (109). Lastly, anti-IgM MoAbs 4-3 and 196.6b were derived in a distinct fusion which used the same immunogen as that for the generation of MoAbs P19 and P24. MoAbs Mu18, P19, 4-3, and 196.6b as well as MoAbs Mu53 and P24 (109) were kindly donated by Drs. David Posnett and Henry Kunkel. All MoAbs were cloned two times at limiting dilution (percent positive wells between 2-7%) before further characterization. The MoAbs described will be made available to interested investigators.

Characterization and purification. The above newly described anti-IgM MoAbs were characterized for unique specificity to IgMk and IgM by solid phase RIA as well as by immunofluorescence on membrane IgM-positive B cells using techniques previously described (109). Purification of these MoAbs (all of mouse gamma 1 isotype) was achieved by affinity chromatography over protein-A Sepharose (Pharmacia). γ1 Ab was specifically eluted with 0.1 M citrate buffer, pH 6.0 (92). Alternatively, some of these Abs were purified by the use of a commercial protein-A Sepharose gel specially optimized for the purification of murine γ1 Abs (MAPS, Bio-Rad, Richmond, VA). Analysis by immunoelectrophoresis with antiserum against murine serum constituents as well as SDS-PAGE indicated that the level of purity of each MoAb preparation exceeded 90%. In addition, all but four (Mu18, IIF5, 2B11, and VIIIB5) of the nineteen purified anti-IgM MoAbs were evaluated for purity by measurement of their ability to bind to affinity columns of IgM-Sepharose. With the exception of MoAbs IX11, XG4, P19, P24 and IF11, greater than 95% of the protein applied to the IgM-Sepharose column remained bound, even after extensive PBS washing. In the case of the exceptional five MoAbs, from 60-75% of protein applied in a first

passage through the column bound. The IgM-binding potential of the non-bound protein was found to be identical to that of the original MoAb preparation. In addition, upon re-passage over a fresh IgM-Sepharose column, the non-bound protein exhibited a similar percentage of binding. We consider this low efficiency binding to be indicative of the lower affinity nature of these MoAbs.

N-terminal amino acid sequence determinations. Micro-Edman degradation was performed according to the method of Chang et al. (125) using DABITC-phenylisothiocyanate double coupling followed by thin layer chromatographic identification of the amino acid derivatives liberated.

Hydrophilicity analysis of μHCD protein BOT. The method of Hopp and Woods (126) was used to analyze the hydrophilicity of mu heavy chain disease protein BOT.

RESULTS

Human IgM fragments. Fig. 7A illustrates the IgM moieties and fragments prepared and used in this study. Molecular weights of these fragments, determined by SDS-PAGE (Fig. 8), generally agree with previously published values (116,117,127-129). In nearly all cases the fragment preparations are free of major contaminants. The second major non-light chain band seen in the $F(ab')_2\mu$ preparation under reducing conditions (Fig. 8B, lane f, approx. 36 kDa) likely reflects cleavage of the μ heavy chain at a trypsin site more N-terminal to the main site which generates the 45 kDa Fd'μ band. This additional band remained upon affinity purification of $F(ab')_2\mu$ over a monoclonal anti-human kappa light chain-Sepharose column (data not shown).

N-terminal amino acid sequence analysis was performed to confirm the identity of certain fragments. Analysis of the $C\mu_2$ fragment revealed a N-terminal sequence of Val-Ile-Ala-Glu-etc (Val_{223} - Val_{235}) which corresponds exactly to the first 13 residues of the $C\mu_2$ fragment (Gal numbering, [122]). Analysis of the amino-terminal 10 residues of the $C\mu_3$ preparation revealed two distinct sequences: one beginning at Gly_{321} (corresponding to $C\mu_3$a of Bubb and Conradie [116]) and a second, smaller fragment which had a sequence that began at Asp_{337}, very near to

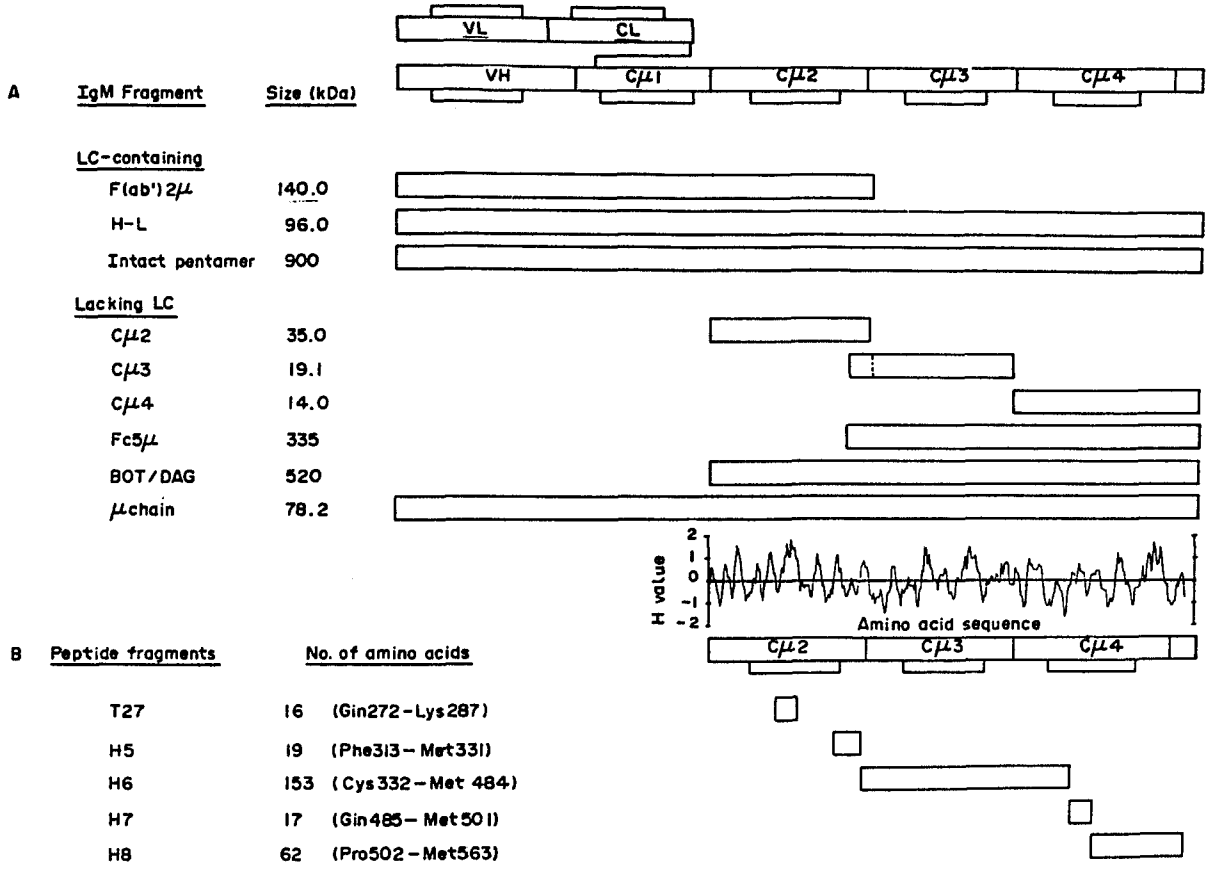

Fig. 7. (A) IgM enzymatic fragments used in this study, their size (in kDa, as determined from non-reducing SDS-PAGE) and their approximate location on the IgM molecule. The dotted line in the $C\mu_3$ fragment indicates the additional amino-terminal sequence found in this domain preparation. (B) Sequenced peptides from BOT μHCD protein. The number of amino acids comprising each peptide, as well as the first and last amino acid for each, are indicated. Also shown is a hydrophilicity profile of the parent protein BOT, computed using the method of Hopp and Woods (126).

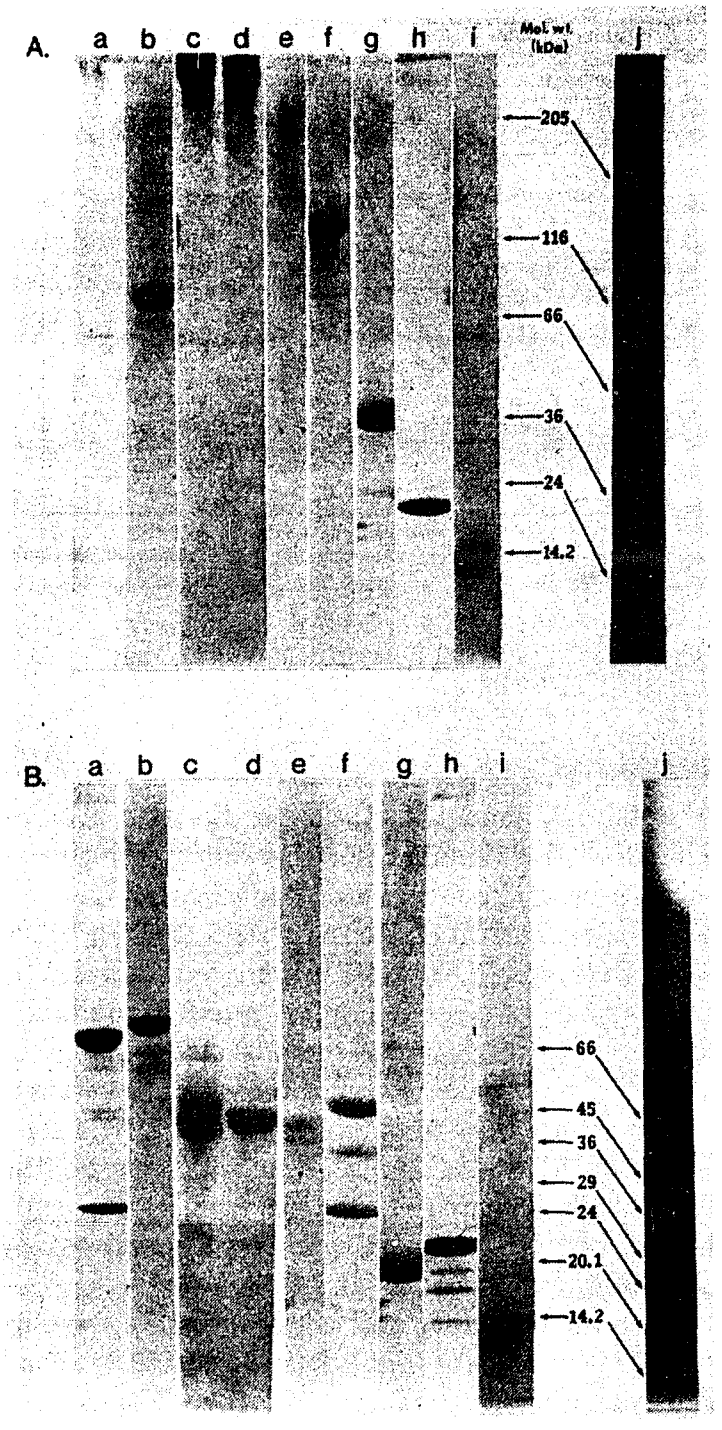

Fig. 8. SDS-PAGE analysis of IgM fragments under non-reducing (A) and reducing (B) conditions. Lane a: intact IgM, b: μ chain, c: BOT μHCD, d: DAG μHCD, e: $Fc_5\mu$, f: $F(ab')_2\mu$, g: $C\mu_2$, h: $C\mu_3$, i: $C\mu_4$, j: H-L. Results in lanes a–i were compiled from three separate 5–20% gradient gels, lane j was obtained from a 5–15% gradient gel. No band is seen in lane a of (A) since intact IgM is completely retained in the gel comb.

the true beginning of the $C\mu_3$ domain as determined by nucleotide sequence homology (130). Because of limitations in the amount of material available, only the N-terminal amino acid of the $C\mu_4$ domain was determined. This amino acid, Val, is consistent with the N-terminal amino acid Val_{442} of $C\mu_4$ (128).

Fig. 7B shows several additional fully sequenced IgM peptide fragments used in this work. These include the BOT μHCD protein tryptic cleavage product T27 as well as BOT CNBr cleavage fragments H5, H6, H7, and H8. The hydrophilicity profile of IgM constant region domains $C\mu_2$, $C\mu_3$, and $C\mu_4$ from BOT is also shown as an indicator of the degree of external exposure and hence predicted relative antigenicity of the amino acid sequences from which these fragments derive (126).

Analysis of Fc5 μ- and F(ab')2 μ-binding specifcity of anti-IgM MoAbs. Nineteen anti-IgM MoAbs were evaluated by inhibition RIA for binding to $Fc_5\mu$ and $F(ab')_2\mu$, two major enzymatic fragments of IgM which together encompass the entire sequence of the human IgM molecule (see Fig. 7A). This analysis revealed that while intact IgM can inhibit labeled IgM binding to all 19 MoAbs (Fig. 9A), $Fc_5\mu$ and $F(ab')_2\mu$ exhibit reciprocal patterns of inhibition with these MoAbs (Fig. 9B and 9C). Thus, the $Fc_5\mu$-binding MoAbs, 4-3, 1G6, 5D7, 196.6b, and IF11, showed no binding to $F(ab')_2\mu$ with the exception of MoAb 4-3, which was inhibited by the highest dose of $F(ab')_2\mu$ tested (26.8 pmoles/25 μl = 150 μg/ml). The $Fc_5\mu$-non-reactive MoAbs, XG9, VIIIF10, IIF5, 2B11, VIIIB5, XG4, IX11, VIIIE11, P19, Bα1, HB57, P24, Mu18, and Mu53, all showed binding specificity for $F(ab')_2\mu$. However, the efficiency with which these MoAbs bound $F(ab')_2\mu$ showed considerable variation. With regard to their binding avidity for $F(ab')_2\mu$, as determined by the concentration of fragment required for 50% inhibition of MoAb binding to labeled IgM, these MoAbs showed the following hierarchy: XG4 = IX11 > IIF5 = VIIIB5 = VIIIF10 > XG9 = 2B11 » HB57 > Mu18 > Mu53 ≈ VIIIE11 > Bα1 ≈ P19 > P24.

Distinction between Cμ1 and Cμ2 domain specificity of F(ab')2 μ -binding anti-IgM MoAbs. $C\mu_1$-specific, $F(ab')_2\mu$-binding anti-IgM MoAbs were distinguished from $C\mu_2$-specific, $F(ab')_2\mu$-binding anti-IgM MoAbs by their relative ability to bind the μHCD proteins BOT and DAG

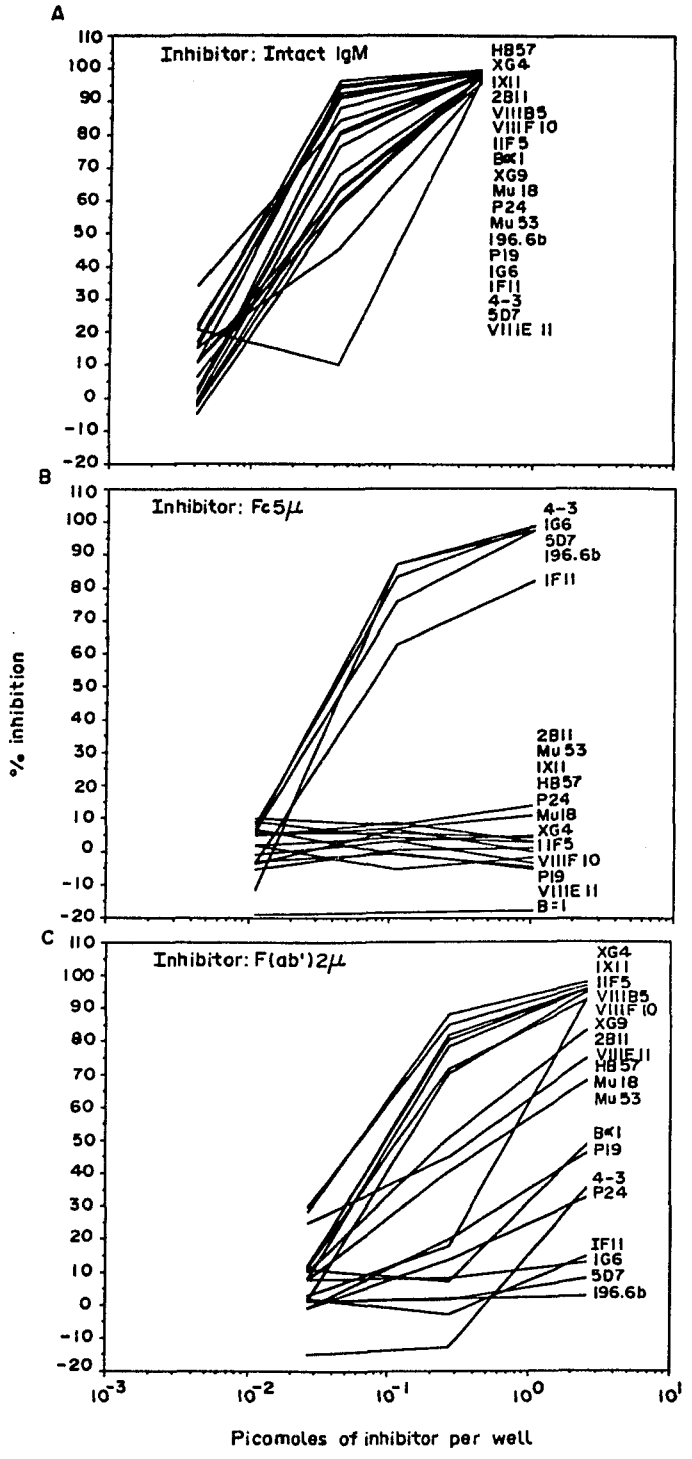

Fig. 9. Competitive inhibition of the binding of ^{125}I-IgM to immobilized anti-IgM MoAbs by intact IgM, $Fc_5\mu$, and $F(ab')_2\mu$. Abscissa values are picomoles of inhibitor per well (25 μl). In this and the remaining figures, MoAbs are listed from top to bottom according to increasing amount of inhibitor required to produce 50% inhibition of ^{125}I-IgM binding to the well-bound MoAb.

with deleted $C\mu_1$ domains in an inhibition RIA. As illustrated in Fig. 10, binding of MoAbs HB57, Mu18, Mu53, and 4-3 to labeled IgM was inhibited by very low concentrations of both BOT and DAG. MoAb P24 was also inhibited by these mutant proteins but higher concentrations were necessary to achieve comparable levels of inhibition. MoAbs XG9, VIIIF10, IIF5, 2B11, VIIIB5, XG4, IX11, VIIIE11, and P19 were not inhibited by any concentration of DAG μHCD protein tested. Inhibition with these latter MoAbs seen with high concentrations of BOT (7.2 pmoles/25 μl = 150 μg/ml) most probably reflects the very minor contamination with intact IgM, barely visible on SDS-PAGE gels (Fig. 8B, lane c). The $Fc_5\mu$-binding anti-IgM MoAbs 4-3, 1G6, 5D7, 196.6b, and IF11 were all significantly inhibited by both BOT and DAG, as expected.

In an attempt to further confirm the $C\mu_2$-binding specificity of MoAbs HB57, Mu18, Mu53, Bα1, and P24, the inhibitory effect of isolated $C\mu_2$ on the binding of these MoAbs to radiolabeled IgM was evaluated. The data in Fig. 11A indicate that most of these MoAbs bind very well to isolated $C\mu_2$ domain. While the binding of MoAbs HB57, Mu18, and Mu53 to labeled IgM was inhibited by ≤0.10 pmoles of $C\mu_2$, the inhibition of binding with MoAb Bα1 required considerably greater amounts. MoAb P24 was inhibited by only the highest amount of $C\mu_2$ tested (214 pmoles = 300 μg/ml). The $C\mu_1$-specific anti-IgM MoAbs XG9, VIIIF10, IIF5, 2B11, VIIIB5, XG4, IX11, VIIIE11, and P19 were not reactive with the $C\mu_2$ domain, with the exception of MoAb XG4, which was only inhibited by very high amounts of this fragment. This may reflect a slight contamination of the $C\mu_2$ preparation with a fragment containing the XG4 epitope or a very low affinity interaction between this $C\mu_1$-binding Ab and $C\mu_2$.

The inefficient binding of MoAb P24 to isolated $C\mu_2$ domain may be in part related to its uniquely compromised ability to bind pentameric IgM molecules with deletions of the $C\mu_1$ domain, ie. BOT and DAG, as demonstrated in Table VI. The diminished binding of P24 to these μHCD proteins was evidenced by an increase in the pmoles of μHCD protein relative to pmoles of intact IgM needed for 50% inhibition.

Distinction between Cμ3 and Cμ4 domain specificity of Fc5 μ-binding anti-IgM MoAbs.

$Fc_5\mu$-binding anti-IgM MoAbs 5D7, 196.6b, 4-3, 1G6, and IF11 were tested for their ability to bind

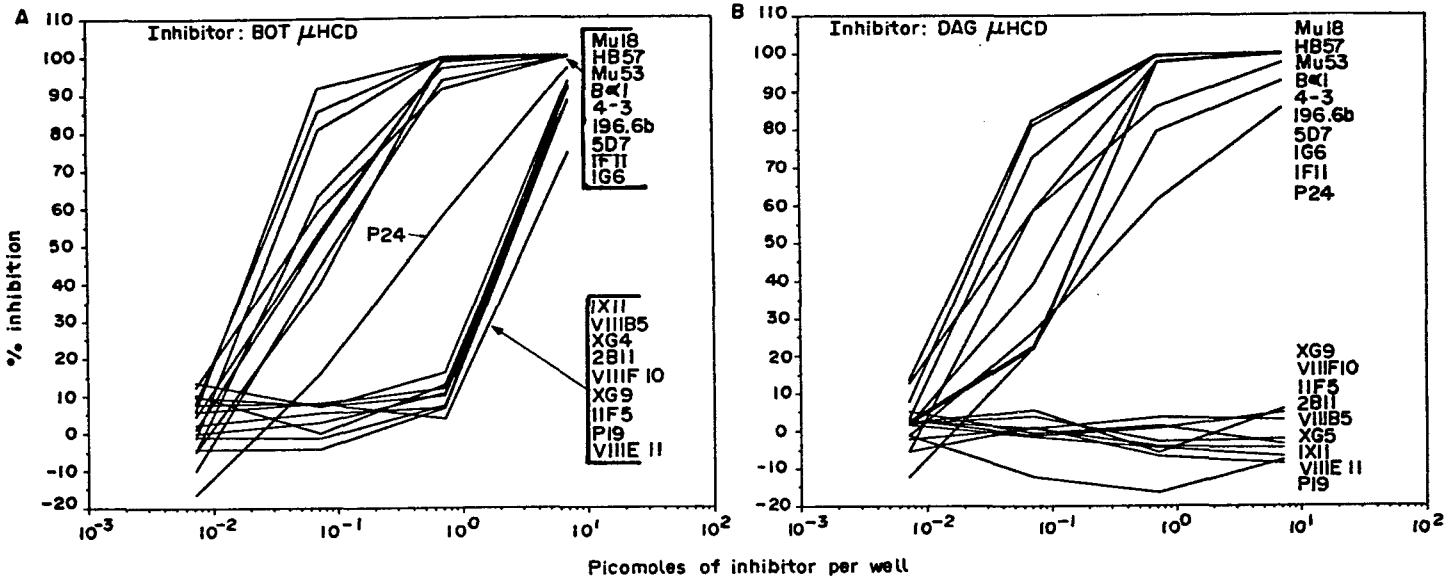

Fig.10. Competitive inhibition of the binding of ^{125}I-IgM to immobilized anti-IgM MoAbs by mu heavy chain disease proteins BOT and DAG, which lack light chain and V_H and $C\mu_1$ heavy chain domains, but retain IgM structure (Mihaesco *et al.*, 113).

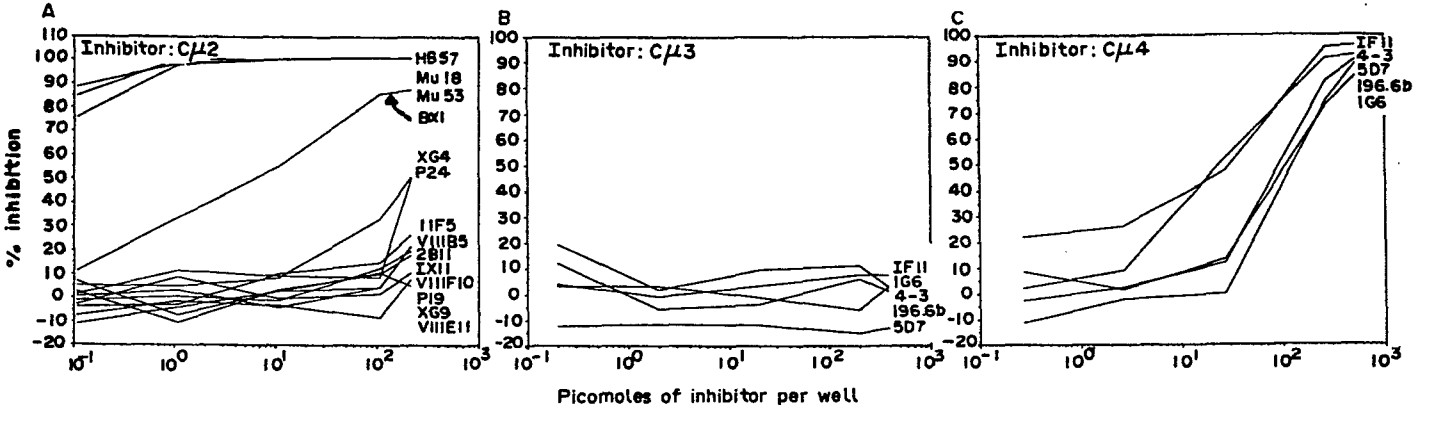

Fig.11. Competitive inhibition of the binding of ^{125}I-IgM to immobilized anti-IgM MoAbs by isolated IgM C_H domains. (A) $C\mu_2$ is used as an inhibitor of ^{125}I-IgM binding to F(ab')$_2\mu$-reactive MoAbs, (B) $C\mu_3$ and (C) $C\mu_4$ as inhibitors of ^{125}I-IgM binding to $Fc_5\mu$-reactive anti-IgM MoAbs.

