

Several pilot studies were undertaken to examine the use of lipofection as a means of transfecting rearranged immunoglobulin genes into stem cells. Several studies are currently in progress to analyze the ability of the "transfected" stem cells to engraft in irradiated SCID mice. Bone marrow cells from 5-FU-treated SCID mice were cultured for 4 days with SCF, IL-3, IL-6, and/or IL-7 prior to incubation with cDNAs for PC-specific heavy and light chain or neo-resistance. Following lipofection, the transfected cells were injected into irradiated SCID mice. At various times post-injection, mice were harvested and assayed for the presence of the PC-specific antibodies and B cells expressing the transfected gene product. Preliminary studies have not shown evidence of PC-specific antibody production in two separate experiments. Experiments currently in progress are examining the efficiency of electroporation versus lipofection in the uptake of PC-specific and neo-resistance cDNAs into 5-FU-treated SCID bone marrow following culture in IL-7. Results from this experiment are pending.

Section 2: Expression of RA3-6B2 epitope for murine CD45 on human NK cells: Biochemical and functional characterization of molecule.

As mentioned in previous reports, the CD45 antigen (Leukocyte Common Antigen) is expressed on all lympho-hematopoietic cells except for platelets and mature red blood cells. It is the product of a single gene, but through differential splicing of mRNA, eight mRNA species can be formed. The CD45 gene encodes a tyrosine phosphatase which has been shown to be involved in the de-phosphorylation of important src-like tyrosine kinases following activation of T cells. Our observation that an antibody generated against the murine CD45 molecule expressed on B cells (B220) is also selectively expressed on subpopulation of human cells is quite unique. Of interest, is that NK cells (CD56+) are 100% positive for the epitope recognized by the anti-murine CD45 antibody, RA3-6B2. Immunoprecipitation of ¹²⁵I- surface labeled human NK cells have indicated that this antibody recognizes a molecule of approximately 220,000 daltons. A molecule of the same molecular weight is also immunoprecipitated using an antibody directed against the human CD45 molecules. Studies to determine whether these two antibodies are recognizing the same molecule as well as studies to determine whether the molecule recognized by RA3-6B2 has phosphatase activity are currently underway.

In addition to physical characterization of this molecule, studies to determine the functional relevance of RA3-6B2 expression on NK cells is being performed in collaboration with Dr. John Ortaldo (NCI/FCRDC). Preliminary studies show that treatment with RA3-6B2 by itself does not appear to effect total protein phosphorylation or induce cytokine production. Examination of specific substrates such as the src-like tyrosine kinase lck will be investigated as well as effects following co-stimulation using anti-CD16 or anti-CD56. Studies examining the effects of RA3-6B2 on cytolytic activity are in progress. Initial studies show an inhibition in both ADCC and NK cytolytic activity. However, this phenomenon is dose-dependent, i.e. 50% inhibition of cytolytic activity occurs at doses below 1 ug/ml, and requires that the NK cells be pretreated with the antibody. Further studies to verify the dose-dependence as well as examine the interaction of RA3-6B2 during co-stimulation with additional molecules will be performed.

Section 3: Modulation of Signal Transduction in Phosphocholine-Specific B Cells from μ K Transgenic Mice