Table VI. The avidity of $C\mu_2$-specific MoAb P24 to IgM is substantially reduced upon deletion of the $C\mu_1$ domain

		Ratio of amount of inhibitor required for 50% inhibition[a]	
		BOT μHCD	DAG μHCD
Anti-IgM MoAb	Experiment	Intact IgM	Intact IgM
Bα1	A	1.42	ND[b]
	B	1.58	2.01
HB57	A	2.27	ND
	B	1.01	1.54
Mu18	A	0.99	ND
	B	0.73	0.99
Mu53	A	1.27	ND
	B	0.91	1.26
P24	A	7.66	ND
	B	13.12	34.83

[a]Ratio determined from the quotient of pmole amount of μHCD protein ÷ picomole amount of intact IgM needed for 50% inhibition of ^{125}I-IgM binding to anti-IgM MoAb.

[b]ND: not determined.

isolated $C\mu_3$ and $C\mu_4$ IgM domains in an inhibition RIA (Fig. 11B and 11C). None of these Abs bound to the $C\mu_3$ domain, yet they all bound to the $C\mu_4$ moiety. Although the absolute amounts of $C\mu_4$ required for inhibition of MoAb binding to labeled IgM was considerably greater than the amounts of $Fc_5\mu$ inhibitor required (see Fig. 9B), the inhibition was specific in that none of the $C\mu_1$- or $C\mu_2$-binding anti-IgM MoAbs were inhibited to any degree by this fragment (Table VII).

Analysis of the dependency of light chain for epitope expression in $F(ab')_2\mu$ - binding anti-IgM MoAbs. To determine what role, if any, light chain may play in forming the determinant bound by the $F(ab')_2\mu$-specific anti-IgM MoAbs, we tested the ability of both μ chain (which lacks light chain) and half-molecules of IgM monomer (H-L) to inhibit the binding of labeled IgM to these MoAbs. H-L was chosen as the light chain-containing moiety because it should more closely resemble μ chain in terms of valency than intact pentameric IgM. Fig. 12 shows that while the $C\mu_1$-binding anti-IgM MoAbs XG9, VIIIF10, IIF5, 2B11, VIIIB5, XG4, IX11, VIIIE11, and P19 were all effectively inhibited in their binding to labeled IgM by H-L, μ chain was ineffective at causing inhibition. In contrast to the $C\mu_1$-specific anti-IgM MoAbs, all of the $C\mu_2$-specific anti-IgM MoAbs were inhibited by both H-L and μ chain. MoAbs HB57, Mu18, and Mu53 were more effectively inhibited than Bα1 and P24 MoAbs. The less effective inhibition observed with these latter MoAbs may, in part, reflect their lower affinity. However, other considerations, such as modification of their respective epitopes following the loss of disulphide linkage between two μ chains or ineffective non-covalent re-association of the μ chain or μk chain units may also contribute.

Table VII summarizes results from inhibition RIAs used to determine the domain specificity of the nineteen anti-IgM MoAbs.

Analysis of anti-IgM MoAb binding to tryptic peptide and CNBr cleavage fragments of μHCD protein BOT. In an attempt to more precisely define the determinants to which our $C\mu_2$- and $C\mu_4$-specific anti-IgM MoAbs bind, use was made of both tryptic peptide and CNBr cleavage fragments of μHCD protein BOT in inhibition RIAs. CNBr cleavage fragments H5 (Phe_{313}-Met_{331}), H6 (Cys_{332}-Met_{484}), H7 (Gln_{485}-Met_{501}), and H8 (Pro_{502}-Met_{563}) (121), which

Table VII. Summary of the efficacy of various soluble IgM moieties at inhibiting ^{125}I-IgM binding to well-bound anti-IgM MoAb

	Picomoles of inhibitor required for 50% inhibition of ^{125}I-IgM binding to well-bound anti-IgM MoAb										
		Soluble inhibitors									
	Well-bound anti-IgM MoAb	Intact IgM	H-L	μ chain	BOT	DAG	$Fc_5\mu$	$F(ab')_2\mu$	$C\mu_2$	$C\mu_3$	$C\mu_4$
$C\mu_1$-binders	XG9	0.028	1.003	$\gg$100	2.375	$\gg$10	$\gg$15	0.140	$\gg$250	$\gg$50	$\gg$1000
	VIIIF10	0.017	0.482	> 100	2.370	$\gg$10	$\gg$15	0.110	$\gg$250	$\gg$50	$\gg$1000
	IIF5	0.017	0.323	> 100	2.450	$\gg$10	$\gg$15	0.098	$\gg$250	$\gg$50	$\gg$1000
	2B11	0.014	0.815	> 100	2.330	$\gg$10	$\gg$15	0.124	$\gg$250	$\gg$50	$\gg$1000
	VIIIB5	0.015	0.505	81.500	2.230	$\gg$10	$\gg$15	0.100	$\gg$250	$\gg$50	$\gg$1000
	XG4	0.013	0.211	> 100	2.310	$\gg$10	$\gg$15	0.062	208.072	$\gg$50	$\gg$1000
	IX11	0.013	0.354	> 100	2.050	$\gg$10	> 15	0.062	$\gg$250	$\gg$50	$\gg$1000
	VIIIE11	0.110	2.057	$\gg$100	3.310	$\gg$10	> 15	0.715	$\gg$250	$\gg$50	$\gg$1000
	P19	0.050	> 80	$\gg$100	2.500	$\gg$10	$\gg$15	> 3	$\gg$250	$\gg$50	$\gg$1000
$C\mu_2$-binders	Bα1	0.025	53.711	7.850	0.047	0.048	$\gg$15	2.800	6.150	$\gg$50	$\gg$1000
	HB57	0.012	1.003	0.910	0.023	0.028	> 15	0.270	$\ll$0.1	$\gg$50	$\gg$1000
	P24	0.037	> 80	38.500	0.470	0.350	$\gg$15	> 5	215.000	$\gg$50	$\gg$1000
	Mu18	0.035	1.784	0.810	0.025	0.025	> 15	0.400	$\ll$0.1	$\gg$50	$\gg$1000
	Mu53	0.044	2.669	1.200	0.031	0.035	> 15	0.600	$\ll$0.1	$\gg$50	$\gg$1000
$C\mu_4$-binders	5D7	0.087	ND[a]	1.750	0.067	0.172	0.046	$\gg$5	$\gg$250	$\gg$50	265.000
	196.6b	0.044	ND	1.950	0.063	0.110	0.046	$\gg$5	$\gg$250	$\gg$50	290.000
	4-3	0.068	ND	0.910	0.047	0.054	0.038	> 3	$\gg$250	$\gg$50	100.000
	IG6	0.051	ND	1.750	0.113	0.175	0.040	$\gg$5	$\gg$250	$\gg$50	340.000
	IF11	0.060	1.221	1.490	0.096	0.225	0.070	$\gg$5	$\gg$250	$\gg$50	71.500

[a]ND: not determined.

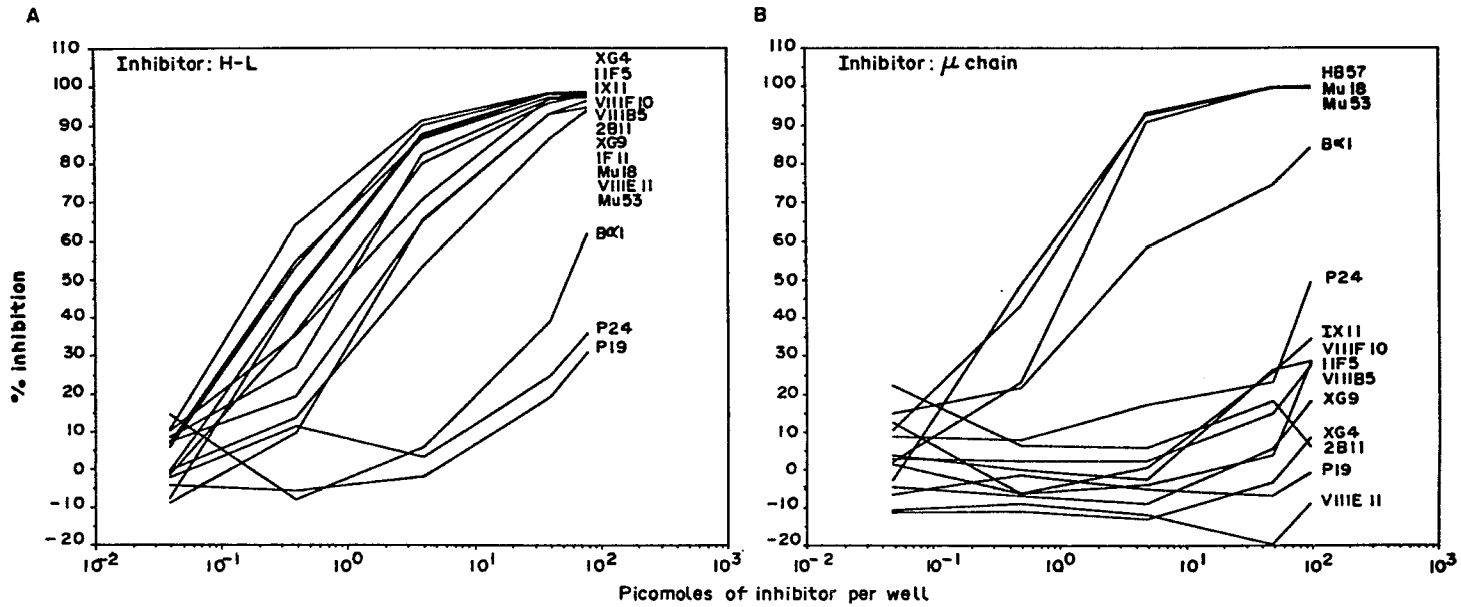

Fig.12. Competitive inhibition of the binding of ^{125}I-IgM to immobilized anti-IgM MoAbs by H-L (half IgM monomers) and purified μ chain.

constitute all amino acids from the last 22 in the $C\mu_2$ domain to 9 amino acid residues inside the secretory domain, were assayed for their ability to interfere with the binding of well-bound $C\mu_2$- and $C\mu_4$-specific anti-IgM MoAbs to ^{125}I-IgM. In this assay, neither the $C\mu_2$-binding Abs HB57, Mu18, Mu53, Bα1, and P24 nor the $C\mu_4$-binding Abs 5D7, 196.6b, 4-3, 1G6, and IF11, showed specificity for any of these CNBr cleavage fragments, even when fragment concentrations as high as 500 µg/ml of BOT equivalent were used (data not shown).

$C\mu_2$ tryptic peptide fragment T27 (Gln_{272}-Lys_{287}) was also tested for its ability to inhibit the binding of ^{125}I-IgM to well-bound $C\mu_2$-binding anti-IgM MoAbs. T27, whose sequence is located within the intra-domain disulphide bond loop of $C\mu_2$, was found to correspond to the most hydrophilic region of the human IgM molecule as determined by the method of Hopp and Woods (see Fig. 7B; 126). Even when used at a concentration of 1000 pmoles/25 µl, tryptic peptide fragment T27 caused no inhibition of labeled IgM binding to any anti-IgM MoAb (data not shown).

Cross-inhibition analysis of anti-IgM MoAbs. Cross-inhibition RIAs were performed in an attempt to determine whether individual anti-IgM MoAbs which bind to the same domain recognize distinct epitopes. The results with the $C\mu_1$-specific anti-IgM MoAbs shown in Table VIII indicate the following: a) MoAbs XG9, VIIF10, IIF5, 2B11, VIIIB5, XG4, IX11, and VIIIE11 all bind to the same or very proximal epitope on the $C\mu_1$ domain since these eight Abs strongly inhibit each other's binding. The amount of Ab required for inhibition of ^{3}H-IgM binding to homologous well-bound Ab or other well-bound Abs of this group likely reflects the relative affinity of each of these MoAbs; b) MoAb P19 probably binds to a distinct but proximal epitope since it shows reciprocal inhibition not only with the former MoAb group but also with MoAb Bα1.

With regard to $C\mu_2$-specific anti-IgM MoAbs, the following are indicated: a) Mu18 and Mu53 MoAbs appear to bind to the same epitope since they reciprocally inhibit each other's binding at low concentrations and each act similarly with the entire panel of MoAbs whether they are used as immobilized ligands or soluble inhibitors. b) MoAb Bα1 very likely binds to a site distinct on the $C\mu_2$ domain than the other four members of this $C\mu_2$-specific MoAb group. It is completely non-competitive with HB57 and competes just weakly with both Mu18 and Mu53 - in a

Table VIII. Anti-IgM MoAb cross-competition radioimmunoassay to determine if MoAbs that bind to the same domain bind to the same or distinct epitope

	Concentration (μg/ml) of soluble anti-IgM MoAb needed for 50% inhibition of 3H-IgM binding to well-bound anti-IgM MoAb[a]																		
		Soluble anti-IgM MoAbs																	
	Well-bound anti-IgM MoAb	XG9	VIIIF10	IIF5	2B11	VIIIB5	XG4	IX11	VIIIE11	P19	Bα1	HB57	P24	Mu18	Mu53	IF11	196.6b	4-3	1G6	5D7
$C\mu_1$-binding	XG9	0.33	0.38	0.61	1.10	0.59	0.69	1.28	1.37	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50
	VIIIF10	0.30	0.42	0.38	0.32	0.39	0.58	1.01	1.88	> 50	> 50	> 50	> 50	> 50	> 50	30.10	> 50	> 50	> 50	> 50
	IIF5	0.28	0.27	0.31	0.57	0.42	0.49	0.95	1.82	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50
	2B11	0.31	0.53	0.48	0.54	0.50	0.61	1.34	1.40	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50
	VIIIB5	0.17	0.33	0.27	0.34	0.22	0.62	0.99	0.72	46.25	> 50	> 50	> 50	> 50	> 50	24.64	> 50	> 50	> 50	> 50
	XG4	0.21	0.21	0.26	0.35	0.26	0.72	1.08	0.45	15.50	> 50	> 50	> 50	> 50	> 50	25.05	> 50	> 50	> 50	> 50
	IX11	0.29	0.30	0.26	0.48	0.24	0.49	1.02	0.28	17.50	> 50	> 50	> 50	> 50	> 50	29.80	> 50	> 50	> 50	> 50
	VIIIE11	0.29	0.33	0.42	0.74	0.53	0.54	0.99	12.37	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50
	P19	0.18	0.19	0.20	0.33	0.23	0.43	0.82	0.41	4.80	11.73	> 50	> 50	> 50	> 50	33.35	> 50	> 50	> 50	> 50
$C\mu_2$-binding	Bα1	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	21.30	1.86	> 50	> 50	8.53	10.17	> 50	> 50	> 50	> 50	> 50
	HB57	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	0.35	19.00	25.20	11.63	> 50	> 50	> 50	> 50	> 50
	P24	0.37	0.62	2.00	> 50	8.90	> 50	50.00	> 50	0.26	0.30	0.25	6.18	0.69	1.70	20.00	0.38	0.24	6.88	6.70
	Mu18	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	5.80	> 50	3.44	7.65	> 50	> 50	> 50	> 50	> 50
	Mu53	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	23.50	0.48	> 50	0.52	1.39	> 50	> 50	> 50	> 50	> 50
$C\mu_4$-binding	IF11	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	8.78	> 50	0.21	1.22	> 50
	196.6b	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	0.25	0.47	> 50	> 50
	4-3	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	4.00	0.20	0.27	> 50
	1G6	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	49.00	0.39	1.71	> 50
	5D7	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	17.00	> 50	19.00	0.71

[a]See Materials and Methods for details on assay procedure.

manner that would imply steric hindrance. B∝1 also competes for ^{3}H-IgM with P19, whereas HB57, P24, Mu18, and Mu53 MoAbs do not. c) MoAbs HB57 and P24 may bind to the same or very near sites on the $C\mu_2$ domain since they inhibit each others binding in a reciprocal manner. The necessity for high soluble P24 concentrations for homologous inhibition and the propensity of well-bound MoAb P24 to be inhibited by nearly every soluble inhibitor in the Ab panel attest to its low affinity nature. When conditions are chosen such that the avidity of MoAb HB57 is compromised, ie. by coating the HB57 MoAb on wells at a lower concentration, it shows a pattern of inhibition by other soluble MoAb inhibitors similar to that of MoAb P24 (109). d) Mu18 and Mu53 likely bind to a distinct, although proximal epitope on the $C\mu_2$ domain from that bound by MoAbs HB57 and P24. This is in part indicated by their discordant reactivity with B∝1. Thus, whereas both HB57 and P24 are poor inhibitors of ^{3}H-IgM binding to well-bound B∝1, both Mu18 and Mu53 can induce significant levels of inhibition. A further indication that Mu53 binds an epitope distinct from HB57 comes from the previous observation (109) that, when coated to wells at the low ligand concentration of 10 μg/ml, HB57 is significantly inhibited in binding to ^{3}H-IgM by a number of MoAbs which do not affect Mu53 binding. The sensitivity of this well-bound HB57 to inhibition by MoAbs which we now know bind to distinct domains likely reflects an unusual sensitivity of this Ab to blocking by steric hindrance, since HB57 appears to be of very high affinity (data not shown; Rudich and Mongini, in preparation).

Cross-inhibition analysis with the $C\mu_4$-specific anti-IgM MoAbs show: a) MoAb 5D7 likely binds to a unique determinant on the $C\mu_4$ domain. It is completely non-competitive with the entire panel of MoAbs when used as a soluble inhibitor and is inhibited only weakly, and non-reciprocally by 196.6b and 1G6, in a manner consistent with steric hindrance. b) MoAb IF11 also likely has specificity for a unique determinant on the $C\mu_4$ domain. Thus, only two $C\mu_4$-specific anti-IgM MoAbs, 4-3 and 1G6, interfere with IF11 binding to labeled IgM but this is non-reciprocal in nature. In addition, IF11, in multiple experiments, consistently caused some titratable inhibition of ^{3}H-IgM binding to well-bound $C\mu_1$-specific MoAbs while the remaining $C\mu_4$-specific MoAbs caused no inhibition with $C\mu_1$-specific Abs, even at the highest doses tested. c) Three MoAbs, 196.6b, 4-3,

and 1G6, all likely bind to distinct but very near determinants on the $C\mu_4$ domain. This is indicated by the observations that i) MoAb 4-3 competes strongly with both 1G6 and 196.6b MoAbs in a reciprocal fashion, but MoAbs 196.6b and 1G6 show very negligible reciprocal inhibition with one another, ii) both soluble 4-3 and 1G6 compete very effectively with well-bound IF11, whereas 196.6b does not, and iii) both soluble 196.6b and 1G6 MoAbs, but not 4-3, appear to sterically interfere with the binding of labeled IgM to well-bound 5D7 MoAb.

DISCUSSION

The domain specificity of nineteen murine monoclonal anti-human IgM antibodies was determined by competition solid-phase RIA methods. These MoAbs have been shown previously to differ significantly in their capacity for B cell signal transduction in both normal B lymphocytes (109) and B leukemic populations (111). Using a combination of enzymatic cleavage fragments, $C\mu_1$-deleted heavy chain disease proteins and isolated C_H domains of human IgM, we have shown $C\mu_1$ domain specificity for nine of the MoAbs, $C\mu_2$ specificity for five MoAbs, and $C\mu_4$ specificity for an additional five MoAbs.

Interestingly, of the nineteen MoAbs, which derived from eight different fusions, none were found to have specificity for $C\mu_3$. Perhaps the large amount of carbohydrate in this domain obscures IgM antigenic sites, or alternatively predisposes for carbohydrate-specific MoAbs cross-reactive with other Ig classes. MoAbs of these latter specificity would have been lost in the selection process.

The $C\mu_1$-binding ability of anti-IgM MoAbs XG9, VIIIF10, IIF5, 2B11, VIIIB5, XG4, IX11, VIIIE11, and P19 was most clearly illustrated by the inability of these reagents to bind the $C\mu_1$ domain-deleted μHCD proteins BOT and DAG. Additional evidence that these MoAbs bind to epitopes on the $C\mu_1$ domain was provided by the light chain requirement for epitope expression. This suggests that the determinants are conformational in nature, since two distinct polypeptide chains are needed for their expression. Whether the actual epitopes are comprised of amino acid residues from both chains or whether one chain strongly influences the amino acid conformation

of the other cannot be determined from these data. All of the $C\mu_1$-specific MoAbs, except P19, appear to bind the same epitope or very proximate epitopes since they all strongly cross-inhibit one another and show similar patterns of weak inhibition with certain other MoAbs.

The $C\mu_2$-binding specificity of anti-IgM MoAbs HB57, Mu18, Mu53, Bα1, and P24 was confirmed by a number of observations. Each of these MoAbs bound both to μHCD proteins with $C\mu_1$ deletions and to $F(ab')_2\mu$: two IgM moieties which have in common the entire $C\mu_2$ domain and the seven N-terminal amino acids of the $C\mu_3$ domain. Specificity for the amino terminus of $C\mu_3$ appeared unlikely since $Fc_5\mu$ (which contains the entire $C\mu_3$ domain), as well as isolated $C\mu_3$, did not bind to these five anti-IgM MoAbs. Additional corroboration of $C\mu_2$-binding specificity came from the fact that, with the exception of P24 and Bα1, these MoAbs showed strong interaction with isolated $C\mu_2$ domain.

The relatively poor binding of $C\mu_2$-specific anti-IgM MoAbs Bα1 and P24 to isolated $C\mu_2$ may be due to one or more of the following: a) an intrinsically lower avidity of these MoAbs for IgM; b) an obfuscation or loss of the epitopes as a result of the aggregation of isolated $C\mu_2$ domain (118); or c) a loss of the epitopes for these MoAbs because of proximity to the pepsin cleavage sites. In addition, as Table VI demonstrates, the absence of the $C\mu_1$ domain in μHCD proteins appears to diminish the avidity of P24 for its determinant, suggesting that this epitope may be significantly influenced by interactions between the $C\mu_1$and $C\mu_2$ domains. We should note that this less efficient binding of MoAb P24 to the $C\mu_1$-deleted μHCD proteins relative to intact IgM should not represent differences in binding avidity due to changes in epitope number since these μHCD proteins are pentameric, like intact IgM.

Results from MoAb cross-inhibition analyses suggest that the five $C\mu_2$-specific MoAbs recognize at least three distinct epitopes. None of these MoAbs showed binding to tryptic peptide T27, a sequence of amino acids which by hydrophilicity analysis (see Fig. 7B) was predicted as having a high probability of constituting an antigenic determinant in the human IgM molecule. It remains possible that one of the $C\mu_2$ determinants encompasses this sequence but that, in the

T27 peptide, an insufficient number of amino acids on either side of the hydrophilic region prevents effective epitope presentation.

It is of interest to note that MoAbs P19 and Bα1, with $C\mu_1$ and $C\mu_2$ specificity, respectively, show a mild degree of reciprocal cross-inhibition. Two possible explanations for this finding are: a) the epitopes for these two MoAbs are very near to one another in the three-dimensional structure of the IgM molecule and thus allow for steric hindrance or b) the binding of one MoAb induces a conformational change in the IgM molecule which is manifest by a decrease in the binding avidity of the other MoAb for a distinct epitope (105).

The $C\mu_4$-binding specificity of MoAbs 5D7, 196.6b, 4-3, 1G6, and IF11 was demonstrated by the binding of these five MoAbs to $Fc_5\mu$ and isolated $C\mu_4$ but lack of binding to $F(ab')_2\mu$ and isolated $C\mu_3$. A weak inhibition of labeled IgM binding to MoAb 4-3 by $F(ab')_2\mu$ may reflect this MoAb's higher avidity for possible contaminating, albeit undetectable, native IgM or its very low affinity interaction with an $F(ab')_2\mu$ epitope. The exceptionally high picomolar quantities of $C\mu_4$ required for inhibition of $Fc_5\mu$-specific MoAb binding to labeled IgM may reflect both a lowered valency of determinants expressed on this isolated fragment as well as a potential loss of true conformation during the preparation of $C\mu_4$.

The determinants to which the five $C\mu_4$-specific MoAbs bind are likely all distinct and may be conformational in nature. Evidence for the latter comes from the fact that three consecutive CNBr cleavage peptides, encompassing the entire $C\mu_4$ domain, did not bind to these anti-IgM MoAbs. Although it is possible that the epitopes were located at the cleavage sites, the fact that both hydrophilicity and β-turn analyses of this domain (which have been used to predict antigenic sites in protein molecules [126,131]), revealed that none of the methionine cleavage sites occurred at major hydrophilic or β-turn regions of the $C\mu_4$ domain (Fig. 7 and Rudich, unpublished observations), suggests that this might be unlikely.

Of the $C\mu_4$-specific Abs, MoAb IF11 was unique in its weak inhibition of the binding of all the $C\mu_1$-specific MoAbs to labeled IgM. The reason for this may be: a) steric interference of $C\mu_1$-specific MoAb binding to IgM by IF11 after this latter MoAb has bound to its $C\mu_4$ epitope;

b) induction of a conformational change in the IgM molecule following IF11 binding which alters the ability of $C\mu_1$-specific MoAbs to bind to their determinant; c) presence of a weak cross-reactivity of $C\mu_4$-binding IF11 with an epitope on the $C\mu_1$ domain; and d) possible bi-clonality of the MoAb IF11 preparation. The third possibility appears unlikely since $F(ab')_2\mu$ showed no reactivity with IF11. The fourth possibility is considered highly unlikely since the IF11 hybridoma was cloned twice at limiting dilution, with a positive frequency of only 2-5%.

These studies designating the domain specificity of the anti-IgM MoAbs, combined with known information on the functional properties of the MoAbs, allow us to make some conclusions regarding the role of domain specificity in the signal transduction capabilities of Abs to membrane IgM. We find that there is no absolute association between the domain specificity of anti-IgM MoAbs and the ability to elicit signals for human B cell proliferation since those unique MoAbs which we have reported to be mitogenic for B cells, ie. HB57, 5D7, Mu53, and VIIIE11 (109) show specificity for either $C\mu_1$, $C\mu_2$, or $C\mu_4$. In addition, inhibitory signal transduction does not appear to have an obligatory requirement for ligand binding to a particular domain since all 19 anti-IgM MoAbs can transduce inhibitory signals which abrogate the spontaneous or induced DNA synthesis of certain human B cell leukemias (111). Clearly, specificity for the μ chain domain nearest the membrane, ie. $C\mu_4$, does not reduce the signaling properties of anti-IgM Abs. This is consistent with our observations that all the MoAbs with $C\mu_4$ specificity, as well as those with $C\mu_1$ and $C\mu_2$ specificity, bind membrane IgM (109 and unpublished results).

In the absence of any indication that ligand domain specificity is exclusively responsible for the heterogeneity observed in the ability of anti-IgM MoAbs to elicit B cell proliferation, it is likely that other parameters of ligand-receptor interaction such as affinity between anti-IgM and membrane IgM and the stoichiometry of ligand binding are involved. The contribution of these binding characteristics to the elicitation of effective signal transduction in human B lymphocytes will be the topic of a subsequent report (Rudich and Mongini, in preparation).

SUMMARY

The domain binding specificity of nineteen murine anti-human IgM monoclonal antibodies that have shown considerable heterogeneity in the transduction of stimulatory and inhibitory signals to B lymphocytes was evaluated by competition radioimmunoassays. Through the use of: a) enzymatic fragments of IgM which each encompass more than a single C_H domain, ie. $Fc_5\mu$ and $F(ab')_2\mu$, b) isolated single domains, $C\mu_2$, $C\mu_3$, and $C\mu_4$, and c) mu heavy chain disease proteins, nine anti-IgM MoAbs were found to have $C\mu_1$ domain specificity, five to have $C\mu_2$-specificity, and five others to have $C\mu_4$-specificity. Ineffective binding to isolated μ chain demonstrated that $C\mu_1$-specific MoAbs were directed to epitopes which require light chain for expression. The lack of binding of the $C\mu_4$-specific MoAbs to CNBr cleavage fragments of $Fc_5\mu$ suggest that the determinants recognized by these MoAbs may also be conformational in nature. Cross-inhibition analyses were used to determine the number of unique epitopes recognized by the anti-IgM MoAbs. Results from these experiments showed that: a) eight of the nine MoAbs specific for $C\mu_1$ likely bind to a single epitope, or very proximate epitopes, b) the five $C\mu_2$-specific MoAbs recognize at least three distinct epitopes, and c) the five $C\mu_4$-specific MoAbs each recognize a separate determinant. A comparison of the known B cell activating properties of these MoAbs with their specificity for the various segments of the IgM molecule indicate that mitogenicity cannot be attributed to selective binding to any one domain.

CHAPTER FOUR

Human Leukemic B Cell Activation: Functional Consequence of Membrane IgM Interaction with Anti-IgM Ligand is an Alterable Cell Characteristic*

INTRODUCTION

Recent studies suggest that the phenotypic diversity of B cell leukemias and lymphomas corresponds to diversity within the pool of normal B lymphocytes (132,133). Thus, the membrane antigen expression of distinct malignant B cell populations appears to reflect the pattern of cell surface marker acquisition during B lymphocyte maturation (134-136) and many functional characteristics of malignant B cells likely mimic those of defined subsets of normal B lymphocytes (133). It has been suggested that the phenotype of normal B cells at each stage of differentiation will be best elucidated by studies of clonal populations of malignant B cells of the corresponding differentiation state. This is supported by the fact that many features of malignant cells initially thought to be aberrant were ultimately shown to be reflective of those of normal, but at the time undiscovered, cells or functions (133,137-141).

One particular functional feature of normal polyclonal B cells which has been mimicked in occasional leukemia or lymphoma cell populations is sensitivity to stimulatory or inhibitory signal transduction by ligands that cross-link membrane IgM. The relatively infrequent examples of mIgM-mediated stimulatory signal transduction that result in enhanced in vitro DNA synthesis of malignant B cell clones are thought to represent an activation phenomenon characteristic of mature resting B cells (8,28). In contrast, signaling through membrane IgM which results in inhibition of DNA synthesis has been thought to represent a form of tolergenic signal transduction most clearly manifest in normal immature B lymphocytes (7,9,10,142).

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We have attempted to identify malignant B cell populations that will facilitate clonal studies of the mechanisms of stimulatory and inhibitory signal transduction following ligand binding to mIgM. This has involved a screen of diverse membrane IgM-positive B cell leukemias for responsiveness to soluble anti-IgM antibody following in vitro culture. In this report we document examples of positive and negative signal transduction by soluble anti-IgM antibodies in a form of leukemia thought to represent a malignancy of mature B lymphocytes, i.e. hairy cell leukemia (134,135). We further demonstrate the unique finding that soluble factors from activated T cells can modulate the responsiveness of one of the leukemic cell populations to anti-Ig ligands such that an inhibitory rather than a stimulatory signal is transduced following membrane Ig cross-linking. These findings indicate that the nature of membrane IgM-mediated signal transduction in certain B cells with mature functional phenotype can be changed following exposure to certain T cell factors. This modulated response may mimic that of an infrequent subpopulation of normal mature B lymphocytes which is masked in activation studies utilizing polyclonal B cells. Both the heterogeneity in responsiveness to membrane Ig binding ligands observed with these leukemic populations and the ability to change the functional phenotype of a given leukemic clone may have important implications for the use of anti-Ig ligands in immunotherapy.

MATERIALS AND METHODS

Leukemic and normal B cells. The malignant B cell specimens used in this study were obtained from Dr. Janet Cuttner of the Mount Sinai School of Medicine. Patients were afflicted with either chronic lymphocytic leukemia (CLL), poorly differentiated lymphocytic lymphoma (PCLL) or hairy cell leukemia (HCL). Neoplastic cells were classified as HCL based on morphology and tartrate resistant acid phosphatase positivity (143). Patient samples consisted in 12 cases of peripheral blood (PB) or leukophoresis specimens and in 1 case (SAP) of a spleen specimen. In all instances, specimens were obtained prior to therapy. Cells were stored in the vapor phase of liquid nitrogen until use. The cell populations selected for functional studies consisted of those with relatively high leukemic cell counts to minimize effects of contaminating cells as well as to

insure availability of substantial numbers of cells for extended study. Prior to being functionally tested for receptivity to anti-IgM Ab-mediated signaling, the cell populations were screened for expression of membrane IgM by visual immunofluorescence with HB57 anti-human IgM monoclonal antibody (109) and fluorescein-conjugated goat $F(ab')_2$ anti-mouse Ig.

Normal B cell and T cell preparations were obtained from normal volunteer human PB by twice rosetting Ficoll-Hypaque isolated cells with neuraminidase-treated sheep red blood cells, as previously described (109). Sheep red blood cells were removed from the rosette-positive (ER-positive) cells by lysis with ammonium chloride in Tris buffer.

Cytotoxic depletion of monocytes and T cells from leukemic cell preparations. Potential contaminating monocytes and T cells were removed from leukemic cell populations by treatment with the pan-T cell MoAbs, BW 264/50 (CD7) and T3/2ad 2A2 (CD3) (144), both of IgM isotype, the pan-monocyte MoAbs, MθS-1 and MθP-9 (Leu M3) (99) of IgG2a and IgG2b isotypes respectively, and complement (C). Briefly, the procedure entailed incubating leukemic cells at 10×10^6/ml in either medium (see culture medium formulation), or medium supplemented with concentrations of the pan T MoAbs and/or the pan-monocyte MoAbs four times the concentration shown to be saturating for normal PB T cells or monocytes, respectively by previous immunofluorescence analyses. Following 45 mins of incubation with occasional shaking, the cells were centrifuged and resuspended in a 1:5 dilution of low background cytotoxicity rabbit serum (Pel-Freez, Rogers, AR) as the source of C. After incubation at 37°C for 45 mins, the cells were washed and placed in culture at a concentration of 2×10^5/200 µl. The effectiveness of this procedure at depleting possible contaminating T cells and monocytes, which were virtually undetectable in the original leukemic cell preparations by immunofluorescence staining, was evidenced by 68% specific cytotoxicity of normal PB cells with the pan-T cell MoAb cocktail + C and a 52% cytotoxicity of PB cells from a patient with myeloproliferative disease with the pan-monocyte MoAb cocktail + C. This latter cell population had been shown to be 58% positive for MθS-1 and MθP-9 by visual immunofluorescence microscopy while normal PB cells routinely contain 50-70% T cells by immunofluorescence analysis.

Immunofluorescence. Binding of MoAbs to the surface of leukemic cells was determined by indirect immunofluorescence assays with the fluorescence-activated cell sorter, FACS IV (Becton Dickinson) as described (98,99,109).

The identity, specificity, and murine isotype of the MoAbs used for the immunofluorescence assay are as follows: HB57 anti-human IgM (IgG1) obtained from American Type Culture Collection (ATCC) (109); 4E5 anti-human IgD (IgG1) (109); HB61 anti-human kappa light chain (IgG1) from ATCC (109); 4C6 anti-lambda light chain (IgG3) (109); MθP-9 (Leu M3) anti-monocyte (IgG2b) (99); 89bl anti-pan T cell (IgG1) (145); Leu 1 (IgG2a) with specificity for all T cells and a subset of B cells (138) from Becton Dickinson; 91d4 (Leu 1-like) (IgG1) (138,145); FMC7 anti-limited B cell (IgM), a gift of Dr. Heddy Zola (146,147); 4F2 with specificity for an activation marker on lymphocytes and monocytes (IgG2a) from ATCC (101); 5E9 anti-transferrin receptor (IgG1) from ATCC (148); S157 monomorphic anti-DR (IgG2a) (149); B1 (IgG2a) and B4 (IgG1) each anti-pan B cell (150,151); B2 anti-limited B cell (IgM) (152,153); J5 anti-CALLA (IgG2a) (154); and PC-1 anti-plasma cell (IgM) (155). The latter five Abs, i.e. B1, B2, B4, J5, and PC-1 were obtained from participation in the Second International Congress of Leukocyte Antigens. HB57, 4E5, HB61, 4C6, 89bl, 91d4, and FMC7 were used in supernatant form for staining. MθP-9, B1, B2, B4, J5, PC-1, and 5E9 were used in diluted ascites form. 4F2, Leu 1, and S157 were used as purified proteins diluted to 50 μg/ml in assay buffer, i.e. PBS + 1% bovine serum albumin (BSA) + 0.1% sodium azide. The isotype control Igs consisted of purified myeloma proteins, MOPC-21 (IgG1), UPC-10 (IgG2a), J606 (IgG3), MOPC-195S (IgG2b) and MOPC-104E (IgM) at a concentration of 50 μg/ml in assay buffer. The IgG MoAbs were ultracentrifuged in a airfuge (Beckman Instruments, Palo Alto, CA) at 100,000 x g for 30 min, and the IgM MoAbs at 100,000 x g for 10 min prior to use.

Reagents for culture. The anti-human IgM murine MoAbs HB57 and XG9 and the murine IgG myeloma proteins MOPC-21 and MOPC-245, of undefined specificity, were purified from murine ascitic fluid as described elsewhere (109). $F(ab)'_2$ fragments of HB57 were prepared by pepsin digestion. Briefly, thirty mg of purified MoAb in 0.1 M citrate, pH 3.6, was treated with

pepsin (Sigma Chemical Co.) at 37°C with an enzyme to substrate ratio of 1:60 for 1 hour. Digestion was terminated by raising the pH >8 by the addition of solid Tris. Potential undigested Ab was removed by passing the digest over protein-A-Sepharose (Pharmacia) by the method of Ey et al. (92). Protein-A-Sepharose nonbound Ab was vacuum-concentrated and sieved through a Sephacryl S-200 (Pharmacia) gel filtration column. Fractions from a single observed peak were concentrated and analyzed by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for purity. Analysis of the HB57 pepsin-digested Ab in a 5-20% gradient gel under non-reducing conditions revealed only one band of molecular weight 110,000 in contrast to the 150,000 mol. wt band of intact MoAb. Goat anti-human IgM Ab in $F(ab)'_2$ form was the kind gift of Dr. Nicholas Chiorazzi.

T cell supernatants used in these studies were derived from cultures of PHA-stimulated mixtures of PB cells of allogeneic individuals as has been described previously (109). Briefly, each supernatant lot was generated by pooling PB cells from four to ten different healthy donors and culturing them at a concentration of 1.5×10^6/ml in 24-well plates in the presence of 2 µg/ml phytohemagglutinin (PHA-P; Difco Laboratories, Detroit, MI). Supernatants were removed after three days of incubation and dialyzed in Iscove's-Ham's supplemented culture medium before being frozen in aliquots at -70°C. The experiments shown in this report were reproduced with three distinct lots of activated T cell supernatants. In some experiments, purified anti-PHA-P Ab (Collaborative Research, Lexington, MA) was added to test for possible effects of contaminating PHA-P. 4ß-phorbol 12ß myristate 13∝ acetate (PMA) was obtained from Sigma Chemical Co. Pokeweed mitogen (PWM) was obtained from Gibco Laboratories, Chagrin Falls, OH.

Cell Culture. Cells were routinely cultured in 96-well microtiter plates (Flow Laboratories, McLean, VA) at 2×10^5 cells per 0.2 ml culture volume in a humidified 37°C incubator with 5% CO_2. Medium used for culture was a Ham's-Iscove's mixture with 15% fetal calf serum and additional supplements described elsewhere (109). DNA synthesis was assessed by uptake of an 18 hr pulse of 1 µCi ^{3}H-thymidine (New England Nuclear). Cultures were harvested from two to eight days after initiation by previously described techniques (109). The data are shown as the

mean ^{3}H-thymidine uptake in three to five replicate cultures ± standard error of the mean (SEM).

Assay for Ig secretion. Cultures of certain leukemic cells were assessed for Ig secretion by radioimmunoassay (RIA) of culture supernatants. Following culture from d0 to d4 (d5) in the presence or absence of anti-IgM MoAb and /or T cell supernatant, cells were washed four times with warm medium while in culture wells and subsequently recultured for an additional three to four days in medium without anti-IgM MoAb or T cell supernatant. The washing and reculture procedure was performed to remove soluble anti-IgM Ab from the first culture period that might interfere with detection of secreted IgM by the RIA. Evidence for the removal of anti-IgM Ab was obtained upon observing that the addition of anti-IgM Ab to IgM secreting cultures just prior to the wash and reculture period resulted in no loss in amount of IgM detected by RIA. At the end of the second culture period, the pooled supernatants from quintuplicate cultures were evaluated for the presence of IgM, IgG, and IgA1 antibody by a modification of a solid phase RIA described previously (97,156). Briefly, polyvinylchloride wells were coated with purified anti-human kappa MoAb at 20 µg/ml in PBS. Serial dilutions of culture supernatant in 1% BSA-containing PBS were then incubated on the coated wells. Various concentrations of purified human IgM kappa, IgG1 kappa, and IgA1 kappa myeloma proteins were also incubated on anti-kappa coated wells. Bound Ig was detected with ^{3}H-labeled MoAb probes to human Ig classes which have been described elsewhere (^{3}H-HB57 anti-IgM, ^{3}H-DC anti-pan IgG, and ^{3}H-CBA1C-7B10 anti-IgAl [156]). The amount of Ig of each isotype in culture supernatant was determined by use of standard curves established with the appropriate human Ig myeloma proteins. The thresholds of sensitivity of these RIAs for IgM, IgG, and IgA1, were 4, 31, and 8 ng/ml, respectively.

RESULTS

Identification of two leukemic clones with receptivity to signal transduction by soluble anti-IgM ligand. Membrane IgM-positive malignant cell preparations from five patients with hairy cell leukemia, five patients with chronic lymphocytic leukemia, and one patient with poorly differentiated lymphocytic lymphoma were tested for possible modulation of in vitro DNA synthesis by a

murine monoclonal anti-human IgM MoAb (HB57). This ligand has previously been shown to be mitogenic in soluble form for normal human B lymphocytes (109). The data in Table IX indicate that within this leukemic panel, two HCL clones showed striking and distinctive responses to culture with a 100 µg/ml concentration of the soluble anti-IgM Ab. KON leukemic cells, which exhibited no background DNA synthesis, showed very significant increases in ^{3}H-thymidine incorporation in the presence of the ligand. In contrast, LUB cells exhibited a high level of background DNA synthesis which was suppressed, i.e. 94% inhibition, by this MoAb. The above responses were not restricted to the use of the HB57 MoAb. Identical stimulatory and inhibitory phenomena were observed with a polyclonal F(ab)'$_2$ preparation of affinity-purified goat anti-human IgM antibody (data not shown). The two hairy cell leukemia clones which manifest responsiveness to anti-IgM ligands were both characterized by an unusual hyperleukocytosis for this form of leukemia. It remains to be determined whether receptivity to anti-IgM mediated signals is characteristic of such forms of this malignancy.

An extended kinetic analysis of the in vitro DNA synthesis observed with KON and LUB leukemic cells both in the presence and absence of anti-IgM ligand is shown in Fig.13. KON cells stimulated with HB57 Ab showed maximal and sustained levels of ^{3}H-thymidine uptake from d4-d8 of culture. This DNA synthesis did not result in extensive and prolonged in vitro proliferation, however (data not shown). The spontaneous DNA synthesis characteristic of LUB cells peaked at d4 of culture. This also was a transient phenomenon in that cell lines of these cells could not be established without transformation by Epstein Barr Virus (data not shown). LUB cells cultured in the presence of anti-IgM ligand showed suppressed levels of DNA synthesis throughout the one to seven days of culture (Fig. 13), which did not reflect a ligand-induced decrease in viability (data not shown).

As indicated in Fig. 14, a striking difference was noted in dose requirements for stimulatory signal transduction in KON cells versus inhibitory signal transduction in LUB cells. While greater than 95% inhibition of DNA synthesis was achieved by concentrations of HB57 ligand as low as 10 ng/ml, stimulation of DNA synthesis in KON cells was best achieved by the highest

Table IX. Responses of Membrane IgM-positive Malignant B Cell Populations to Soluble Anti-IgM Antibody

Donor	Leukemic Classification	Cells/mm^3	cpm ^{3}H-Thymidine Uptake ($\bar{X}$ ± SEM)		
			Medium	MOPC-21 (MOPC-245)	HB-57 Anti-IgM MoAb
KON	HCL	340,000	353 ± 48	445 ± 99	15,936 ± 1,049
LUB	HCL	262,000	11,147 ± 137	11,350 ± 440	655 ± 43
LIE	HCL	35,400	1,632 ± 183	2,380 ± 166	2,350 ± 56
ZAL	HCL	89,000	255 ± 30	ND	363 ± 22
SAP	HCL	Spleen	2,131 ± 324	2,234 ± 522	4,212 ± 350
LAN	CLL	324,000	219 ± 2	161 ± 6	175 ± 11
SCH	CLL	66,800	94 ± 7	235 ± 22	157 ± 6
DAV	CLL	506,000	470 ± 123	732 ± 17	143 ± 42
WHI	CLL	80,000	399 ± 44	ND	753 ± 423
BRE	CLL	330,000	118 ± 12	ND	140 ± 44
SCH	PDLL	149,000	395 ± 61	ND	259 ± 24

Populations of peripheral blood cells (exception = SAP spleen) of either HCL, CLL, PDLL classification were characterized as positive for mIgM by visual immunofluorescence microscopy and cultured at 2×10^5 per well in the presence of 100 μg/mL HB-57 anti-IgM MoAb or MOPC-21 (MOPC-245) myeloma protein as a murine IgG1 control. After 48 to 72 hours, the cultures were pulsed with 1 μCi per well ^{3}H-thymidine and cultures continued for an additional 18 hours. Cells were harvested onto glass filters and counts per minute of ^{3}H-thymidine incorporation was determined by scintillation counting techniques.

Abbreviation: ND, not done.

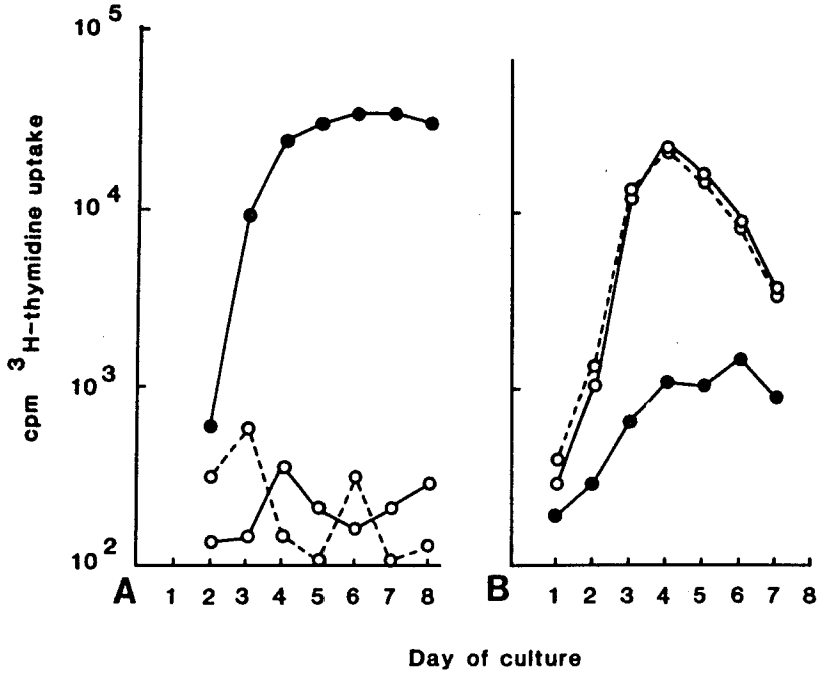


Fig 13. Time course of the positive and negative effects of soluble anti-IgM Ab on DNA synthesis of cultured KON and LUB HCL B cells. Cells were incubated with 100 μg/mL of soluble HB-57 anti-IgM MoAb, control MOPC-245 myeloma protein or medium alone for various intervals. DNA synthesis was assessed by uptake of an 18-hour pulse of 1 μCi ^{3}H-thymidine at the termination of the culture period. The data represent the mean of cpm ^{3}H-thymidine uptake in quintuplicate cultures. O---O, Medium only; O—O, +100 μg/mL MOPC-245; ●—●, +100 μg/mL HB-57 anti IgM.

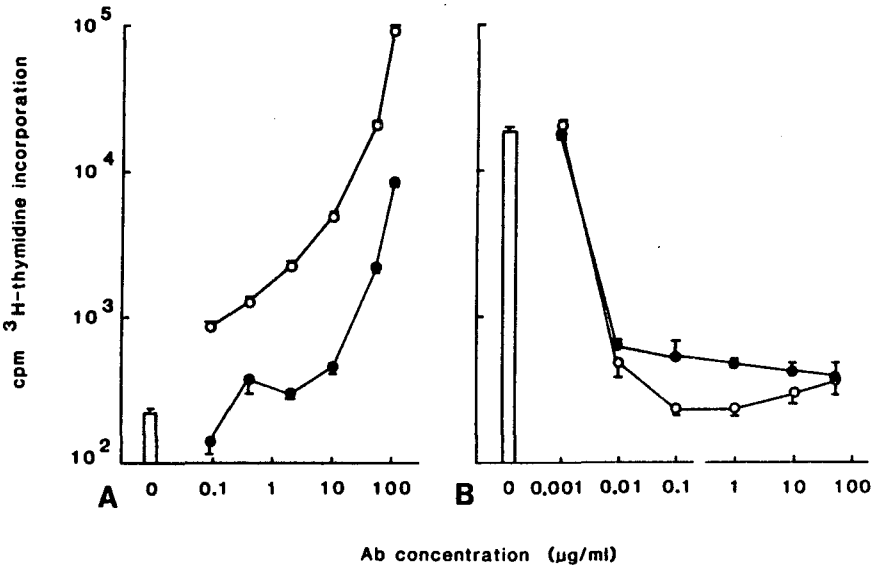


Fig 14. Comparison of the response of KON and LUB leukemic B cells to various concentrations of intact (●) or F(ab)$_2$ fragments (O) of HB-57 anti-IgM MoAb. The incorporation of an 18-hour pulse of ^{3}H-thymidine was measured on d5 (KON cells) or d4 (LUB cells).

concentration of Ab tested, i.e. 100 µg/ml. The Fc portion of the antibody ligand did not contribute to the transduction of inhibitory signals in LUB cells as indicated by the identical inhibitory effects of intact and $F(ab)'_2$ forms of HB57 anti-IgM antibody (Fig. 14). However, the presence of the Fc portion of the ligand did dampen its stimulatory effects for KON cells since $F(ab)'_2$ fragments consistently induced higher levels of ^{3}H-thymidine uptake than the intact Ab (Fig. 14). Similar Fc-mediated down regulation of the mitogenic properties of anti-Ig ligands has been previously observed with normal B cells (75,157). Membrane IgM cross-linking was necessary for both forms of signaling in that Fab' fragments of HB57 were functionally compromised (data not shown).

Modulatory effects of activated T cell supernatant on signal transduction through membrane IgM in KON leukemic cells. Activated T cell supernatants and purified T cell factors, such as B cell growth factor (BCGF) and interleukin 2 (IL2) have been shown to exhibit synergy with soluble anti-IgM Ab in inducing the proliferation of normal resting B cells (70,86,109,158) and some malignant B cell populations (137,159,160). The T cell factors are thought to promote the cell cycle progression of B cells which have received activation signals from membrane IgM cross-linking events. Our studies with KON leukemic cells, however, showed that the behavior of these cells in culture with activated T cell supernatants differed significantly from that of normal polyclonal populations of peripheral blood B cells (Table X).

Firstly, unlike normal B cell populations, KON cells showed significant increases in ^{3}H-thymidine uptake when cultured with T cell supernatant alone. The increases were manifest as early as two days of culture and peaked on day 4 of culture. T cell supernatants from three distinct lots tested all had maximal mitogenic effects at the highest concentration tested, i.e. 25%, but could induce significant DNA synthesis at concentrations as low as 1% (data not shown). The KON cell activating moiety in the T cell supernatants is not due to residual PHA-P used to stimulate T cell factor production since, as shown in Table XI, a concentration of anti-PHA-P, which can completely abrogate the response of peripheral blood T cells to PHA-P, did not inhibit the leukemic cell response to T cell supernatant. Furthermore, KON cells showed no response to a

Table X. Effect of Activated T Cell Supernatant on DNA Synthesis of Normal and Leukemic B Cells in the Presence and Absence of Anti-IgM Ab

Cells	100 μg/mL HB-57 Anti-IgM	cpm ^{3}H-Thymidine Uptake ($\bar{X}$ ± SEM)	
		No T Cell Supnt	+25% T Cell Supnt
Normal ER-peripheral blood cells	−	1,680 ± 57	1,961 ± 256
	+	4,343 ± 210	11,615 ± 883
KON leukemic B cells	−	145 ± 32	14,586 ± 948
	+	24,311 ± 898	3,855 ± 941
LUB leukemic B cells	−	32,643 ± 3,574	29,438 ± 1,045
	+	1,688 ± 176	2,209 ± 180

Normal human peripheral blood B cell preparation (ER negative cells) and KON and LUB leukemic B cells (2×10^5 per culture) were tested for responsiveness to soluble anti-IgM Ab in the presence and absence of 25% supernatant from activated T cell cultures. The data were obtained from three to five replicate cultures harvested on d4.

Abbreviation: Supnt, supernatant.

Table XI. Mitogenic Activity of T Cell Supernatant for KON Leukemic B Cells Is Not Due to Contaminating PHA-P*

Cells	Stimulant	cpm ^{3}H-Thymidine uptake ($\bar{X}$ ± SEM)	
		No Ab	+ Anti-PHA-P (50 μg/mL)
KON	None	177 ± 39	197 ± 42
	T cell supnt (25%)	4,267 ± 157	4,041 ± 141
	PHA-P (0.5 μg/mL)	299 ± 64	252 ± 32
PB T cells (ER+)	None	560 ± 216	295 ± 79
	T cell supnt (25%)	556 ± 139	445 ± 44
	PHA-P (0.5 μg/mL)	13,720 ± 781	246 ± 65
	PWM (1:400 vol/vol)	18,242 ± 1,543	17,247 ± 3,697

*KON leukemic PB cells or ER positive cells from normal PB were cultured at 2×10^5 per well with the various activators in the presence or absence of 50 μg/mL purified anti-PHA-P Ab. The concentration of PHA-P mitogen tested was that which would be maximally expected to be present in cultures containing 25% activated T cell supernatant. KON cells were harvested on d4, normal T cells were harvested on d3 following an 18-hour pulse with 1 μCi ^{3}H-thymidine.

concentration of PHA-P which would be maximally present in cultures containing 25% T cell supernatant.

A second and more surprising finding with KON cells, shown in Table X, was that co-culture with both anti-IgM Ab and T cell supernatant resulted in an antagonism in the induction of DNA synthesis, i.e. the ^{3}H-thymidine uptake was significantly below that observed with either activator alone. The suppressed DNA synthesis noted on d4 of culture (Table X) did not reflect a shift in the kinetics of peak ^{3}H-thymidine uptake in that significant inhibition was noted from d2-d8 of culture (data not shown). In contrast to KON cells, LUB cells did not respond to culture alone with T cell supernatant by further increases in ^{3}H-thymidine uptake nor did the addition of T cell supernatant modulate the inhibitory effect of anti-IgM Ab on the background DNA synthesis of these cells.

The data shown in Table X suggest that, after exposure to factor(s) from activated T cells, KON cells become receptive to inhibitory rather than stimulatory signals following membrane IgM cross-linking. Further analysis of this phenomenon revealed that inhibitory signal transduction could be achieved by HB57 anti-IgM MoAb concentrations which were orders of magnitude lower than those required for transduction of stimulatory signals in the absence of T cell supernatant (Table XII). Although the DNA synthesis in T cell supernatant-stimulated cultures containing high concentrations (200 μg/ml) of HB57 anti-IgM Ab was significantly less than that observed in cultures with either activator alone, significant levels of DNA synthesis were still detectable in these cultures, i.e. 7,689 ± 258 cpm ^{3}H-thymidine uptake. This likely reflects a residual, although compromised, stimulatory signal from the high concentration of HB57 anti-IgM Ab, which in the absence of T cell supernatant, induced 72,789 ± 2,400 cpm ^{3}H-thymidine uptake. Supportive of this is the fact that anti-IgM MoAb XG9, which is not mitogenic for KON cells, causes >95% inhibition of T cell supernatant-induced DNA synthesis at ligand concentrations ranging from 0.5 to 200 μg/ml. It should be noted parenthetically that the difference in stimulatory signaling properties of HB57 and XG9 MoAbs has also been observed with normal B lymphocytes (109) and reflects the distinct epitope specificity and cross-linking potential of these antibodies

Table XII. Inhibitory Signal Transduction in T Cell Supernatant Stimulated KON Leukemic Cells Is Achieved by Low as Well as High Concentrations of Anti-IgM Ligands

MoAb	Conc (μg/mL)	cpm ^{3}H-Thymidine Uptake ($\bar{X}$ ± SEM)	
		No T Cell Supnt	+25% T Cell Supnt
None	—	155 ± 19	16,927 ± 690
HB57 anti-IgM	200	72,789 ± 2,400	7,689 ± 258
	50	12,462 ± 352	2,205 ± 132
	10	575 ± 82	753 ± 176
	0.5	375 ± 126	557 ± 5
	0.05	265 ± 88	2,908 ± 29
	0.005	188 ± 34	12,594 ± 912
XG9 anti-IgM	200	395 ± 26	627 ± 64
	50	319 ± 16	506 ± 57
	5	273 ± 59	549 ± 57
	0.5	108 ± 18	646 ± 45
	0.05	144 ± 22	2,438 ± 59
	0.005	102 ± 49	15,221 ± 395

2×10^5 KON leukemic B cells were incubated in the presence or absence of activated T cell supernatant with various concentrations of HB-57 or XG9 anti-IgM MoAbs. Cultures were terminated on day 5 of culture after an 18-hour pulse with ^{3}H-thymidine.

(S. Rudich and P. Mongini, manuscript in preparation).

The anti-IgM Ab-mediated inhibitory signal transduction observed in T cell supernatant-activated KON cells mimics that observed with LUB cells (Fig. 14). Thus, inhibition in both cases was mediated by very low ligand concentrations, and in both cases $F(ab)'_2$ fragments of anti-IgM MoAb were as efficient at transducing inhibitory signals as intact Ig (data not shown with T cell supernatant-activated KON cells).

Anti-IgM Ab does not induce terminal differentiation of leukemic cells. The negative effect of anti-IgM Ab on T cell supernatant-induced KON DNA synthesis and spontaneous LUB cell DNA synthesis does not reflect an induced differentation to immunoglobulin-secreting plasma cells. Thus, KON cells did not secrete IgM, IgG, or IgA1, above the threshold of detection of the radioimmunoassays described in Materials and Methods, when precultured either in the presence or absence of anti-IgM ligand and/or T cell supernatant. While LUB cells did secrete a substantial amount of IgM after preculture in medium without Ab (2,920 ng/ml), the preculture of these cells with 0.010 to 100 μg/ml HB57 resulted in a significant reduction in subsequent IgM production (≤6 ng/ml). Comparable inhibition of Ig secretion from CLL lymphocytes by anti-idiotype Ab has been previously reported (27). There was no indication that the inhibition of IgM secretion in LUB cells was accompanied by a shift in the isotype of Ab synthesized (data not shown).

Culture of KON cells with both anti-IgM MoAb and mitogenic phorbol ester does not result in inhibition of DNA synthesis. Anti-IgM Ab did not induce a negative signal for DNA synthesis when added to KON cell cultures with another independent activator of these leukemic cells, i.e. 0.8 μM phorbol myristate acetate (PMA). Rather, the co-culture of 1 μg/ml HB57 anti-IgM Ab and 0.8 μM PMA resulted in ^{3}H-thymidine uptake somewhat greater than that seen with either stimulus alone. Thus, the ^{3}H-thymidine incorporation (cpm) above background in cultures containing anti-IgM MoAb alone, PMA alone, or the combination of anti-IgM MoAb and PMA was 730 ± 22; 17, 981 ± 907; and 29,264 ± 767, respectively (background = 318 ± 49). Similar phenomena were observed when doses of PMA as low as 0.0008 μM were tested (data not shown).

Comparison of cell surface antigens on KON and LUB hairy cell leukemia lymphocytes.

Cell membrane antigen expression on the two HCL cell populations which, in the absence of T cell factors, displayed contrasting responses to anti-IgM ligands was evaluated by use of the FACS. The data shown in Table XIII indicate that both KON and LUB leukemic cell populations were positive for IgM, kappa. While the KON cell population was brighter than the LUB population, both HCL clones expressed substantially greater amounts of mIgM than all studied CLL clones (data not shown). A very low level of normal B cell contamination in the KON cell preparation was indicated by an incidence of lambda light chain positive cells of only 1.4% above the gate set for 5% positive cells with an isotype matched control MoAb, J606. By this same comparison, the LUB cell preparation contained 6.1% lambda-positive cells above background.

Both KON and LUB leukemic cells clearly expressed CD20 pan B cell molecules and HLA-DR molecules as indicated by strong positive staining with MoAb B1 and MoAb S157, respectively. In addition, they both showed positive expression of an activation-associated molecule recognized by 4F2 and expression of CD19 (B4) and CD5 (Leu1-91d4). The latter is a membrane molecule found on all T lymphocytes, a small subpopulation of normal B cells and most chronic lymphocytic leukemias (138,145). Both cell populations were very weakly stained by B2 (CD21), a marker most clearly expressed on less mature B cells (153). Neither KON or LUB cells showed significant staining with J5 (CD10) or PC-1, indicators of precursor B cells and plasma cells, respectively (154,155).

The major surface marker differences between KON and LUB cells appeared to be in the expression of membrane IgD and the B cell subpopulation marker FMC7, which is found on 50% of normal peripheral blood B cells and on most HCL (146,147). While KON cells were consistently positive for mIgD and FMC7, the expression of these molecules on LUB cells was so weak as to be questionable.

It should be noted that in multiple instances the fluorescence intensity of leukemic cells positively stained with the B cell-directed Abs overlapped with that of control populations and thus the percentage of cells gated as positive was less than 100%. Since the staining profiles of both

Table XIII. FACS Analysis of Membrane Antigen Expression on KON and LUB Leukemic Cells

Ab Tested	Specificity	KON Leukemic Cells		LUB Leukemic Cells	
		Percent Positive*	ΔMFI†	Percent Positive*	ΔMFI†
HB57	IgM	91.9	92	84.2	75
CBDA-4E5	IgD	80.6	45	7.5	10
HB61	Kappa light chain	94.3	102	80.2	64
PMG3A-4C6	Lambda light chain	1.4	−3	6.1	9
MθP-9 (LeuM3)	Pan monocyte	2.4	−11	3.7	6
89bl (CD3)	Pan T cell	−2.3	−6	1.0	0
B1 (CD20)	Pan B cell	92.1	125	85.9	76
B2 (CD21)	Limited B cell	26.8	15	8.7	14
B4 (CD19)	Pan B cell	76.9	37	57.1	32
FMC7	Limited B cell	37.7	20	13.2	7
J5 (CD10)	CALLA	3.2	0	−0.6	−1
PC-1	Plasma cell	7.7	5	12.6	15
Leu 1‡ (CD5)	Pan T and limited B	88.3	48	57.5	37
4F2	Activation antigen	85.3	43	31.4	26
S157	HLA-DR	84.1	75	89.1	72
5E9	Transferrin receptor	0.0	3	2.7	6

*The percent positive values represent the percent of cells that showed staining above background with the fluorescence threshold (gate) set at 5% positivity with each respective isotype control Ab. The values shown are the mean from two experiments in which frozen aliquots of the same leukemic cell preparation were used for each replicate experiment.

†The ΔMFI (mean fluorescence intensity) values were determined by subtracting the mean channel fluorescence observed with isotype matched control MoAb from that observed with the test MoAb. The values shown are the means from two experiments in which the logarithmic amplification of the fluorescence signal was similar (60 channels per decade in first experiment; 71 channels per decade in the second experiment on a 255 channel scale).

‡Leu 1 MoAb was used in one experiment while 91d4 MoAb, which recognizes the same molecule as Leu 1 and gave identical profiles to Leu1, was used in the second experiment.

Table XIV. Functional Responses of KON and LUB Leukemic B Cells Depleted of Contaminating T Cells and Monocytes

		^{3}H-Thymidine Uptake ($\bar{X}$ ± SEM) Pre-culture Treatment With				
Cells	D 0-4 Culture In	Medium	C	Pan T MoAb	Pan Monocyte MoAb	Pan T + Pan Monocyte MoAbs + C
KON	Medium	615 ± 84	509 ± 48	250 ± 22	149 ± 32	252 ± 73
	1 μg/mL anti-IgM	589 ± 100	1,037 ± 144	439 ± 14	283 ± 120	556 ± 42
	100 μg/mL anti-IgM	14,327 ± 1950	36,398 ± 2705	23,357 ± 314	20,912 ± 131	31,355 ± 1572
	25% T cell supnt	15,924 ± 772	21,324 ± 1108	17,208 ± 636	12,758 ± 4214	15,846 ± 399
	1 μg/mL anti-IgM + 25% T cell supnt	691 ± 26	1,787 ± 254	676 ± 74	712 ± 25	806 ± 36
LUB	Medium	34,456 ± 1386	46,826 ± 1419	55,545 ± 1640	45,738 ± 4820	40,327 ± 836
	1 μg/mL anti-IgM	298 ± 64	318 ± 84	420 ± 35	331 ± 46	217 ± 13

KON and LUB cells were depleted of possible contaminating T cells and monocytes by treatment with pan T cell MoAb, and pan monocyte MoAbs and complement as described in Materials and Methods. 2×10^5 treated cells were then cultured with medium, 1 or 100 μg/mL HB-57 anti-IgM MoAb, and/or 25% activated T cell supernatant for four days. DNA synthesis was assessed by uptake of ^{3}H-thymidine during the last 18 hours of culture.

LUB and KON cells with these Abs never indicated a bimodal distribution (data not shown), the percent positivity in these cases likely represents the proportion of cells bearing the antigen above a certain threshold level of detection.

The functional characteristics of KON and LUB leukemic cell populations are not due to contaminating T cells and monocytes. The absence of consistently measurable numbers of contaminating T cells and monocytes by FACS analysis was characteristic of both the KON and LUB cell preparations (Table XIII). Nevertheless, to eliminate the possibility that a low level contamination with these cells might contribute to the functional characteristics of the leukemic cell population, we tested the functional reactivity of KON and LUB cells following treatment with monocyte-specific and T cell-specific cytotoxic Abs plus complement. For depletion, conditions were used that had been established to specifically deplete monocytes and T cells from non-leukemic peripheral blood (see Materials and Methods section). The data in Table XIV indicate that all the phenomena seen with the untreated cell populations remain intact in the cell populations treated with the specific MoAbs and C.

DISCUSSION

The data presented here document that certain clonal populations of hairy cell leukemia B lymphocytes can receive either stimulatory or inhibitory signals for DNA synthesis when cultured in the presence of soluble anti-IgM antibody-supplemented medium. Inherent susceptibility to inhibitory signal transduction was evidenced in a leukemic clone, LUB, which exhibited significant background DNA synthesis. In contrast, stimulatory signal transduction was detected in a distinct clone, KON, which showed no background DNA synthesis. These studies substantiate a growing set of observations that certain transformed B cell populations are extraordinarily susceptible to in vitro growth regulation by membrane Ig cross-linking ligands (8,9,28,159-163). The particular finding that one hairy cell leukemic clone, KON, receives stimulatory signals for DNA synthesis from soluble anti-Ig ligands without the need for accessory T cell-derived factors is uncommon but is in agreement with the recent report of Baeker and Rothstein with a poorly differentiated

lymphocytic lymphoma (28). Since B cell malignancies likely represent clonal expansion of normal B cell subsets, these latter observations are of particular importance in that they strongly imply that ancillary T cell factors are not obligatory for the proliferation of all human B cells and that membrane IgM cross-linking can, at least in some instances, suffice as the activating signal.

It should be emphasized that the DNA synthesis observed in KON leukemic cell preparations is highly unlikely due to the proliferation of contaminating normal B lymphocytes. First, as just mentioned, the anti-IgM Ab-induced KON cell DNA synthesis was independent of accessory T cell factors while normal peripheral blood B cells required ancillary T cell factors for optimal stimulation by this ligand. Second, FACS analysis, as well as visual cell surface immunofluorescence analysis, showed KON cells to contain insignificant numbers of lambda light chain positive cells, i.e. $\leq 2\%$. This implies a maximal normal B lymphocyte contamination of 4%. Third, when normal B cells are a cultured at 2×10^4 cells/culture (the number expected if normal B cells constituted even 10% of the leukemic cell preparation) no ^{3}H-thymidine incorporation above background is observed (unpublished results).

A unique observation made in this study was that activated T cell supernatants could modulate the outcome of membrane IgM-mediated signal transduction in the KON leukemic B cell population such that negative rather than positive effects on DNA synthesis were observed. This phenomenon became initially apparent on noting that KON cells responded with DNA synthesis to activated T cell supernatant alone or to high concentrations of soluble anti-IgM antibody alone but that they exhibited significantly depressed levels of DNA synthesis when cultured with both activating moieties simultaneously. Although observed at all ligand concentrations, anti-IgM Ab-mediated inhibitory signal transduction was most pronounced at very low Ab concentrations that were non-mitogenic in the absence of T cell supernatant. Interestingly, the phorbol ester-induced DNA synthesis of KON cells was completely resistant to anti-IgM Ab-mediated inhibitory signal transduction. This resistance may reflect the recently described capacity of PMA to block anti-IgM-mediated phosphatidylinositol metabolism and cytoplasmic Ca^{2+} increases in murine B cells (164,165), although other explanations are also possible.

The antagonistic phenomenon exhibited when anti-IgM Ab and T cell supernatant were combined in culture was not due to supra-optimal signaling via a common activation pathway since (a) non-mitogenic anti-IgM MoAbs were capable of inhibiting T cell supernatant-induced KON cell DNA synthesis, and since (b) suboptimally mitogenic concentrations of anti-IgM MoAb HB57 inhibited the DNA synthesis induced by suboptimally mitogenic concentrations of T cell supernatant (unpublished results). Furthermore, the T cell supernatant-induced change in the nature of mIgM-mediated signaling in KON cells did not reflect a T cell factor-induced modulation of mIgM expression since FACS analysis of KON cells incubated with T cell supernatant for 2, 6, 24, 36, or 44 hours revealed levels of mIgM not significantly different from control cells (unpublished results).

Although it has previously been suggested that negative regulation of DNA synthesis through mIgM cross-linking is a phenomenon intrinsic to immature B cells and that positive signaling of DNA synthesis through mIgM is a phenomenon representative of more mature B cell subpopulations (10,158), our observations with KON cells indicate that certain B cells expressing a mature functional phenotype can also be negatively regulated in cell cycle turnover by membrane IgM cross-linking events. In the KON clone, susceptibility to inhibitory or stimulatory signal transduction appears to be determined by the presence or absence of a T cell-derived factor(s) that can independently activate the B cells. In this context, the observations of Linton and Klinman that recently stimulated secondary B cells exhibit a period of tolerance susceptibility to membrane Ig-binding ligands (166,167) may be particularly relevant.

The negative signal transduction in T cell supernatant-activated KON cells appears to be analagous to the mIgM-mediated inhibitory signaling inherent to LUB leukemic cells. We have considered it possible that LUB cells secrete an autocrine growth factor comparable to the KON cell stimulatory growth factor in activated T cell supernatants and that the mechanism of inhibition exhibited in both cell types is identical. However, extensive experiments have revealed no evidence for synthesis of such a growth factor by LUB cells. Thus, unlike lines which secrete autocrine growth factors (107,140,168-170), conditioned medium from LUB cells, as well as cell

lysates, did not augment the growth of limiting concentrations of LUB cells and did not induce S phase entry of either KON cells or anti-IgM Ab-stimulated normal B lymphocytes (unpublished results). It is alternatively possible that LUB leukemic cells express certain oncogenic elements that maintain them in a state analagous to the activation state reached by KON cells after exposure to T cell factors. In this regard, the leukemic cells described here could provide interesting models for testing the hypothesis that oncogenes mimic the biochemical events associated with certain ligand-receptor interactions (171-173).

Analysis of cell membrane antigen expression on peripheral blood isolated KON and LUB leukemic cells indicated clearly that these hairy cell leukemia cells are of the B cell lineage. Since normal B lymphocytes have been shown to decrease their mIgD expression following activation (174), the presence of significant mIgD on KON cells but little or no mIgD on LUB cells is consistent with LUB cells representing a slightly more activated state. It is of interest that neither cell population exhibited a true resting B cell phenotype in that both expressed substantial amounts of the activation associated molecule, 4F2 (101). However, these leukemic lymphocytes may represent activated cells which have reverted to a resting "secondary" 4F2 - positive state as recently described in normal T cells by Suomalainen (175). Consistent with this is their lack of significant transferrin receptor expression.

Although we have not yet identified the precise cytokine(s) from activated human T cells responsible for both activating the cells and modulating membrane IgM-mediated signaling, these effects of unseparated factors from polyclonally activated T cells on an isolated hairy cell leukemic B cell clone are likely relevant to the regulation of leukemic growth in vivo. The findings imply that activated T cells may have major roles in expanding the in vivo growth of certain leukemic B cell clones and furthermore, that in vivo treatment with mIg-binding ligands may be particularly useful for negatively regulating the T cell factor-enhanced growth of some of these leukemias as well as the spontaneous growth of certain other leukemias.

Since not all mIgM-positive leukemic B cell clones exhibit such exquisite sensitivity to mIg-mediated signal transduction, it would appear that in vitro screening of the responsiveness of

distinct leukemic cell populations to anti-Ig ligands, both in the presence and absence of T cell factors, might increase the likelihood of effective in vivo anti-idiotype immunotherapy (26). Clearly the efficacy of these in vivo Ab treatments at diminishing tumor load is likely to be greatly influenced by the characteristic way in which a malignant B cell population responds to anti-Ig ligands.

SUMMARY

A functional study of several human malignant B cell populations has indicated that occasional leukemic clones are extraordinarily sensitive to signal transduction through membrane IgM. One isolated hairy cell leukemia (HCL) with low background DNA synthesis was stimulated to significant levels of DNA synthesis when cultured with high (100 μg/ml) concentrations of soluble anti-IgM ligands. In contrast to the activation of normal peripheral blood polyclonal B cells, this DNA synthesis was completely independent of accessory T cell factors. Although the HCL clone could also be induced to enter S phase by incubation in media supplemented with only activated T cell supernatant, culture of the clone with activated T cell supernatant plus anti-IgM Ab resulted in DNA synthesis that was significantly less than that induced by either activator alone. Factor(s) in T cell supernatant appear to modulate the leukemic clone so that the binding of ligand to membrane IgM is perceived as an inhibitory rather than a stimulatory signal for DNA synthesis. In terms of Ig Fc independence and low ligand dose requirements, anti-IgM Ab-mediated inhibitory signal transduction in the T cell supernatant-activated HCL clone was found to mimic anti-IgM Ab-mediated suppression of the spontaneous DNA synthesis of an alternative HCL clone. The functional results suggest that the type of signal transduced by anti-Ig ligands may reflect differences in the activation state of receptive leukemic B cells.

CHAPTER FIVE

Anti-IgM-Mediated B Cell Signaling: Molecular Analysis of Ligand Binding Requisites for Human B Cell Activation and Tolerance

INTRODUCTION

The precise role of membrane Ig in regulating the physiology of B lymphocytes has been a topic of considerable debate for over a decade. The ensuing investigations have indicated that no single mechanism exists by which mIg communicates the presence of extracellular signals to the B cell. Rather, depending upon the nature of the extracellular ligand, mIg appears able to function in at least three distinct fashions. If the ligand contains a polyclonal B cell activating moiety as part of its structure, mIg can passively focus the activating molecule onto the B cell surface and enable it to directly stimulate the B cell (1). Alternatively, if the ligand has T cell-reactive epitopes, mIg can focus the ligand onto B cells bearing class II major histocompatibility antigens and aid in the presentation of such epitopes to T lymphocytes (20,21,176,177). In this case, the T cells and their products directly stimulate the B cells. Finally, mIg can directly signal biochemical changes within B lymphocytes. This latter phenomenon appears to occur only following interaction with ligands which can cross-link mIg molecules (22,23).

Studies aimed at elucidating the mechanism of direct signal transduction through mIg clearly indicate that not all mIg cross-linking events result in B cell activation, defined as any change in lymphocyte physiology which increases the cells' capacity to mount an immune response (14). Instead, the physiological outcome of the biochemical signal cascade initiated after mIgM cross-linking appears to depend upon the prior differentiation or activation state of the receptive B cell. Immature B cells and certain activated B cell populations can receive inhibitory

(tolerogenic) signals for their clonal expansion or function (15,23,167,178-180), while mature, resting B cells are susceptible to stimulation (69,70).

The mechanism of direct transmembrane signaling through mIg has been predominantly studied with anti-Ig Abs which have the capacity to signal B cells independently of their antigen specificities. Both stimulatory and inhibitory signal transduction with these ligands appear to involve the activation of phospholipase C, which in turn cleaves phosphatidylinositol 4,5-diphosphate (PtdInsP2) (32,45,181,182). Following PtdInsP2 hydrolysis, the induction of both stimulatory and inhibitory signals involves the generation of at least some common second messengers (14,22,23). It has been suggested that the disparate effects on cell proliferation of this common signaling pathway may reflect either subtle variations in the biproducts of inositol phosphate metabolism in different cell types, or alternatively, the presence of distinct elements in the diverse B cell populations which interpret the receptor-generated signals in different ways (23).

Because both stimulatory and inhibitory signal transduction through mIgM involve similar early biochemical reactions, it is intriguing that the dose requirements for achieving stimulation or inhibition have generally been found to differ by one or more orders of magnitude (6,9,42,43,49,63,72,73,178,183). Thus, while inhibition of plasma cell differentiation and DNA synthesis of many transformed B cells can be observed with ng/ml concentrations of anti-Ig ligands, stimulation of DNA synthesis of resting mature B cells by such ligands requires µg/ml concentrations, even in the presence of ancillary T cell growth factors. This suggests that the ligand binding requisites for triggering B cell tolerance may be significantly different from those for triggering B cell clonal expansion.

In this report, we have attempted to rigorously evaluate the binding requisites for eliciting the activation and alternatively, the inactivation of B lymphocytes through mIg cross-linking. By employing a large panel of anti-human IgM monoclonal Abs, we examine how the site specificity, the affinity, and the valency of epitopes bound on mIgM affect the capacity for stimulation or

inhibition of DNA synthesis in the appropriately-sensitive human B lymphocytes. The data shown clearly indicate that the binding requisites for inducing inhibition of B cell DNA synthesis are much less stringent than those for stimulation. This may reflect differences in the size of the cross-linked mIgM cluster and/or differences in the necessity for continuous mIgM cross-linking for achieving these distinct functional events. Since the capacity of human B cells to be actively signaled by murine anti-IgM MoAbs likely extends to autologous anti-Ig Abs, these studies provide considerable insight into the factors controlling the potential of anti-idiotype Abs and rheumatoid factors to act as positive or negative regulators of human B cell function.

MATERIALS AND METHODS

Murine Anti-Human IgM MoAbs. The preparation, isolation, purification, and characterization of all ten murine anti-human IgM MoAbs used in these studies, ie. XG9, P19, HB57, P24, Mu18, Mu53, 5D7, 4-3, 1G6, and IF11 have been detailed in previous publications (109,184). With the exception of 5D7 (IgG2a murine isotype), all MoAbs are of the IgG1 isotype.

Preparation of Anti-IgM MoAb Fab' and $F(ab')_2$ Fragments. Fab' fragments of most anti-IgM MoAbs were obtained from the reduction of the corresponding $F(ab')_2$ fragments prepared by pepsin digestion (185). Optimal conditions for $F(ab')_2$ isolation for each MoAb were initially determined by 'mini-prep' analysis in which the temperature, time, and pH of the digestion as well as the enzyme-to-substrate (E:S) ratio was varied. Mini-preps were run on 5-20% SDS-PAGE gels (184) as a first indicator of the efficiency of the reaction. Those preps which were acceptable by SDS-PAGE, ie. showing little or no undigested IgG and predominately one $F(ab')_2$ band, were then analyzed for IgM-binding ability by solid-phase RIA (109) using a tritiated rat anti-mouse kappa light chain MoAb as probe (kindly provided by Dr. Constantin Bona). Reaction conditions which yielded optimum results on both the gel and RIA assays were found to vary between the different MoAbs. In general, for $\gamma 1$ Abs, the conditions were: buffer of 0.1M citrate,

pH 3.6, temperature of 37°C, an E:S ratio of 1:60, and a reaction time of between 1 - 1 1/2 h. However, MoAb IF11, under such conditions, only yielded Fab' fragments. No set of conditions were identified for optimal production of $F(ab')_2$ fragments from the IgG2a Ab 5D7. This MoAb had a propensity for digestion to Fab' fragments only. Conditions used for Fab' fragment production of this Ab were: buffer of 0.1 M citrate, pH 4.2, temp of 37°C, E:S ratio of 1:20 , and a reaction time of 3 h. Large scale preparation of the fragments used in this study involved digesting > 25 mg of purified MoAb with the appropriate volume of a 5 mg/ml solution of pepsin (Sigma Chemical Co.). Digestions were terminated by raising the pH of the mixture to >8 by the addition of solid Tris (Sigma Chemical Co.). Any potentially undigested intact Ab was removed by passage of the digest over protein-A Sepharose (Pharmacia) as described (92). Non-bound MoAb was vacuum concentrated in 0.01 M Tris-HCl, 0.15 M NaCl, pH 7.3 (TBS). $F(ab')_2$ fragments (or Fab' fragments in the cases of MoAbs 5D7 and IF11) were further purified by sieving the protein-A non-bound material through a calibrated Sephacryl S-200 gel filtration column (Pharmacia) eluted in TBS and run at < 3 ml/cm^2-h. In all cases a single sharp peak was obtained and vacuum concentrated in PBS- 0.01% azide (PBS-A) to ≥1 mg/ml. Analysis of fragments in a 5-20% gradient SDS-PAGE gel under non-reducing conditions revealed only one major band at an average mol. wt of 110 kD (or 52.4 kD for the 5D7 and IF11 MoAbs).

Production of Fab' fragments required the reduction of the isolated $F(ab')_2$ fragments with cysteine (Sigma Chemical Co.; added as a solid to a final conc of 10 mM after sparging the solution with N_2) for 1 - 1/2 h at 37°C. The reduced protein was then alkylated for 1 - 1/2 h at room temp in the dark by adding solid iodoacteamide (Sigma Chemical Co.) to a final concentration of 30 mM. The reduced and alkylated solution was then sieved through a S-200 gel filtration column. Analysis of the single peak obtained by 5-20% gradient SDS-PAGE under non-reducing conditions revealed just one major band at an average mol. wt of 52.4 kD. Calculations based

upon densitometric tracings of the Coomassie Blue stained gels revealed that this main peak accounted for over 95% of stained deflections.

Iodination of MoAb Fab' Fragments. MoAb Fab' fragments were iodinated with carrier-free ^{125}Iodine (New England Nuclear) using a modification of the chloramine-T method (124). In a typical iodination, ~250 µg of freshly-thawed Fab' protein (at a concentration of ≥1 mg/ml in PBS-A) was labeled with 1 to 1.25 mCi of ^{125}I. After the protein and iodine were added to the reaction vessel (12 x 75 mm plastic test tube), the mixture was buffered by the addition of a volume of 0.25 M phosphate buffer, pH 7.5, equal to that of iodine added. 10 µl of a freshly prepared solution of chloramine-T (Eastman Kodak Chemicals, Rochester, NY) at a concentration of 1 mg/ml in 0.05 M phosphate buffer, pH 7.5 (iodination buffer) was added to the reaction vessel, which was vortexed vigorously for 30 s. After 30 s, 10 µl of freshly prepared sodium metabisulfite (Eastman Kodak Chemicals; 2 mg/ml in iodination buffer) was added to the vessel and again vigorously vortexed. After an additional 60 s, 100 µl of potassium iodide (Fisher Scientific Co., Fair Lawn, NJ; 5 mg/ml in iodination buffer) was added to the vessel. After a final vortexing, the labeled Fab' protein, as well as free ^{125}I, were separated by immediate passage over a PD-10 gel filtration column (Pharmacia) which had been previously equilibrated with 10% BSA-PBS-A and then washed extensively with PBS-A until the OD_{280} = 0.

Immediately after pooling the large mol. wt fractions, trichloroacetic acid (TCA) precipitable protein was measured. The percent TCA precipitable counts for all but one of the iodinated Fab' MoAbs was >97%. The exception, MoAb 5D7, gave a value of ~ 90%.

The specific activity (sp act) of each iodinated MoAb Fab' fragment was determined by quantitating the labeled protein with the Bio-Rad Protein Assay Kit as per the manufacturers' instructions (Bio-Rad, Richmond, CA). Bovine gamma globulin was used as the protein standard. Typical sp acts obtained using this method were between 2 to 6 x 10^6 cpm/µg . After determining the sp act of each iodinated MoAb Fab', the pooled fractions were made 1% with respect to BSA.

Determination of IgM-bindable activity of each labeled Fab' preparation was determined by passing each labeled fragment over an IgM-Sepharose column containing a vast excess of IgM. One ml of packed human IgM-coupled Sepharose (~ 6 mg IgM/ml gel), or one ml of Sepharose beads treated in a identical manner but containing no coupled protein, were packed into Pasteur pipettes. Approximately 80,000 cpm (~26 ng) of each iodinated MoAb Fab' fragment was then slowly loaded and passed through the columns. 98-100% of the labeled protein was consistently retrieved from the control columns. The percentages of IgM-Sepharose-bindable protein were as follows: XG9 = 88.84, P19 = 89.41, HB57 = 91.13, P24 = 87.76, Mu18 = 92.14, Mu53 = 85.12, 5D7 = 92.74, 4-3 = 87.54, 1G6 = 94.21, and IF11 = 84.88.

Most of the iodinated MoAb Fab' fragments were also assayed to compare their relative binding affinities for human IgM with that of the unlabeled parent Fab'. A competition solid-phase RIA (184) was devised on polyvinylchloride (PVC) wells using a limiting amount of human IgM as coating Ag (2 μg/ml in PBS-A). After pre-coating the wells with IgM, a constant, saturating concentration of labeled Fab' was added. Test wells also contained defined amounts of unlabeled Fab' to achieve various ratios of labeled to unlabeled Fab' (1:8, 1:4 .... 4:1, 8:1). After an overnight incubation at 4^{o}C, the plates were washed and the amount of bound radiolabeled ligand was measured with a gamma counter (Beckman Instruments, Palo Alto, CA). The expected as well as observed cpm bound were then compared and used as an indicator of the relative binding efficiency of the iodinated MoAb vs. that of the unlabeled parent. In all cases tested, the observed values did not differ from the predicted by more than 50%, indicating that the iodination procedure did not appreciably diminish the native binding affinity of the MoAb Fab' fragments.

Pentameric and Monomeric Human IgM Isolation.

Pentameric IgM. The IgM myeloma protein "Pan" was used as the source of human pentameric IgM in these studies. Its isolation and purification have been detailed elsewhere (184).

Monomeric IgM. Monomeric units of the IgM macroglobulin Pan were prepared for use in electron microscopy studies by 2-mercaptoethylamine (2-MEA) reduction of the pentamer (186). 'Mini-prep' analysis was first performed to determine optimum 2-MEA concentrations as well as reaction times. The mini-preps were analyzed on 5-15% gradient SDS-PAGE gels. Conditions were never found which gave only IgM monomer. Variables were optimized, however, for a reduction product which contained ~35% monomer (mol. wt 212 kD), ~55% unreacted and high mol. wt reduction products of pentameric IgM (mol. wt >500 kD), and ~10% lower mol. wt reduction products (mol. wt <100 kD). For preparation of IgM monomer, 166 mg of purified IgM pentamer (16.6 mg/ml) was dialyzed vs. 0.05 M Tris-HCl, 0.5 M NaCl, pH 8.2 (TBS-pH 8.2) overnight at 4^{o}C. After warming the protein to 30^{o}C, nitrogen was bubbled through the solution and solid 2-MEA (Calbiochem Biochemicals, La Jolla, CA) was then immediately added to a final conc of 0.003 M. The protein was rotated slowly for 1/2 h at 30^{o}C and then alkylated by the addition of solid iodoacetamide to a final conc of 0.006 M. Alkylation was performed in the dark for 1 h on ice. At the end of 1 h, the reduced protein was dialyzed in the cold vs. TBS, pH 8.2 containing 0.012 M iodoacetamide for 5 h. Monomer IgM was separated from both higher and lower mol. wt reduction products by sieving through a calibrated Sephacryl S-300 gel filtration column eluted with 0.1 M Tris-HCl, 0.15 M NaCl, pH 8.0 and run at < 2.5 ml/cm^{2}-h. Two main peaks and one very minor peak were obtained. Individual fractions from these peaks were analyzed on 5-15% gradient SDS-PAGE gels under both reducing and non-reducing conditions. The ascending limb of the second peak consisted of nearly pure IgM monomer, with a very slight amount of H-L (half-molecules of monomer, mol. wt 97.7 kD). These fractions were pooled, vacuum concentrated vs. PBS-A and stored at -70^{o}C. Densitometric tracings of SDS-PAGE analyses of the pooled protein revealed that > 90% of Coomassie Blue stained deflections were from the monomer band, the remaining 10% were derived almost entirely from a weak H-L band. Final yield of IgM monomer was ~ 8% of theoretical maximum.

Cell sources. Non-malignant human B lymphocytes were obtained as residual surgical splenic fragments from a normal patient having a spleen removed during an operative procedure, from a patient with a splenic cyst, and from a patient suffering from Felty's syndrome (109). Isolated B cell enriched populations were obtained by twice rosetting out T cells with neuraminidase-treated SRBCs as described (109). When analyzed for cell surface markers by indirect immunoflourescence staining with the FACS (FACS IV, Bectin Dickinson), these populations were found to contain >85% Ig-positive and Leu 16 (CD-20)-positive B cells, <1% 89b1 (CD3)-positive T cells (145), and < 2% P9 (Leu M3)-positive monocytic cells (99). In addition, the T cell-depleted cell populations from the spleens were found to contain <4% cells positive for the activation-association molecule bound by MoAb 4F2 (101).

The leukemic B cell specimens used in this study were contributed by Drs. Ilona Szer, Stephanie Seremetis, and Janet Cuttner. The Bia and Lan clonal cell populations were obtained by leukophoresis of patients with chronic lymphocytic leukemia (CLL). Kon and Lub clonal cell populations were obtained from patients with hairy cell leukemia (HCL) and have been described elsewhere (111). At the time of sampling, all donor patients had peripheral white blood cell counts $\geq$ 262,000 cells/mm^3, insuring little or no contamination with non-leukemic cells. This was confirmed by indirect immunofluorescence staining with T cell, monocyte, and B cell-specific MoAb probes (111). FACS analysis of the leukemic cell populations with Ig heavy and light chain-specific MoAbs and a fluorescein-conjugated goat $F(ab')_2$ anti-mouse Ig-probe revealed the following membrane Ig phenotypes: Bia (μ,δ,λ), Lan (μ,δ,k), Kon (μ,δ,k) and Lub (μ,δ,k). B cells from the lymphoblastoid cell line Daudi (ATCC; μ,δ,k) were also used for some ligand-binding experiments. With the exception of Daudi, the individual B cell populations used in these studies were frozen at the same time in multiple aliquots and stored in the vapor phase of liquid nitrogen until use. The T lymphoblastoid cell line KE37 (mIg-negative) was used as a control for non-specific binding in the ligand-binding experiments.

Cell culture conditions. T cell-depleted splenic lymphocytes and leukemic B cells were cultured in a humidified 37°C incubator at 5% CO_2, in flat-bottomed wells of 96-well microculture plates at 2 to 2.5 x 10^5 cells per well in a volume of 0.2 ml. Medium used for all functional assays was a Ham's F-12/Iscove's modified Dulbecco's medium mixture with 15% fetal calf serum and additional supplements as described elsewhere (109). DNA synthesis was assessed by uptake of an 18 h pulse of 1μCi ^{3}H-thymidine (72.5 Ci/mmol; New England Nuclear). Cultures were harvested and ^{3}H-thymidine incorporation was measured (109). Where raw data is illustrated, cpm ± SEM from three identical cultures are reported. Data indicated as Δcpm represents the difference between the cpm in test vs. control cultures. SI values reported represent the quotient obtained by dividing the cpm measured in test wells by the cpm observed in control cultures.

T cell factors. Activated T cell supernatants (supnts) containing growth factors for B cells were obtained from cultures of PHA-stimulated mixtures of peripheral blood cells of allogeneic individuals as described previously (109,111). In some experiments, a partially-purified B cell growth factor (BCGF) preparation was utilized (Cellular Products, Inc., Buffalo, NY) and found to mimic the effects of activated T cell supnts.

Equilibrium-Binding Analysis. Ligand cell-binding assays used for all Scatchard analyses were performed using the phthalate oil method of separating cell-bound from free ligand (187). This method entails a single, rapid sedimentation of cells through a viscous oil and thus has the decided advantage of minimally perturbing the binding equilibrium of label to cells, since no cell washings are necessary. The assay was performed in small (400 μl) eppendorf tubes which were previously coated with DMEM + 1 % BSA + 15 mM sodium azide overnight at 4°C and then dried. This pre-coating was done to decrease the non-specific binding of label to assay tubes. In addition, immediately prior to the start of an experiment, each iodinated MoAb Fab' fragment was ultracentrifuged at ~100,000 x g for 8 mins (Beckman Airfuge, Beckman Instruments) with only the top one-half volume being used for ligand-binding analysis. Media used for the assay

consisted of the above coating medium plus 50 mM 2-deoxy-D-glucose (ICN Biochemicals, Cleveland, O.) to block potential anti-IgM Fab' fragment internalization (188).

Cells used in these assays, which included the leukemic B cells Bia, Lan, and Lub, the B lymphoblastoid cell line Dau, and the T cell line KE37, were purified by centrifugation through Ficoll/Hypaque at 4°C. Interface layers were harvested and washed twice with DMEM + 10% FCS at 4°C. The final cell pellet was then resuspended in assay media and trypan blue viability was determined. In almost all cases, the average viability of the starting cell population was >95%. (The Lan cell population had a starting viability of ≤ 90%). The number of cells used for analyses varied, depending upon the mIgM density of the cell population, the sp act of the MoAb Fab' fragment, and its affinity for IgM. Assays with Bia cells utilized 1.3 to 3.55 x 10^7 cells/ml; those with Dau = 1.22 to 2.5 x 10^7/ml; Lan = 0.6 to 1.75 x 10^7/ml; Lub = 2.54 to 11.85 x 10^7/ml; and KE37 = 2.5 x 10^7/ml.

Non-specific binding of label to cells was always determined in two different ways: a) by assaying the binding of ligand to cells in tubes containing a 100:1 picomolar ratio of pentamer IgM to Fab' ligand, and b) by measuring the binding of iodinated Fab' fragments to the T cell line KE37. Both gave levels of background binding which were not significantly different. In addition, in many experiments, non-specific binding was evaluated by substituting unlabeled Fab' for pentameric IgM. In these instances, the degree of background binding did not significantly change.

Prior to the equilibrium-binding analyses, each MoAb Fab' fragment was assayed to determine the kinetics for reaching equilibrium on the Dau cell line at 4°C (data not shown). Equilibrium was assumed to be attained when a two-fold increase in reaction time did not give more than a 10% increase in the amount of ligand bound to the cell (189). Although equilibrium for most MoAb Fab' fragments was reached by 1/2 to 1 h at 4°C, an incubation time of 2 h was used for each Ab studied.

The equilibrium-binding experiments were performed as follows: 2:3 serial dilutions of iodinated MoAb Fab' fragments in assay media were added with cells to pre-coated eppendorf tubes to a final volume of 200 µl. Triplicates were run when label was used alone, and duplicates were used for assessing background binding with excess IgM. The tubes were slowly rotated for 2 h at 4°C to prevent cell settling. 150 µl of the cell mixture was then quickly removed and gently layered over 150 µl of pre-cooled phthalate oils (a 2:1 vol/vol mixture of dibutyl: dioctyl phthalate [Eastman Kodak Chemicals]) in 7 x 50 mm (400µl) polyethylene microfuge tubes (Beckman) and centrifuged in a microfuge (Beckman Instruments) at 12,000 x g for <1 min at 22°C. Separation of the cell pellet from the non-bound ligand in the aqueous layer was achieved by freezing the tubes at -70°C and sawing off the tube tip. The cell-bound cpm in the pellet was determined with the use of gamma counter. The data were plotted as simple binding isotherms and were analyzed by the LIGAND computer program (190) to give the equilibrium binding constants (Ka) and the number of epitopes bound by MoAb Fab' ligand, in addition to the percent coefficient of variance of these values (190,191).

Propidium iodide analysis. The DNA content of individual nuclei from cultured Kon leukemic cells was measured with the FACS IV using propidium iodide (PI) as a marker (69). Briefly, cells were centrifuged and stained with 50 µg/ml PI (Sigma Chemical Co.) in 0.1% sodium citrate and 0.1% Nonidet P-40 detergent (Sigma Chemical Co.). Approximately 10,000 nuclei were analyzed for each sample. Debris was excluded from analysis by gating based upon forward light scatter. The amount of DNA was assessed by the level of fluorescence emission at 608-655 nm following excitation at 488 nm. Segregation of cells in Go (G1) vs. S, G2, and M was based upon slope extrapolation as described (36). Briefly, Go (G1) cells were considered to be those falling between the gates set by linear extrapolation of the ascending and descending limbs of each diploid cell peak to baseline. Cells above the gate set by extrapolation of the descending limb of the diploid peak were considered to be in S, G2, or M.

Immunoelectron microscopy. Immunoelectron microscopy was performed as previously detailed elsewhere (192,193). Briefly, freshly cleaved mica sheets, onto which a thin film of carbon was coated, were serially floated onto: a 1% tryptophan solution; H_2O; MoAb $F(ab')_2$ fragments, IgM monomer or their immune complexes generated by interacting the individual components in a 1:1 ratio, each at 1 mg/ml in 0.15 M ammonium acetate; 0.3 M ammonium acetate; and a 2 % uranyl formate staining solution. All reagents were placed as large droplets onto a Telfon ring slide and kept at 4°C. The polished down side of a copper grid was gently placed upon the carbon film floating on top of the stain. After insuring that the carbon membrane had adhered to the grid, the grid-membrane complex was quickly removed from the surface of the staining well and excess stain was removed by capillary action. In such a procedure, the carbon membrane folds back upon itself and thus traps a thin film of stain between two folds of the protein-coated film on the grid. The grids were examined on a JEOL CEM CX-100 electron microscope and photographed at 100,000 diameters magnification. Negatives were projected to a total magnification of 260,000 x for analysis.

RESULTS

Domain and Epitope Specificity of Murine Anti-Human IgM MoAbs. Table XV lists the murine anti-human IgM MoAbs used in this study and shows the μ chain domain specificity as well as a designation of the number of unique epitopes bound by these MoAbs. Previous competition RIAs suggested that the two $C\mu_1$-specific MoAbs bind unique determinants on the IgM $C\mu_1$ domain; the four $C\mu_2$-specific MoAbs bind to at least two distinct epitopes; and each of the four $C\mu_4$-specific MoAbs bind a distinct epitope (184).

Evaluation of Anti-IgM MoAb Affinity for mIgM on Human B Cell Populations. In order to relate binding affinity to the potential for transmembrane signaling, binding constants were obtained for anti-IgM MoAb interactions with membrane IgM. Cellular equilibrium-binding ex-

TABLE XV. Human IgM Domain and Epitope Specificity of Murine anti-IgM MoAbs

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

* IgM μ chain specificity was established by inhibition RIAs using both enzymatically-derived IgM domain fragments and domain-deletion mutant proteins (184).

** MoAbs were defined as binding to the same or distinct epitopes based upon competition RIAs (184). The designations c (c') and d (d') indicate that these MoAbs cannot be unequivocally distinguished as binding to the same or very proximate sites as the MoAbs binding to c and d epitopes, respectively.

periments using MoAb Fab' fragments were performed with four different IgM-positive clonal B cell populations to obtain a binding constant (Ka) that was likely representative of MoAb binding to polyclonal B cell populations.

Table XVI summarizes data from equilibrium-binding experiments analyzed by the method of Scatchard (190,194,195) and shown as Ka values measured at 4°C. The MoAbs are listed in descending order of Fab' affinity: HB57 having the greatest average Ka with MoAb IF11 having the lowest. In general, affinities measured on different B cell populations were quite consistent with one another (average variation = 25.2 ± 4.8% [$\bar{x}$ ± SEM]). (The day-to-day average variation of separate determinations made on the same cell population was 21.3 ± 5.6% [$\bar{x}$ ± SEM]). One MoAb, XG9, however, did have a significantly lower affinity on Lub leukemic cells (1.03 ± 0.31 x 10^7 M^{-1}) than on the remaining three cell populations (mean Ka = 6.95 ± 1.59 x 10^7 M^{-1}).

Ka values shown for the lower affinity MoAbs (P19, P24, and IF11) were determined with less precision than those of the remaining Abs. This was in part due to the fact that only cell populations expressing high density mIgM (Dau and Lan) yielded useful Scatchard data for P24 and IF11, and in part due to the availability of fewer reliable data points for Scatchard analysis with these low affinity MoAbs, ie. fewer data points fulfilled the requirement of being 100-fold above background binding observed with excess IgM added to assay tubes (189).

Analysis of the stoichiometry of MoAb Fab' binding to mIgM. Although each mIgM molecule consists of two identical heavy chains, it cannot be unequivocally concluded that all anti-IgM MoAbs recognize epitopes which are divalently expressed on each mIgM molecule. Alternatively, it is possible that some MoAbs bind to univalently expressed epitopes (196,197) or perhaps to determinants redundantly expressed on each heavy chain. Because the number of bindable epitopes on mIgM might have important implications for the cross-linking potential of anti-IgM MoAbs, the binding stoichiometry of MoAb Fab' for mIgM was evaluated.

TABLE XVI. Analysis of anti-IgM MoAb affinity for B cell Membrane IgM

MoAb Fab'	Ka (x 10^{-7}) values for Fab' affinity on various B cell populations*				Mean affinity Ka (x 10^{-7}) ± SEM
	Bia	Dau	Lan	Lub	
HB57	64.72±17.74 [6]°	48.78 [5]	51.34 [12]	48.85 [4]	55.68 ± 6.73
5D7	15.15 [11]	12.21 [5]	11.39 [11]	10.84±0.13 [8]	12.06 ± 0.66
XG9	4.73±0.41 [5]	6.09±0.83 [13]	10.04 [4]	1.03±0.31[34]**	6.34 ± 1.02
Mu18	4.48 [5]	4.04 [4]	5.45 [6]	4.66 [5]	4.66 ± 0.30
4-3	1.63 [9]	1.85 [12]	2.52 [1]	1.84 [5]	1.96 ± 0.20
Mu53	1.30 [5]	1.96 [12]	2.01 [3]	1.45 [9]	1.68 ± 0.18
1G6	0.72 [6]	0.65 [11]	0.77 [9]	0.67±0.08 [15]	0.69 ± 0.03
P19	~ 0.17 [9]	~ 0.31 [10]	~ 0.74 [9]	~ 0.25 [26]	~ 0.37 ± 0.13
P24	ND†	~ 0.14 [67]	~ 0.27 [28]	ND	~ 0.20 ± 0.06
IF11	ND	~ 0.02 [26]	ND	ND	~ 0.02

* The data represent the Ka x 10^{-7} values from 1 to 3 independent determinations on each clonal B cell population. In instances where >1 determination was made, the mean value ± SEM of the Kaas well as the mean of the percent coefficient of variation is shown.

° Percent coefficient of variation of the Scatchard plot as determined by the computer program LIGAND (190,191).

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

† not determined.

Scatchard plot analyses of equilibrium binding data allows a derivation of the number of bound epitopes at ligand saturation (n) (190,198,199). The left hand portion of Table XVII shows values for the total number of bound mIgM epitopes per cell under saturating conditions with Fab' fragments of each of the seven highest affinity MoAbs. Data, shown only for three clonal B cell populations, did not include the B cell line Dau because Dau cells used in binding studies were derived from multiple freezes of the in vitro cultured line and any variation in the average mIgM density of these cells could have led to error in our comparison of MoAb-binding stoichiometry. The data indicate that, with the clear exception of MoAb 5D7, and the possible exception of MoAb Mu18, all of the MoAbs bind to a comparable number of epitopes within each of the cell populations tested. 5D7 consistently bound to an epitope expressed with a two to three fold greater frequency than the epitopes bound by the remaining MoAbs. This difference appears to be significant since replicate n value determinations made on the same cell population but on separate days resulted in an average variation of $35 \pm 7\%$ ($\bar{x} \pm$ SEM, range = 2-63%). The multiple sites bound by MoAb 5D7 Fab' appeared to be identical since all derived Scatchard plots were linear and conformed to a one-site but not two-site receptor complex when evaluated by the LIGAND computer program (190, data not shown).

The right hand portion of Table XVII lists normalized values for the number of epitopes bound per mIgM molecule. This normalization was based upon the assumption that the $C\mu_1$-specific MoAb XG9 binds to two determinants per mIgM molecule. This was considered a valid assumption for two reasons: a) the two $C\mu_1$ domains are widely spaced apart on the Fab arms of IgM (200), and b) immunoelectron microscopic data, to be presented later, supported the existence of a XG9-binding determinant on each Fab arm of mIgM.

The normalization of data allowed comparison of the relative number of MoAb-definable epitopes per mIgM molecule in the three B cell populations tested. Analysis of the mean mIgM epitope valency obtained from each MoAb indicates that the $C\mu_2$-specific MoAbs HB57 and

TABLE XVII. Evaluation of the valency of MoAb-definable epitopes on human membrane IgM

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

¶ Because of the lesser reliability of cell-binding data from low affinity ligands, MoAbs P19, P24 and IF11 have been excluded from this analysis.

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

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

° In instances in which >1 equilibrium-binding experiment was performed, the average "n" ± SEM are given. Numbers in [ ] indicate the % CV of the "n" value(s).

Mu53, and the $C\mu_4$-specific MoAbs 4-3 and 1G6, likely bind to divalently-expressed determinants on mIgM - the epitopes presumably arising from each of the two μ chains which comprise the intact IgM molecule. By this analysis, the $C\mu_4$-specific MoAb 5D7 appears to recognize an epitope with a valency of four to six per mIgM molecule (ie. two to three per μ heavy chain). The $C\mu_2$-specific MoAb Mu18 consistently yielded a normalized epitope number which was less than that of the $C\mu_1$-directed MoAb XG9. Since this epitope number was always greater than one-half that of XG9, the univalent versus bivalent nature of this determinant cannot be unequivocally established by our present data.

Relationship of anti-IgM domain specificity to mitogenic potential for human B splenocytes. Fig. 15 evaluates the importance of IgM domain specificity to the relative capacity of soluble anti-IgM MoAbs to trigger splenic B lymphocyte proliferation in the presence of 30% T cell supnt as a source of ancillary growth factors. The data indicate that neither of the $C\mu_1$- specific MoAbs, directed to two distinct epitopes, were able to elicit substantial DNA synthesis at any dose tested (concentration range: 2 to 1000 μg/ml). Further functional studies with an additional seven $C\mu_1$-specific MoAbs, that have been defined as binding to the same or very proximal determinant as that of MoAb XG9 (184), have indicated that these MoAbs are as a whole either non-mitogenic or only weakly mitogenic for human B cells (109; data not shown). In contrast, all but one of the $C\mu_2$-binding MoAbs tested were able to induce substantial DNA synthesis, although the doses required to induce proliferation varied considerably. Of the four $C\mu_4$-directed MoAbs, 5D7 was exceptionally mitogenic at low ligand concentrations, MoAb 4-3 required high ligand concentrations for significant B cell stimulation, and MoAbs 1G6 and IF11 were only very weakly mitogenic at the highest doses tested.

The above data indicate that although soluble anti-IgM MoAbs of $C\mu_1$-domain specificity may be impaired in their capacity for triggering human B cell DNA synthesis, the binding of ligands to either the $C\mu_2$ or membrane-proximal $C\mu_4$ domain can result in significant B cell proliferation.

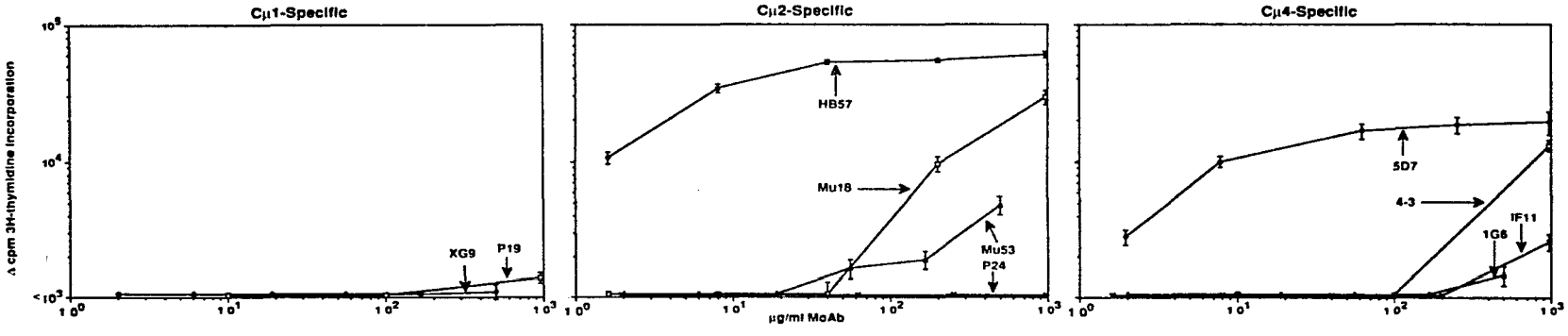


Figure 15. Human B cell proliferation elicited by domain-specific anti-IgM MoAbs. T cell-depleted splenic B lymphocytes were cultured with the indicated concentrations of anti-IgM MoAb in the presence of 30% T cell supnt. The data are expressed as Δcpm ± SE diff from triplicate cultures at 72 hrs and were obtained from three representative experiments in which the average background proliferation in the absence of MoAb was 2120 ± 529 cpm ($\bar{x} \pm$ SEM). While no $C\mu_1$-specific MoAb induced substantial B cell DNA synthesis, there was diversity in the ability of both $C\mu_2$- and $C\mu_4$-binding MoAbs to elicit proliferation.

Since not all MoAbs to the $C\mu_2$and $C\mu_4$ domains elicited the same degree of stimulation, binding characteristics other than IgM domain specificity must play a critical role in determining the triggering potential of these ligands.

Relationship of anti-IgM MoAb affinity to potential for triggering B cell DNA synthesis.

Fig. 16 evaluates the capacity of individual MoAbs of differing affinities to stimulate in vitro B cell DNA synthesis at either low (10 to 20 μg/ml) or high (100 to 300 μg/ml) ligand concentrations. Shown are plots of the mean index of stimulation (SI) achieved by intact forms of the MoAbs as a function of the affinity of the MoAb Fab' fragments. The data clearly indicate that at low ligand concentrations, only the two highest affinity anti-IgM MoAbs, ie. HB57 and 5D7 (Ka = 5.6 and $1.2 \times 10^8\ M^{-1}$, respectively) are able to induce significant DNA synthesis in soluble form. Greater ligand concentrations appeared to compensate for diminished ligand affinity and allow for induction of DNA synthesis by Abs with Fab' binding affinities of 0.38 to $4.66 \times 10^7\ M^{-1}$ (Fig. 16B). MoAbs with univalent Ka $\leq 0.38 \times 10^7\ M^{-1}$ appeared to be non-stimulatory, even at high ligand concentrations. With the very notable exception of MoAb XG9, a direct relationship was observed between MoAb binding affinity and the stimulatory potential of the Ab. Indeed, when a least squares linear regression analysis was performed of the data (excluding MoAb XG9 and the lowest affinity Ab IF11), a regression line was obtained with a correlation coefficient of 0.985 (data not shown).

MoAb XG9 has a propensity for monogamous binding interactions with monomeric IgM.

Since the spacing between duplicated $C\mu_1$ epitopes on the Fab arms of a mIgM molecule might be expected to approximate the allowable spacing between the two antigen-binding sites of an Ab molecule , we considered the possibility that the $C\mu_1$-specific XG9 MoAb might bind to mIgM in a monogamous fashion (199), ie. one intact XG9 Ab molecule binding to both $C\mu_1$ epitopes within the same IgM molecule. This would have important implications for the mitogenic properties of MoAb XG9 since a high proportion of monogamous binding would reduce the capacity of this high

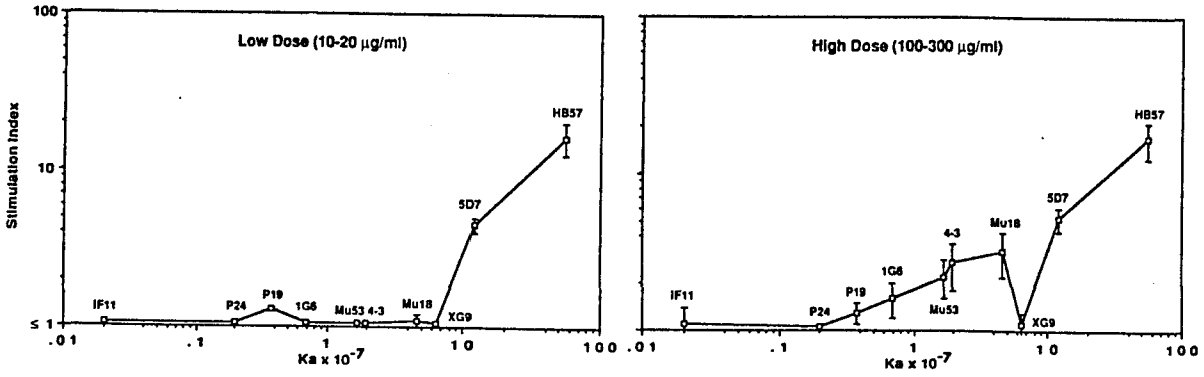


Figure 16. Relationship of MoAb affinity for membrane IgM to mitogenic potential for human B lymphocytes at both low and high ligand concentrations. 2 x 10^5 T cell-depleted splenocytes were cultured in the presence of 30% T cell supnt. The stimulation index (SI) used for each MoAb represents the mean ± SEM of SI values obtained from between 1 to 4 separate experiments in which cells were cultured with 10 to 20 µg/ml of MoAb, and from 4 to 7 separate experiments in which cells were incubated with 100 to 300 µg/ml of MoAb. These indices are plotted as a function of the mean Ka of the MoAb Fab' fragments (see Table XVI). While only the highest affinity MoAbs (HB57 and 5D7) were capable of inducing B cell DNA synthesis at limiting concentrations (10-20 µg/ml), at high ligand input (100-300 µg/ml) there was a direct linear relationship between MoAb Ka and proliferation-inducing capacity. The lowest affinity MoAbs (IF11, P24, and P19) were generally non-stimulatory, whereas MoAbs of progressively higher affinity were increasingly more mitogenic.

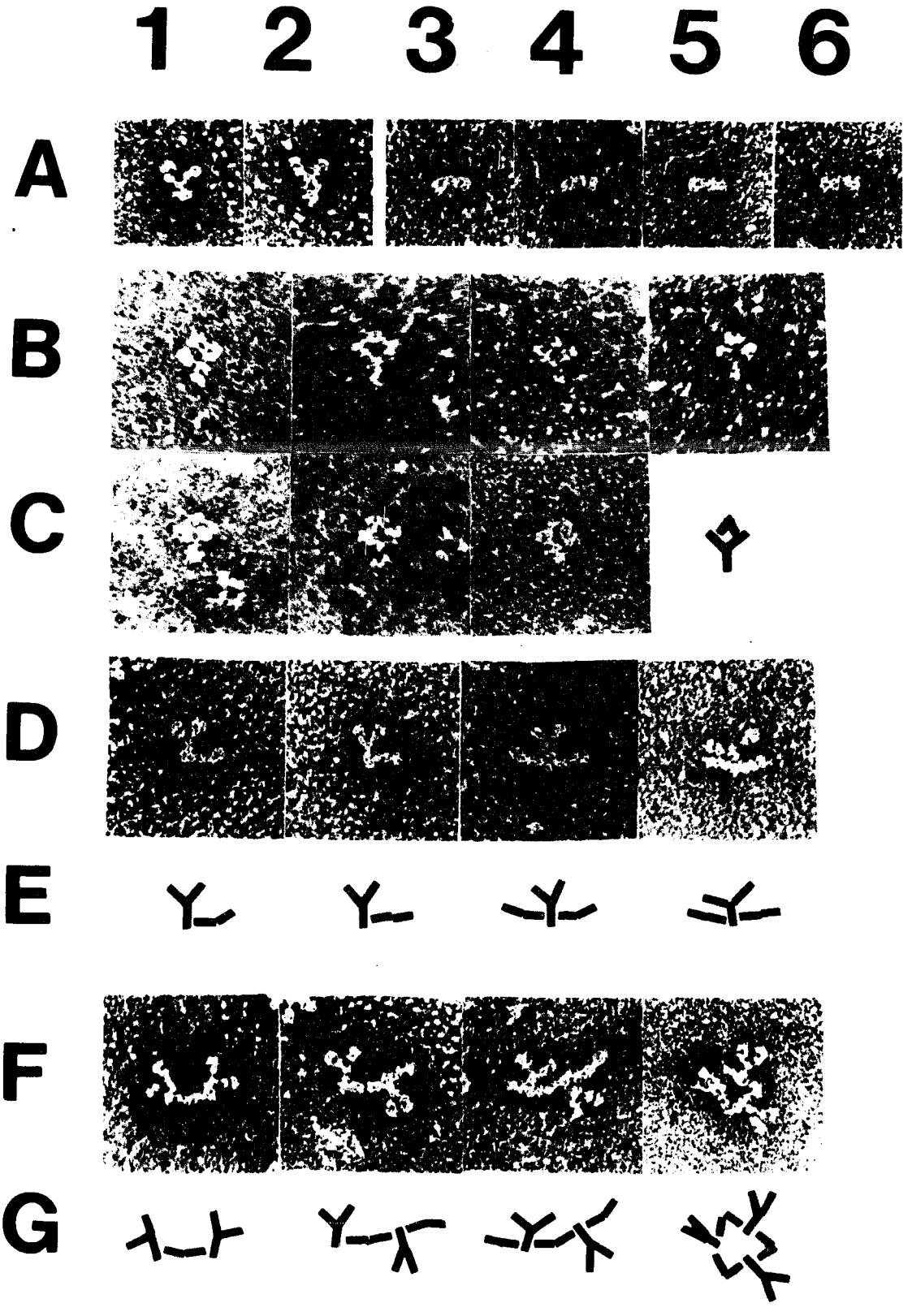
affinity Ab to cross-link mIgM, a process which is obligatory for direct signaling by anti-Ig ligands (34,46).

The capacity for monogamous binding of MoAb XG9 to IgM was evaluated by immuno-electron microscopic analysis of immune complexes formed between monomeric IgM (Fig. 17, panels A1 and A2) and $F(ab')_2$ fragments of XG9 (panels A3 and A4). Of those immune complexes formed between monomeric IgM and XG9 $F(ab')_2$, 72% were indeed found to be in the form of monogamous interactions as indicated in panels B1-B4 and C1-C3, and shown schematically in panel C4. The remaining complexes were observed to have either one XG9 $F(ab')_2$ bound to two monomeric IgM molecules in a bigamous manner, or alternatively, observed to have XG9 $F(ab')_2$ molecules bound to IgM monomer via one arm only (data not shown). The monogamously bound XG9 $F(ab')_2$ molecules appeared to have more compacted dimensions than when found in free, nonbound form. This has been previously noted with anti-kappa MoAb binding to mouse IgG molecules (192) and was considered to be due to geometric constraints that prevent the immune complex from lying on the same flattened plane.

Panels D1-D4 and F1-F4 of Fig.17 show the contrasting types of complexes observed between monomeric IgM and the $F(ab')_2$ fragment of the $C\mu_4$-specific MoAb 1G6 (shown alone in panels A5 and A6). The results clearly indicate the bivalent nature of the 1G6 epitope. Of the immune complexes formed, >75% had two 1G6 $F(ab')_2$ molecules bound per IgM monomer (shown schematically in panels E3 and E4, and G2-G4), whereas the remainder of observed immune complexes had only one $F(ab')_2$ bound per molecule of monomer (panels E1 and E2, and G1). Monogamous binding of 1G6 $F(ab')_2$ to monomeric IgM was not detectable.

Inhibition of leukemic B cell DNA synthesis can be achieved by MoAbs having Cμ1, Cμ2, and Cμ4-binding specificities. The susceptibility of certain malignant B cell populations to negative regulation by anti-Ig ligands (10,23,27,111,167) has been used as a means of evaluating the ligand-binding requisites for inhibitory (tolerogenic) signal transduction and contrasting them with

Figure 17. Immunoelectron photomicrograph of immune complexes formed between MoAb $F(ab')_2$ fragments XG9 and 1G6 and human IgM monomer. Immune complexes were formed from monomeric IgM and the $F(ab')_2$ fragments of MoAbs XG9 or 1G6 and analyzed by immunoelectron microscopy (see Materials and Methods for further details). Row A: Panels A1 and A2 show the human monomeric IgM molecule alone; Panels A3 and A4, and A5 and A6 show $F(ab')_2$ fragments of MoAbs XG9 and 1G6, respectively. Rows B and C show immune complexes formed between monomer IgM and the $F(ab')_2$ fragment of MoAb XG9; panel C4 indicates the interpretation of these photomicrographs in schematic form, ie. monogamous binding of the MoAb $F(ab')_2$ fragment to monomer IgM. Rows D and E, and Rows F and G: Immune complexes formed between monomer IgM and $F(ab')_2$ fragment of MoAb 1G6 (Rows D and F) and interpretive schematic illustrations of those immune complexes (Rows E and G, respectively).


1 2 3 4 5 6
A
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G

the binding requisites for activation of B cell DNA synthesis. Fig. 18 illustrates the ability of the domain-specific anti-IgM MoAbs to inhibit the DNA synthesis of two previously described human HCL B cell populations (111): Kon, which can be induced to in vitro DNA synthesis by culture in exogenous T cell factor-containing supnt, and Lub, which synthesizes DNA spontaneously. The data are plotted as dose-response profiles of μg/ml MoAb added to culture versus the cpm ^{3}H-thymidine incorporation observed. The stippled bars indicate the level of DNA synthesis in the B cell populations in the absence of added MoAb. The data obtained clearly indicate that, in contrast to their extreme diversity in inducing normal B cell proliferation (Fig. 15), all MoAbs, irrespective of their IgM domain specificity, can signal the inhibition of leukemic B cell DNA synthesis. In fact, most Abs, with the exception of MoAb P24 on Kon cells, could inhibit the leukemic response by > 90% . This MoAb-mediated inhibition has been shown previously to be due to the IgM-binding specificity of the ligands and to be independent of Ab Fc (111). While B cell activation to DNA synthesis required μg/ml concentrations of ligand (Fig. 15), inhibition of DNA synthesis in these leukemic clones could be achieved with ng/ml doses.

Affinity requisites for anti-IgM-mediated inhibitory signaling. Although all anti-IgM MoAbs were capable of transducing inhibitory signals, some variation in the concentration of Ab needed to do so was observed. The data in Fig. 19 indicate that the diversity in the inhibitory signaling potential of certain MoAbs reflects their affinity for mIgM. Thus, those MoAbs which required significantly greater amounts of ligand to actuate 50% inhibition of leukemic B cell DNA synthesis, ie. P19, P24, and IF11, have the lowest affinity for mIgM (Ka = 0.37, ~0.20, and ~0.02 x 10^{7} M^{-1}, respectively). With these three ligands, a direct relationship was noted between MoAb affinity and inhibitory potential. However, MoAbs with Fab' binding afinities ≥ 0.69 x 10^{7} M^{-1} did not appear to distinguish themselves in their capacity to cause inhibition. This suggests that a threshold affinity exists for inhibitory signaling by anti-Ig ligands, above which increases in affinity have a minimal effect upon the capacity to induce an inhibitory signal.

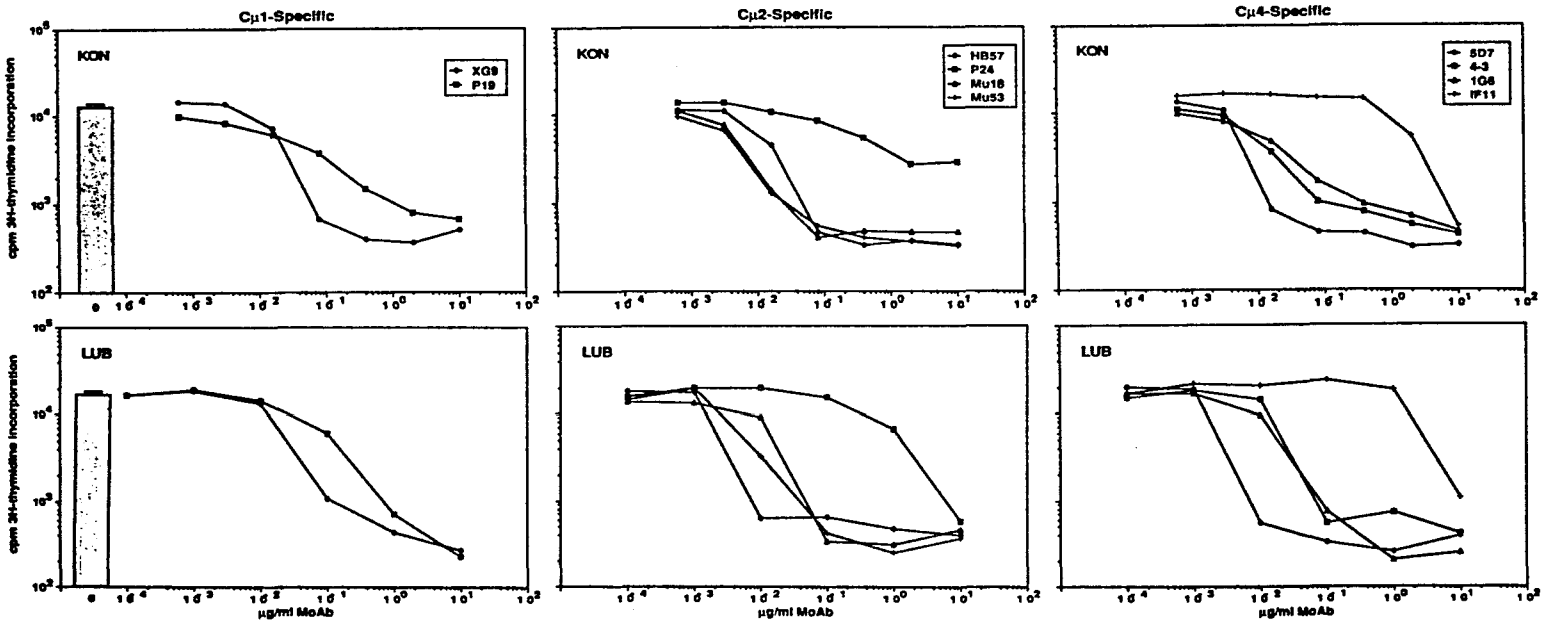

Figure 18. All anti-IgM MoAbs can transduce inhibitory signals for leukemic B cell DNA synthesis. 2 x 10^5 Kon leukemic B cells (111) + 25% T cell supnt or 2 x 10^5 Lub HCL cells in non-T cell supnt supplemented medium, were cultured with the indicated concentrations of anti-IgM MoAb. The data are expressed as cpm ^{3}H-thymidine incorporation on d4 of culture. Stippled bars indicate the level of DNA synthesis obtained when these cells were cultured in the absence of MoAb. All anti-IgM MoAbs, irrespective of their domain specificity, were able to inhibit the DNA synthesis of these leukemic clones.

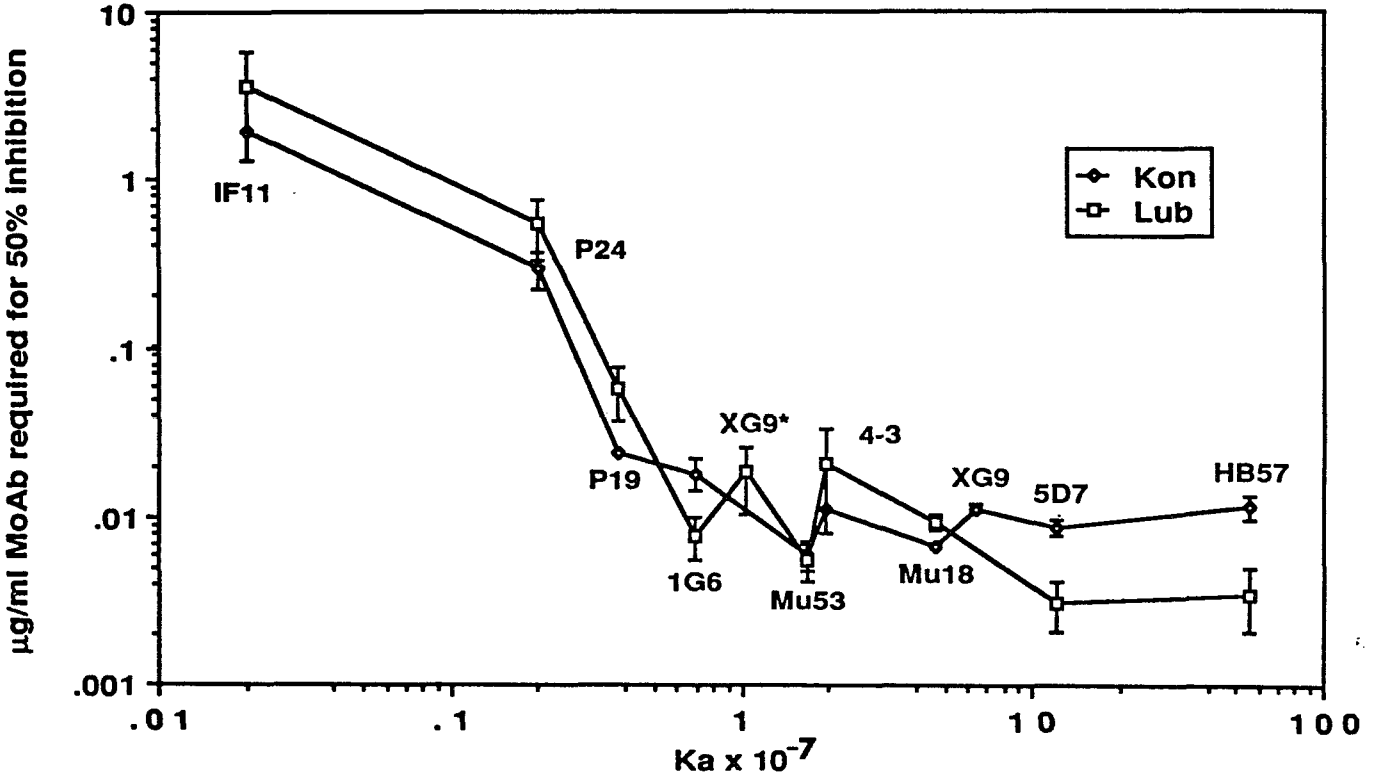

Figure 19. Effect of MoAb affinity upon ability to inhibit leukemic B cell DNA synthesis. The concentration of intact MoAb required for 50% inhibition of leukemic B cell ^{3}H-thymidine incorporation was calculated from dose-response profiles obtained from at least two separate experiments on both Kon and Lub HCL B cells (Fig. 18). The mean values are plotted as a function of MoAb Fab' affinity. In the case of Lub cells, MoAb XG9 is indicated as having a lower affinity, XG9*, than on Kon cells (see legend Table XVI). The composite data indicate that the inhibition of leukemic B cell DNA synthesis by intact Ab is relatively independent of affinity, until an Fab' affinity threshold of approximately 0.4×10^{7} M^{-1}.

Binding requisites for stimulatory and inhibitory signal transduction differ in a clonal B cell population. Our data indicate that, contrary to their compromised signaling capacity for DNA synthesis, MoAbs with a high propensity for monogamous binding interactions with mIgM, as well as MoAbs of intermediate affinity, are not impaired in their tolerogenic potential. These observations, taken together with the substantial difference in ligand concentrations required for each form of signaling, suggest that the ligand binding requisites for triggering inhibition of DNA synthesis through mIgM are significantly less restrictive than those for triggering stimulation of DNA synthesis. However, given that stimulatory signal transduction was evaluated in normal human B lymphocytes, and inhibitory signal transduction was evaluated in malignant B cell clones, it is possible that the distinctions reflect inherent differences in the requisites for transmembrane signaling in normal versus leukemic cell populations rather than differences in the mechanisms of signaling opposing functional phenomena.

To discern between these possibilities, we have utilized the unusual HCL clone, Kon, which can receive stimulatory, or alternatively, inhibitory signals from anti-IgM ligands, depending upon the cells' state of activation. The bi-functional nature of this clone is illustrated in Table XVIII and has been described in greater detail in a previous report (111). The data shown indicate that in the absence of accessory T cell factors, the Kon cell clone can respond with very significant levels of DNA synthesis to 200 μg/ml concentrations of the high affinity MoAb, HB57. However, in the presence of activating factors in T cell supnt, the DNA synthesis of the clone is negatively regulated by low as well as high doses of the anti-IgM ligand.

A clear difference in ligand binding requisites for achieving stimulation versus inhibition of DNA synthesis is indicated when Kon cells are cultured with the $C\mu_1$-specific MoAb XG9, the $C\mu_4$-specific MoAb 1G6, or the bi-clonal mixture of these two Abs either in the presence or absence of T cell supnt. Previous studies with normal B lymphocytes have shown that these two non-mitogenic Abs exhibited striking synergy when mixed together in culture in a 1:1 ratio (109). The

TABLE XVIII. Kon leukemic B cell clone receives either stimulatory or inhibitory signals from anti-IgM MoAb depending upon the cell's activation state.

Anti-IgM MoAb	Total Ab conc (μg/ml)	cpm 3H-thymidine uptake (d5)* ($\bar{x}$ ± SEM)		Percent of cells in S, G2, or M (d4)**	
		no T supnt	+ T supnt	no T supnt	+ T supnt
None	-	460 ± 40	19,929 ± 2178	1.0	8.1
HB57	2	493 ± 13	813 ± 146	2.0	1.4
	200	40,596 ± 5980	6,827 ± 410	10.7	4.2

* 2×10^5 Kon leukemic B cells were cultured with or without HB57 anti-IgM MoAb in the presence or absence of 25% T cell supnt. Proliferation was assessed on day 5 after a 18 h incubation with 3H-thymidine. The mean ± SEM from triplicate cultures are shown.

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

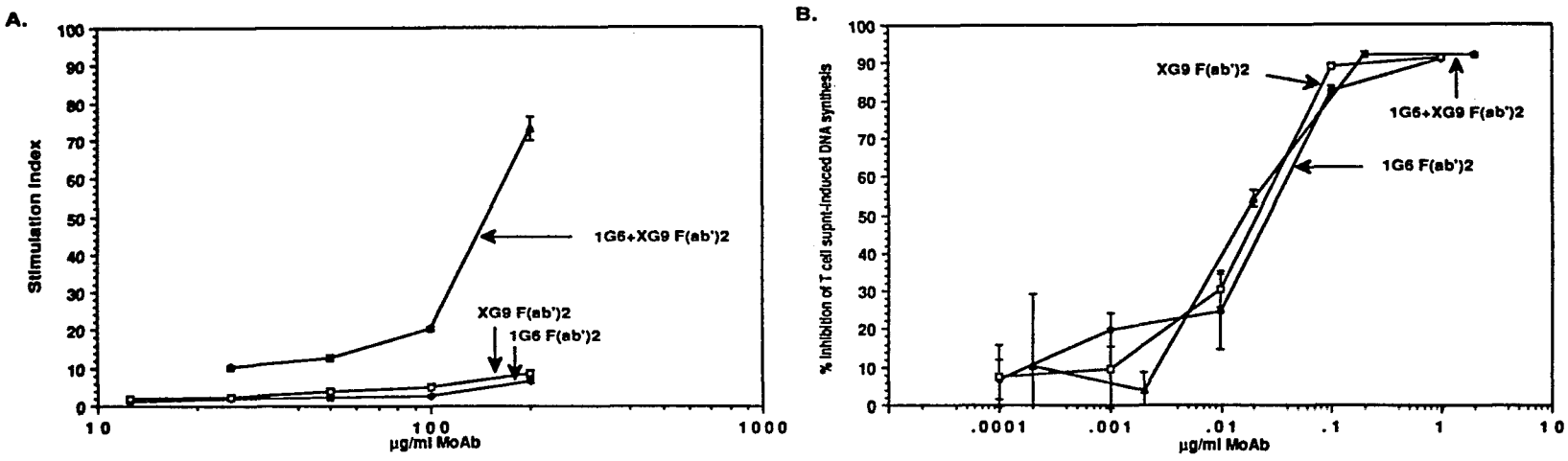

Figure 20. Binding requisites for stimulatory and inhibitory signal transduction through membrane IgM differ in a clonal B cell population. 3×10^5 Kon leukemic B cells were cultured alone (A) or in the presence of 25% activated T cell supnt (B) with either MoAb 1G6 or XG9 F(ab')$_2$ fragments or a 1:1 mixture of the two at the indicated total Ab concentrations. Stimulation index calculated in (A) was based upon a background level of stimulation (Kon with media alone) of 454 ± 93 cpm ($\bar{x}$ ± SEM). Percent inhibition of T cell supnt-induced DNA synthesis in (B) was based on T cell factor-induced DNA synthesis of 5717 ± 304 cpm ($\bar{x}$ ± SEM). In the absence of T cell factors, (A), MoAbs 1G6 and XG9 individually did not induce substantial ^{3}H-thymidine uptake in Kon cells, but the mixture of these Abs at high ligand concentrations showed striking synergy in the stimulation of Kon cell DNA synthesis. In the presence of stimulatory T cell supnt (B), the individual MoAbs and the MoAb mixture were all capable of transducing inhibitory signals at comparable low ligand concentrations.

data in Fig. 20A show that this synergistic effect is reproduced with Kon leukemic cells in the absence of T cell supnt. However, when the action of these ligands on T cell supnt-activated Kon cells (Fig. 20B) is compared with their effect on resting Kon cells (Fig. 20A), several contrasting observations can be made which are of relevance to the mIgM signaling process for activation or inactivation of B cell DNA synthesis: (A) Unlike the synergy observed between MoAbs XG9 and 1G6 at inducing Kon cell DNA synthesis, the individual MoAbs are as effective as the mixture at inhibiting T cell supnt-initiated DNA synthesis, ie. no synergy is observed for inhibitory signaling. (B) Whereas a very high MoAb concentration is required for stimulation of Kon cell DNA synthesis (see also Table XVIII), inhibition of Kon cell T supnt-induced DNA synthesis requires orders of magnitude less ligand.

DISCUSSION

The goal of the work presented in this report was to critically examine the ligand binding requisites for direct induction of DNA synthesis, or alternatively tolerance, through B cell membrane IgM receptors. Functional studies with a well-characterized panel of MoAbs with known domain specificities, affinities, and binding stoichiometries for human mIgM have indicated that although cross-linking of mIgM molecules is necessary for both forms of signaling, the ligand requirements for eliciting B cell DNA synthesis are considerably more demanding than those for inducing inhibition of DNA synthesis in activated B cell populations. This discussion will first focus on an evaluation of the requisites for B cell clonal expansion and later focus on a comparison of these requisites with those for active tolerogenic signal transduction.

The data presented here indicate that the previously described diversity in the ability of the soluble anti-IgM MoAbs to induce B cell DNA synthesis (109) is, for the most part, a direct result of affinity differences among the anti-IgM ligands. With one explainable exception, we have found that MoAbs with a univalent Ka $\geq 1.68 \times 10^7$ M^{-1} were able to induce significant levels of

^{3}H-thymidine uptake in the presence of ancillary T cell factors and that the degree to which these Abs could initiate the DNA synthesis of resting B lymphocytes was directly correlated with their univalent binding affinities. Because stimulatory signal transduction by anti-Ig Abs requires bivalent binding of the ligand (34,46), and bivalent MoAb binding to cell membrane antigens is characterized by at least a 10 to 100 fold enhancement in affinity over that exhibited by univalent Fab' fragments (198,201), we might expect that the minimal binding avidity of a soluble anti-IgM ligand needed to induce human B cell DNA synthesis would be $\geq 1.68 \times 10^{8\text{-}9}\ M^{-1}$.

While the high affinity ligands, HB57 and 5D7, induced maximal B cell ^{3}H-thymidine uptake at relatively low ligand concentrations (10 to 20 μg/ml), the intermediate affinity Abs with univalent binding affinities of 1.68 to $4.66 \times 10^7\ M^{-1}$ elicited DNA synthesis only at much higher ligand concentrations. This inverse relationship between ligand affinity and concentration required for signaling is a manifestation of the law of mass action, $Ab + Ag \Leftrightarrow [Ab \cdot Ag]$. Thus, the greater the number of free Ab molecules, the greater the probability of complex formation (202), particularly if the dissociation rate, k_d, is high, as with low affinity ligands (203). A minimal affinity threshold for signaling the DNA synthesis of B cells does exist, however, since MoAbs with univalent $Ka \leq 1.68 \times 10^7\ M^{-1}$, ie. 1G6, P19, P24, and IF11, were all generally ineffective at inducing S phase entry at concentrations up to 300 μg/ml. It would appear that below this threshold affinity, the ligands do not bind to mIgM long enough to achieve sufficient inter-linkage of mIgM receptors.

The only ligand which deviated from a direct linear relationship between MoAb Ka and proliferation-inducing capability was MoAb XG9. This relatively high affinity Ab ($Ka = 6.34 \times 10^7\ M^{-1}$) was consistently non-mitogenic for normal human B cells, even when cultured at concentrations as high as 1 mg/ml. A likely explanation for the ineffectual stimulatory capability of this MoAb was obtained upon immunoelectron microscopic analysis of the immune complexes formed between $F(ab')_2$ fragments of XG9 and monomeric IgM. This study revealed that MoAb XG9 has a

predilection for monogamous binding to the bivalently-expressed $C\mu_1$ epitopes of IgM molecules. The tendency to bind to IgM in this manner presumably reflects the fact that the spacing between the $C\mu_1$ epitopes approximates the flexible spacing between the bivalent combining sites of the MoAb. Since monogamous binding prevents the cross-linking of mIgM molecules (see Fig. 21A), this MoAb is indeed expected to display a diminished mitogenic phenotype.

We should note that one alternative explanation for the low stimulatory capacity of MoAb XG9 is that the affinity of this Ab for mIgM on the three splenic B cell populations used in functional studies is lower than that measured on three of the four clonal B cell populations used for affinity measurements and is more analogous to the affinity measured on the aberrant Lub HCL B cell population. This might be possible if allotype variation was responsible for the differences in XG9 binding affinity noted between Lub and the remaining B cell populations. We consider this explanation highly unlikely, however, since we have never observed any functional variation indicative of possible allotypic variation when B cell populations from > 15 different peripheral blood or spleen donors were tested, ie. XG9 has been uniformly non-mitogenic. The aberrantly low Ka for MoAb XG9 on Lub may be due to germ-line mutation in the Lub leukemic clone, or alternatively due to unique glycosylation of Lub mIgM which either directly or indirectly lowers the affinity of XG9 binding to $C\mu_1$.

Although evidence for monogamous binding of MoAb XG9 to IgM described above was based upon analysis of its' interaction with soluble monomeric IgM, it is likely that MoAb XG9 also binds in a monogamous fashion to IgM molecules on the B cell surface. This is appreciated if one calculates the average distance between mIgM molecules on the surface of a mature, resting B lymphocyte. Thus, if a B cell sphere of 7 microns in diameter has ~10^5 mIgM molecules on its surface, each mIgM molecule would be expected to be separated by a distance of ~500 Å. Since this distance is much greater than the distance between the duplicated $C\mu_1$ epitopes on the Fab arms of IgM (< 140 Å) (202,204), it is highly probable that monogamous binding events will be

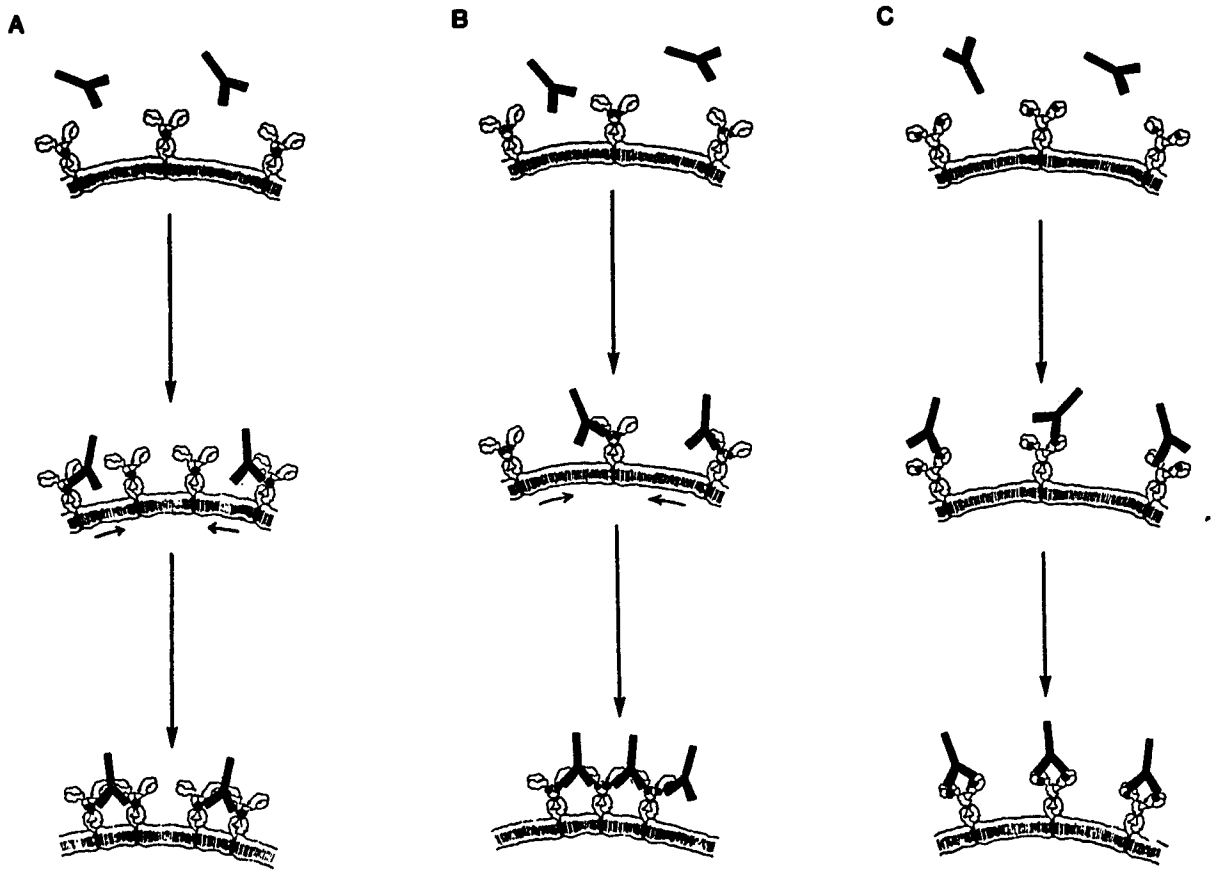

Figure 21. Schematic models for anti-IgM MoAb interaction with membrane IgM epitopes.

A) Monogamous binding to divalently-expressed epitopes on $C\mu_1$; B) Bigamous binding to divalently-expressed epitopes on another mIgM domain; C) Bigamous binding to univalently-expressed epitopes on mIgM.

favored over bigamous binding of MoAb XG9 to distinct mIgM molecules. Furthermore, as the density of mIgM diminishes through gradual capping and the slow re-expression of new mIgM molecules (205,206), this phenomenon likely becomes even more accentuated.

Structural considerations, as well as our immunoelectron microscopy evidence, suggest that $C\mu_2$ and $C\mu_4$-specific Abs have a lower probability of engaging in monogamous binding interactions with mIgM than $C\mu_1$-specific Abs. Duplicated epitopes in the $C\mu_2$ and $C\mu_4$ regions are expected to be topographically more proximal to one another since these domains show considerable homologous interaction (200,207). In these instances, the inappropriately narrow angle necessitated between MoAb Fab' arms for mIgM binding, as well as the topographically polar orientation of epitopes on each side of the globular structure of the paired homologous domains, would be expected to preclude monogamous binding of MoAbs to epitopes on these IgM domains. Empirical capping observations with the mIgM-positive HCL clone Kon support the above conclusions in that the $C\mu_1$-specific MoAb XG9 was much less effective at inducing capping of Kon cell mIgM than non-$C\mu_1$-binding MoAbs of much lower affinity (unpublished results).

The data and conclusions regarding the functional effects of MoAb XG9, and other $C\mu_1$-specific MoAbs which bind to the same or neighboring epitopes (109,184; unpublished results), are indeed consistent with and provide an explanation for the data of Goroff et al. (81) with a set of anti-murine IgD MoAbs. These investigators reported that high affinity MoAbs directed to the Fd portion of murine IgD were ineffective at capping and stimulating mouse B cell DNA synthesis, whereas high affinity MoAbs to the Fc portion of murine IgD were good at inducing capping and were very mitogenic. In addition, since monogamous binding has recently been shown to be characteristic of a high proportion of anti-idiotype interactions with both soluble and cell-bound Ig molecules (193,201,208), our data have strong functional implications for these Abs. Thus, anti-idiotype Abs which have a propensity for monogamous binding interactions with mIg should

be expected to be significantly impaired in their capacity for inducing the clonal expansion of B lymphocytes, regardless of their affinity for their respective idiotopes.

This report has also attempted to address the extent to which valency of expressed epitopes on mIgM influences the stimulatory properties of anti-IgM ligands. As shown in Fig. 21B and C, differences in the valency of recognized determinants could have significant effects on the cross-linking potential of anti-Ig ligands. Thus, MoAbs directed to divalently-expressed epitopes on regions of the mIgM molecule that allow bigamous binding should cross-link neighboring mIgM molecules and ultimately yield linear concatemers of these molecules (see Fig. 21B). In contrast, MoAbs directed to univalently-expressed epitopes should only form dimers of mIgM (see Fig. 21C). Since Dinztis et al. (50) have suggested that a large number, from ten to twenty, mIg molecules must be cross-linked for induction of B cell Ig secretion by a T cell independent antigen, it might be predicted that Abs which bind to univalently-expressed epitopes would be significantly impaired in their signaling potential.

With this in mind, we have analyzed the stoichiometry of MoAb Fab' binding to mIgM. Our analysis strongly suggests that all of the MoAbs, with the exception of 5D7, and perhaps Mu18, bind to two determinants per mIgM molecule. Therefore, most of the MoAbs with distinct mitogenic properties are not distinguishable in the valency of recognized epitopes on mIgM. It is thus expected that, provided their affinity is sufficiently high, these MoAbs should be capable of forming linear cross-linked aggregates of multiple mIgM molecules.

Monoclonal Ab Mu18 consistently bound fewer epitopes at saturation than the prototype Ab to a divalently-expressed epitope, MoAb XG9. However, since the number of epitopes bound by this MoAb was always greater than one-half the number of epitopes bound by XG9, we were not able to conclude that Mu18 unequivocally binds to an univalent determinant. This diminished epitope valency observed with MoAb Mu18 may represent: a) steric interference preventing Mu18 binding to two proximal sites, b) an induced change in the configuration of one epitope

following initial binding to the homologous determinant, or c) the occasional unilateral association of mIgM molecules with other membrane molecules which sterically interfere with MoAb Mu18 binding to one of its two epitopes. Since Scatchard plots of MoAb Mu18 Fab' binding to all four B cell populations assayed were uniformly linear and did not conform to a two receptor-site model (data not shown), possibilities (a) and (b) above, which invoke both a "native" and a "compromised" affinity site, seem unlikely.

Despite the lowered average valency of Mu18-designated epitopes on mIgM, this relatively high affinity Ab was as mitogenic as would have been expected if affinity were the sole indicator of mitogenicity. This may reflect the fact that all Mu18 epitopes on normal splenic B lymphocytes are available for divalent binding and/or that signaling to DNA synthesis can be as effectively induced by formation of multiple stable dimers of mIgM as by formation of linear cross-linked lattices of mIgM. This latter possibility has been recently raised by Perelson (52) as a challenge to the immunon concept of Dintzis et al. (50). Further work with high affinity ligands having unequivocal binding specificity for univalent epitopes should clarify the minimal size of the signaling unit for inducing B cell DNA synthesis.

The $C\mu_4$-specific MoAb 5D7 was unique among the panel of anti-IgM MoAbs in its ability to recognize an epitope with a valency of greater than two per mIgM molecule. Because the hybridoma cloning conditions, the unusual isotype of this MoAb, and the unusual enzymatic fragmentation characteristics of this MoAb all attest to its monoclonality, we do not consider that this Ab represents a mixture of Abs to distinct determinants. Rather, 5D7 may recognize a repeated sequence of linearly-arranged amino acids or repeated conformational epitopes on the IgM $C\mu_4$ domain. A search of the $C\mu_4$ domain sequence for a repeating unit of four to six amino acids provided no evidence for a repeated linear determinant (130,209). Evidence that the 5D7 epitopes may be conformational in nature is supported by the fact that solid-phase competition RIAs with the three cyanogen bromide cleavage fragments of $C\mu_4$ showed no interaction with MoAb 5D7,

while the isolated intact $C\mu_4$ domain was able to bind MoAb 5D7. Although it is also possible that 5D7 binds to repeated epitopes on an IgM-specific carbohydrate, current evidence (209,210) does not support the presence of a $C\mu_4$-associated carbohydrate on human membrane IgM.

Despite the fact that a high epitope valency might be expected to enhance the formation of large, branched lattices of cross-linked mIgM molecules by MoAb 5D7, this Ab did not display mitogenic capabilities above that expected from its Fab' affinity alone. This may reflect a) suppressive signals from the γ2a Fc of this Ab (75,178), b) steric hindrance which prevents effective binding of intact 5D7 Ab molecules to all possible sites, or c) the fact that large cross-linked lattices of mIgM molecules are no more effective at transducing stimulatory signals than smaller, less complex cross-linked clusters of mIgM.

We have elsewhere with normal B cells (109), and here, with Kon leukemic B cells, shown that the non-mitogenic $C\mu_1$-specific MoAb XG9 and the non-mitogenic $C\mu_4$-specific MoAb 1G6 exhibit profound synergy in the induction of B cell DNA synthesis. Several factors may contribute to this synergy. First, these two MoAbs likely show no interference in binding to mIgM because they are directed to the most distal μ heavy chain domains (184). Second, the $C\mu_4$-specific MoAb 1G6 may help promote a greater degree of bigamous XG9 binding interactions with mIgM by cross-linking mIgM and bringing neighboring mIgM molecules into closer proximity. The end result of the stable binding of ligands to multiple isotypic determinants is likely the creation of a more complex receptor-aggregated cluster and an increase in the overall avidity of the multi-ligand complex. In this latter regard, it has been clearly documented that mixtures of MoAbs to distinct sites on the same antigen exhibit a substantially greater avidity than the individual MoAbs to their respective epitopes on the same antigen (104).

Use of the panel of anti-IgM MoAbs for signaling the inhibition of DNA synthesis in certain activated human leukemic B cell populations has revealed that the binding requisites for inhibitory signaling through mIgM are much less rigorous than those for stimulation of normal B cell DNA

synthesis. This finding with leukemic B cells has some physiologic significance for normal B cells since the inhibitory signaling process in leukemic cells may mimic the mechanism involved in the metabolically active phenomenon of neonatal B cell tolerance (10,23,167,179).

Several observations indicated that induction of B cell DNA synthesis and the induction of B cell tolerance have distinct ligand binding requisites. First, the ligand concentrations required to achieve maximal inhibition of B cell DNA synthesis were orders of magnitude lower than the dose requirements for stimulation of B cell DNA synthesis (6,42,43,49,63,72,73,178,183). Second, while the capacity to initiate inhibitory signaling was clearly affinity independent at univalent ligand binding affinities $\geq 0.69 \times 10^7\ M^{-1}$, an upper threshold for achieving maximal stimulation of B cell DNA synthesis was never observed with our individual MoAbs. Third, while a high affinity $C\mu_1$-specific MoAb with a propensity for monogamous binding to mIgM was found to be significantly impaired in capacity to initiate B cell DNA synthesis, the same Ab was quite competent at initiating B cell tolerance. Finally, while anti-IgM MoAbs to distinct Cμ domains manifest significant synergy in the induction of human B cell DNA synthesis, combinations of MoAbs did not show synergy in signaling the inhibition of leukemic B cell DNA synthesis.

It is important to note that the differences in ligand binding requisites for the induction of normal B cell DNA synthesis and the suppression of leukemic DNA synthesis were not due to inherent differences in the way in which mIgM-binding events are transduced into signals in normal versus leukemic cells per se. Evidence for this was obtained from a leukemic B cell clone, Kon, which could receive stimulatory, or alternatively, inhibitory signals following mIgM cross-linking, depending upon its state of activation. The binding requisites for achieving DNA synthesis in this clone were found to be comparable to those for inducing normal B cell proliferation and to differ significantly from the requisites for causing inhibition of the T cell factor-induced DNA synthesis of Kon.

How can we explain the distinction in binding requisites for activation and inhibition of B cell DNA synthesis? We have considered that our results are consistent with two possibilities. A) The triggering of stimulatory signals may require a greater cross-linked receptor cluster, immunon, than the triggering of inhibitory signals. Thus, stimulatory signal transduction may be achieved only with high affinity ligands, or alternatively, high concentrations of intermediate affinity ligands, which are capable of stabilizing such clusters (see Fig. 22A). In this case, combinations of anti-Ig ligands, by increasing the overall avidity of mIgM binding, as well as by creating larger, more complexly-branched cross-linked lattices, would be expected to be even more optimal conduits for stimulatory signal transduction. The suggestion that small receptor aggregates may be adequate for triggering tolerance has been previously made by Teale and Klinman (7,62) upon noting that pharmacological inhibitors of mIg patching did not abrogate the induction of tolerance in immature B lymphocytes.

(B) The second possible explanation for the distinct ligand-binding requisites for stimulatory and inhibitory signal transduction relates to the apparent requirement for multiple rounds of mIgM cross-linking, capping, endocytosis/shedding for stimulatory signal transduction but not for inhibitory signal transduction (see Fig. 22B). Our own unpublished observations, as well as the work of others, has indicated that for S phase entry of anti-Ig-treated resting B cells, ligand must be present for an interval of 24-36 h (42,43,206), while inhibition of DNA synthesis necessitates the presence of anti-IgM ligand for only a few hours (10). This difference in the requirements for continued mIgM cross-linking becomes significant when one considers that, after an initial cycle of capping and endocytosis, the B cell slowly re-expresses its lost mIg (205). Continued signaling via mIgM cross-linkage under such low mIgM density would likely be achieved either by very high affinity ligands or by high doses of intermediate affinity ligands. This explanation has been proposed by DeFranco et al. (42) to help explain the requirements for high concentrations of anti-IgM ligands at late, but not early, intervals in the stimulation of murine B lymphocytes.

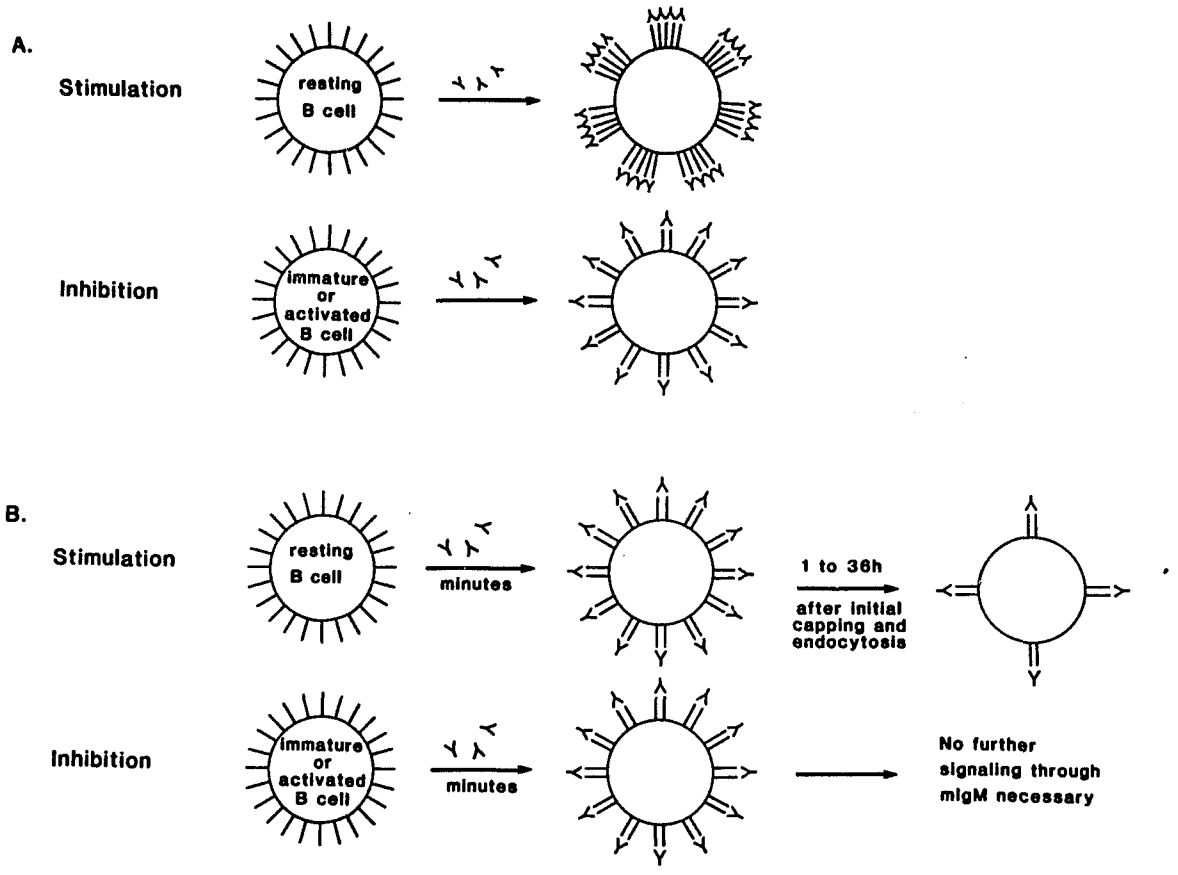

Figure 22. Models for mIgM cross-linking events which lead to stimulatory or inhibitory signals for B cell DNA synthesis. A) Stimulatory and inhibitory signal transduction may differ in the minimal requisite unit of cross-linked receptors. B) Unit signal for activation and suppression of B cell DNA synthesis may be achieved by cross-linking similar numbers of receptors. However, the activation of DNA synthesis may require repeated cycles of mIgM cross-linking under conditions of extremely low receptor density.

The strong influence that mIgM receptor density plays on ligand requisites for signaling may also be relevant to inhibitory signaling if the receptor density is low enough. Thus, our unpublished work has indicated that in order to inhibit the T cell factor-induced DNA synthesis of a B cell chronic lymphocytic leukemic clone which expressed very low levels of mIgM (~2 x 10^3 molecules/cell), orders of magnitude greater amounts of the lower affinity anti-IgM MoAbs were necessary than for inhibitory signaling of Lub and Kon cells (shown here, Fig. 18), which bear ~2 x 10^4 and 10^5 mIgM molecules per cell, respectively. Furthermore, Maruyama et al. (178) have found that significantly greater amounts of anti-IgM MoAbs were required to inhibit T cell factor-driven IgG and IgA large B cell differentiation than to inhibit similarly induced IgM large B cell differentiation. These observations can be explained in light of our above discussion if one considers that the mIgM density on a large B cell that has recently switched isotypes and is poised to differentiate to IgG or IgA plasma cells is very low.

We consider that the second explanation discussed above for diversity in ligand-binding requisites for activation or inhibition of B cell DNA synthesis may be the most physiologically relevant. This bias is based upon the recent observations of many other investigators which indicate that anti-Ig MoAb ligands that are ineffective at inducing the DNA synthesis of resting B cells can nevertheless initiate a number of early activation-related biochemical changes in B cells that preceed, or are concurrent with, an initial cycle of mIg capping. Examples of such early pre-proliferative events include membrane depolarization (36), and increased c-myc (37) and membrane Ia expression (36,81). This suggests that rigorous ligand binding requisites for B cell DNA synthesis must not be met in the first encounter of ligand with resting B cells but rather must be met at some point following an initial cycle of capping and endocytosis. The inference here is that the formation of a large and complex immunon, as shown in Fig. 22A, may not be necessary for the activation of resting B lymphocytes. Although it remains to be determined whether the binding requisites for inducing B cell tolerance are subtly different from those for inducing early

pre-proliferative changes in resting mature B cells, the current data are compatible with the hypothesis that binding requisites for inducing any early biochemical phenomena in B cells with a normal density of mIgM are the same. The inhibitory or stimulatory result of these early signals would depend upon the activation state of the particular B cell population being studied (111,167,178). In order for early pre-proliferative changes in stimulated resting B cells to lead to clonal expansion, one of two events is necessary. These are: a) further mIgM cross-linking by ligands that can form stable complexes of mIgM aggregates under conditions of low mIgM density, or b) signaling by activated class II MHC-restricted helper T cells or their products (14,20,211).

The biological implications of our studies on ligand-binding requisites for B cell signaling would appear to have the most immediate relevance to that role played by autologous rheumatoid factors or anti-idiotype Abs in the regulation of the B cell immune response. Since many anti-idiotype Abs may have a predilection for binding idiotypic determinants in a monogamous, "XG9-like" manner (193,201,208), these Abs may be ineffective at inducing the clonal expansion of B lymphocytes but active in the negative regulation of the maturation or differentiation of idiotype-positive B cell clones (212-214). Furthermore, since most human rheumatoid factors appear to be of relatively low affinity (215), these ligands might be predicted to manifest a more direct role in the negative feedback suppression of B cell responses than in B cell clonal expansion (25,216).

SUMMARY

The ligand binding requistes for membrane IgM-mediated signaling of human B lymphocyte clonal expansion and B cell tolerance were investigated with a well-characterized set of soluble murine monoclonal anti-human IgM antibodies. Evaluation of the impact of μ chain domain specificity, affinity, and binding stoichiometry for membrane IgM on Ab-induced regulation of normal and leukemic B cell DNA synthesis revealed that the ligand binding requisites for

inducing or, alternatively, suppressing B cell DNA synthesis are significantly different. First, while the induction of S phase entry required μg/ml concentrations of ligand, orders of magnitude lower concentrations of ligand sufficed for inhibitory signaling. Second, while an upper affinity threshold for achieving maximal stimulation of B cell DNA synthesis was never detected, inhibitory signaling by bivalent ligands appeared to become relatively affinity independent at Fab' binding affinities > 0.69×10^7 M^{-1}. Third, while a $C\mu_1$-specific MoAb with an enhanced incidence of monogamous binding to mIgM was ineffective at inducing B cell DNA synthesis, the Ab was not significantly compromised in ability to initiate inhibitory signals. Finally, whereas MoAbs to distinct domains could show striking synergy in the induction of B cell DNA synthesis, synergy was not noted for inhibitory signal transduction. These differences could be observed in a clonal B cell population which positively or negatively responded to mIgM ligation depending upon its state of activation. The accumulated observations indicate that the ligand binding requisites for inhibitory signal transduction in human B lymphocytes are much less rigorous than those for stimulatory signal transduction and suggest that many physiologically relevant anti-Ig antibodies are more likely to function in the negative feedback regulation of B cell responses than in the direct triggering of human B cell clonal expansion.

CHAPTER SIX

General Discussion

This thesis has focused on an evaluation of the binding requisites for human B cell clonal expansion and clonal suppression through ligation of the membrane IgM Ag receptor. By utilizing anti-IgM MoAbs of defined site specificity, affinity, and binding stoichiometry for human IgM, we have attempted to distinguish the ligand binding characteristics necessary for stimulatory and inhibitory signaling and further to elucidate the physical nature of the cross-linked unit of mIgM molecules necessary for each form of signaling.

Our analysis to date strongly suggests that affinity of a ligand is of paramount importance in determining the signaling potential of anti-isotype antibodies. Our data furthermore indicate that signal transduction for achieving DNA synthesis has a much higher affinity requisite than inhibitory signal transduction. These conclusions are compatible with and extend the varied reports of other laboratories. Leptin (78), working in the murine system, found that of 17 rat anti-murine IgM MoAbs, directed to each of the four IgM C_H domains, none were able to induce proliferation in soluble form, even in the presence of T cell factors. Yet, they each were able to inhibit LPS-induced B cell proliferation and maturation. Low affinity of the ligands appeared to be one likely explanation for the non-stimulatory properties of the soluble MoAbs since when these MoAbs were coupled onto an inert support, they all induced B cell DNA synthesis. Maruyama et al. (178) were able to observe stimulation of human B cell DNA synthesis by three soluble anti-IgM MoAbs and found that the lowest relative affinity Ab was the least effective at triggering proliferation. Hamano and Asofsky (79) also noted that ability to inhibit B cell hybridoma

proliferation and differentiation correlated with ligand affinity. The lowest affinity of two anti-murine IgM MoAbs was the least efficient signal transducer.

In agreement with Leptin (78), who used anti-IgM MoAbs, Goroff et al. (81) also found that non-mitogenic anti-IgD MoAbs can be made mitogenic by virtue of being made insoluble. In this latter study, non-mitogenicity of soluble anti-IgD MoAbs was found in some cases to be correlated with low relative affinity and, in other cases, with Fd specificity and poor cross-linking ability. A clear explanation for why certain high affinity anti-IgD MoAbs were poor cross-linkers and non-mitogenic was not evident at the time. Our experience with the $C\mu_1$-binding XG9 MoAb suggests that a propensity for monogamous binding to mIgD may have been responsible.

Because mature, resting B lymphocytes bear both IgM and IgD of identical Ag specificity, it has been suggested that these two receptor molecules serve different functions (16,17). Perhaps mIgD, with its longer and more flexible hinge (200), may best serve to capture multivalent, T cell-independent ligands and trigger B cells in a more direct manner. In contrast, mIgM, being rather inflexible and devoid of a hinge (200,207), may play a more important role in binding paucivalent T cell-dependent Ags and focusing them for cognate T cell help. In addition, it has been proposed that these two membrane isotypes may serve different functions for B cell tolerance, with mIgM being more efficient at transducing tolerogenic signals (16,17). The differing structures and possibly different functional roles of mIgM and mIgD make it highly appropriate that rigorous comparisons be made of the ligand signaling requisites through mIgM and mIgD with panels of specific Abs. Further work defining the minimal affinity requisites for signaling with anti-IgD MoAbs may elucidate whether signal transduction through mIgD differs in its ligand binding requisites from signal transduction through mIgM. One might predict that as a result of its more extensive and flexible hinge, the affinity requirements for cross-linking mIgD may be somewhat less restrictive than for cross-linking mIgM.

The phenomenal synergy noted between certain combinations of anti-IgM MoAbs is likely a reflection of the enhanced avidity associated with a multi-ligand complex. Stable binding of the ligands in this complex appeared to be influenced by the domain specificity of these MoAbs. Thus, synergy was most noted between MoAbs directed to distinct domains which were less apt to sterically interfere with one another and more likely to stabilize one anothers' binding to mIgM (217). These observations and conclusions likely relate to the occasional supra-additive signaling observed by Sell (218) with anti-allotypic Abs directed to distinct epitopes on rabbit IgG molecules. The ability of $C\mu_1$-binding MoAbs to effectively synergize with $C\mu_4$-specific MoAbs, despite the fact that when cultured individually, a large percentage of the $C\mu_1$-specific MoAb binding is monogamous, presumably reflects the ability of non-$C\mu_1$-specific Abs to bring mIgM molecules close together such that bigamous binding of $C\mu_1$-binding MoAbs is a more favorable event than in the absence of such binding. We have also noted exceptional synergy between two $C\mu_4$-specific MoAbs, 5D7 and 1G6. The fact that cross-inhibition assays showed these MoAbs to bind epitopes relatively distant from one another on $C\mu_4$ may explain this observation.

In addition to topographical location, affinity of the individual ligands in the mixture appeared to influence the capacity for synergy between pairs of MoAbs. Thus, mixtures, with one ligand of univalent Ka < 0.4×10^7 M^{-1}, in general did not appear to manifest substantial synergy. While MoAbs of Ka ~ 0.7×10^7 M^{-1}, ie. MoAb 1G6, were capable of showing very significant synergy with other higher affinity ligands, the concentration of MoAb 1G6 relative to the high affinity ligand required for maximal synergy was significantly greater. In the mixtures of 1G6 + XG9, and 1G6 + 5D7, greater that 100 μg/ml concentrations of MoAb 1G6, but only 10 μg/ml concentrations of MoAbs XG9 or 5D7 were required for maximal synergy (unpublished results).

A novel observation made during these studies was that certain mixtures of MoAbs were able to signal Go ⇒ S transitions in the absence of T cell factors. The only mixtures capable of achieving very significant Go ⇒ S transitions in the absence of T cell help were those which

included the $C\mu_4$-specific MoAb 1G6 plus a relatively high affinity $C\mu_1$-specific MoAb. With these MoAb combinations, the signal(s) generated to drive Go ⇒ S transitions in the absence of T cell help were almost as efficient as the signals generated in the presence of such help, since the addition of ancillary T cell supnt to these cultures did not induce substantially higher levels of DNA synthesis. The only individual MoAb which could induce significant S phase entry of resting normal B cells, or the Kon leukemic B cell clone, in the absence of T cell growth factors was the highest affinity Ab, HB57. However, Go ⇒ S signaling of resting normal B cells by HB57 alone was substantially compromised over the Go ⇒ S signaling observed by HB57 in the presence of T cell growth factors.

It is considered likely that the capacity of anti-IgM-binding ligands to induce S phase entry without the ancillary signaling provided by T cell factors is a reflection of the affinity of the ligand or ligand mixture. The capacity of high affinity ligands to more effectively cross-link mIgM molecules over a sustained interval, under conditions of low mIgM density, presumably allows for repeated signaling through mIgM. This signaling then bypasses the requirement for activation of ancillary signaling pathways utilized by T cell growth factors.

It is also possible, however, that in the case of the synergistic mixtures of MoAbs containing MoAb 1G6, additional elements come into play to provoke a T cell factor-independent response. Perhaps the binding of MoAb 1G6 to a unique site near the B cell membrane leads to allosteric changes in the mIgM molecule which, when combined with accessory cross-linkage mediated by a $C\mu_1$-specific MoAb, leads to optimal association with other non-Ig membrane constituents which are responsible for direct signal transduction. Recent observations from other laboratories have indicated or have suggested the idea that binding of certain MoAbs to epitopes on MHC molecules (105,219) or T cell receptors (220) can induce conformational changes in these molecules. Since these molecules are all part of the same supergene family (221), it is not unreasonable to consider that similar MoAb-induced conformational changes occur in the human

IgM molecule. It is alternatively possible that the significant T cell-independent signaling associated with mixtures of MoAbs containing MoAb 1G6 may be due to MoAb 1G6 cross-reactivity with a growth factor receptor or other membrane constituent with a signal transduction function.

Although most individual MoAbs were unable to induce resting B cells to enter S phase in the absence of T cell factors, many were capable of causing Go ⇒ G1 transitions in resting B cells. We have used as criteria for Go ⇒ G1 activation the capacity to induce responsiveness to T cell growth factors (13,222), or here specifically, the capacity to induce B cell DNA synthesis in the presence of activated T cell supnts. We have no direct evidence for the capacity of any of our ligands to induce early biochemical events associated with Go*, a pre-G1 activation phase described by Cambier et al. (39) and Klaus et al. (43). However, the fact that all our ligands can initiate tolerogenic signals, taken together with the fact that active signaling of B cell tolerance and B cell activation appear to involve some analagous early biochemical phenomena (14,23), suggests that all our ligands can cause at least some Go ⇒ Go* transitions. Compatible with this conclusion are the findings of others (36,37) that anti-IgM MoAb ligands that cannot induce DNA synthesis either in the presence or absence of growth factors can induce Go ⇒ Go* activation of resting B cells.

As shown in Fig. 23, it would appear that the capacity of ligands to induce each of the activation-associated cell cycle transitions is a direct reflection of their affinity for mIgM. MoAbs of very low affinity, such as IF11, P24, P19, and 1G6, which presumably can just induce minimal perturbation of the mIgM molecule upon ligation, can induce Go ⇒ Go* transitions. While Go* B cells undergo some of the early pre-proliferative changes associated with activation, they are not driven far enough into cycle to express growth factor receptors. However, the mIa hyper-expression which has been shown by others to be induced on B cells at Go*, may make these cells more amenable to direct cognate interaction with Ia-restricted T lymphocytes (14,35). Thus, such ligands, which are compromised in capacity to extensively cross-link mIgM and signal

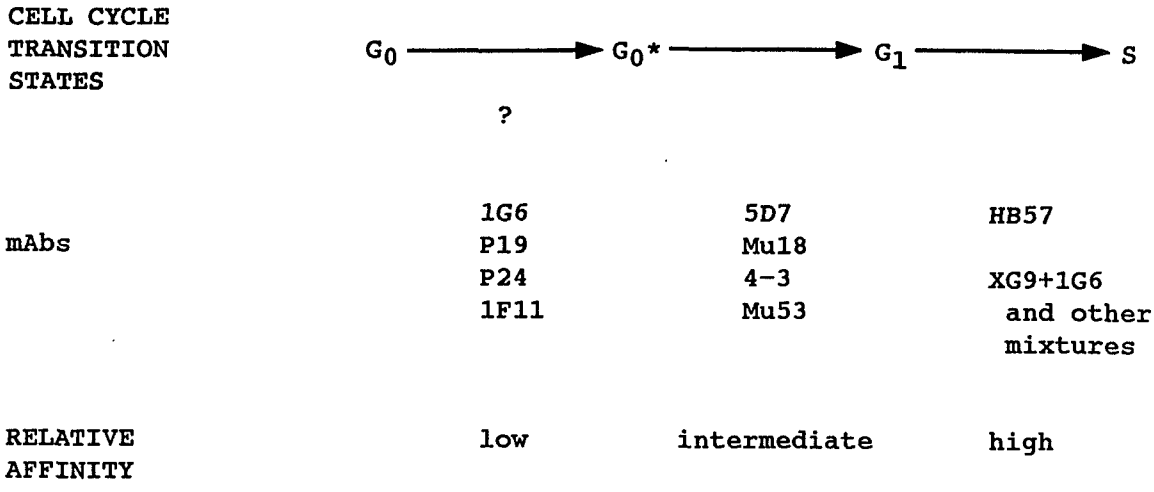

Figure 23. Capacity of anti-IgM MoAbs to induce the various cell cycle transitions appears to be directly related to ligand affinity for mIgM. Capacity to induce Go⇒Go* transition is inferred from ability to transduce signals for inhibition of leukemic DNA synthesis. Capacity to induce Go⇒Go*⇒G1 transition is indicated by induction of B cell DNA synthesis in the presence of ancillary T cell growth factors. Capacity to induce Go⇒Go*⇒G1⇒S transition is indicated by induction of DNA synthesis in the absence of T cell growth factors.

S phase entry, may, in the presence of the appropriate T cells, enhance the clonal expansion of B cells. This situation is most analagous to T cell-dependent signaling by paucivalent Ags with relatively low affinity interactions with mIg.

Intermediate affinity anti-IgM MoAbs, such as Mu53, 4-3, Mu18, and 5D7, all appear capable of eliciting enough mIgM cross-linking to activate the biochemical machinery appropriate to drive resting B lymphocytes from Go ⇒ G1. Here, the cells express receptors for growth factors, and cognate T-B collaboration (211) may become less essential for promoting DNA synthesis. Presumably, more highly multivalent Ags, or divalent Ags with intermediate affinity interactions with mIgM, would mimic the physiological effects of these anti-IgM ligands.

Finally, there are those ligands, which by the nature of their extremely high affinity binding to mIgM, are capable of driving resting B lymphocytes from Go all the way to S phase. These ligands include the MoAb HB57, to a partial degree, and especially MoAb mixtures exemplified by the $C\mu_4$-specific MoAb 1G6 and the $C\mu_1$-specific MoAb XG9, among several others. The threshold univalent affinity necessary to induce Go ⇒ S transitions must exceed that of HB57, ie. $5.6 \times 10^8\ M^{-1}$, since this MoAb was only marginally successful at inducing T cell factor-independent DNA synthesis. Highly multimeric T-independent type 2 Ags (223), and paucivalent Ags with very high affinity interactions with mIg, may mimic these ligands in their mode of action.

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