The B cells that develop in the μ K 207-4 transgenic mice differ from those of normal mice in that they express high levels of the transgene encoded product on their surface, express no sIgD, and endogenous encoded IgM is expressed on less than 20% of these cells. This cell surface phenotype is similar to that of immature B cells that have recently emerged from the bone marrow. B cells exhibiting this phenotype are more susceptible to tolerance induction than mature sIgM:sIgD positive B cells. We have analyzed the B cells from the 207-4 transgenic mice for their ability to respond to anti-Ig signals that induce proliferation in normal B cells. The results of these studies are in the accompanying manuscript which will be published in Current Topics in Microbiology and Immunology. These studies revealed a restricted defect in the ability of B cells from 207-4 mice to proliferate in response to soluble anti-Ig-antibodies even though they proliferate in response to the same antibodies conjugated to Sepharose beads (see Table 2). Treatment of these T⁺ B cells for as little as 1 hr with soluble anti- μ actually results in the death of approximately 2/3 of these B-cells within 24hr of stimulation (see Table 6). The proliferative defect seen in the PC-specific B cells from the 207-4 transgenic mice was not observed in the B cells from the μ K anti-TNP Sp6 transgenic mouse line (Table 5). These results may indicate a selective tolerance mechanism in the PC-specific B cells from the 207-4 transgenic mice. This defect may be the result of a previous encounter with autologous or environmental PC during the early stages of B cell development. Thus, if the PC-specific B cells are activated and expanded by environmental or autologous antigen, their biochemistry may be altered such that massive cross-linking of their receptors now leads to apoptosis and cell death rather than proliferation. On the other hand, the TNP-specific B cells that develop in Sp6 transgenic mice could represent virgin B cells which could utilize different biochemical activation pathways.

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MODULATION OF SIGNAL TRANSDUCTION IN PHOSPHOCHOLINE-SPECIFIC B CELLS FROM $\mu\kappa$ TRANSGENIC MICE

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INTRODUCTION

The bone marrow of an adult mouse produces approximately 16 million new B cells each day (1). The vast majority of these cells appear to be rapidly turned over since 2/3 of the peripheral B cell pool is comprised of long-lived $\mu^+\delta^+$ B cells (2). Recent studies indicate that newly generated B cells are selected into this long-lived pool via an Ig V region-mediated process that appears to involve internal autoantigens or external environmental antigens (3-7); and that this receptor-driven selection process is influenced by both the anatomical and physiological environment of the B cell (5,7) as well as the genetic make up of the host (8). Thus, we have recently demonstrated that phosphocholine-(PC)-specific B cells are positively selected into the peripheral lymphoid tissues of M167 μ -H-chain transgenic mice that express a normal X-chromosome but they are clonally deleted in both M167 μ and $\mu\kappa$ transgenic mice which coexpress these M167 transgenes with the X-linked immune deficiency gene, *xid* (6,8). Idiotypic and antigen binding analysis of antibodies generated via transfection of variant V_H1 genes in conjunction with the $\kappa 8$, $\kappa 22$, and $\kappa 24$ light chain genes suggest that both the positive and negative selection of idiotype-positive, PC-binding B cells are antigen-mediated and not idiotype-mediated processes (6)(Kenny, J.J. et al., submitted). In as much as >97% of the peripheral B cells in M167 $\mu\kappa$ transgenic mice express the transgene-encoded IgM product as an antigen-specific receptor on their surface (9), it was of interest to determine whether or not these antigen-selected B cells had been altered during their positive selection process. The data presented in this paper suggest that the initial encounter with antigen has resulted in a selective form of tolerance in that extensive cross-linking of the IgM receptors with soluble anti-Ig leads to the death of these B cells.

RESULTS AND DISCUSSION

Thymus Dependent Immune Responses Appear to be Normal in M167 Transgenic Mice

To analyze the in vivo and in vitro immune responses of B cells from M167 $\mu\kappa$ transgenic mice to PC, mice were either immunized i.p. with PC-KLH in CFA and their spleen cells assayed 5 days later for PC-specific PFC, or unprimed splenic B cells were set up in vitro with PC-KLH and a KLH-specific T helper cell line. The data in table 1 demonstrate that large numbers of M167-id⁺ PFC were generated in the spleens of transgene positive (TG⁺) mice while the TG⁻ littermates produced the expected T15-id dominant response. In as much as TG⁺ mice have more than 10×10^6 PC-specific B cells in their spleens prior to immunization (6,9), it is somewhat surprising that the TG⁺ mice produce less than 10^6 PC-specific PFC/spleen following immunization with

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PC-KLH. This could indicate that: 1) very few of these B cells are capable of developing into antibody secreting cells; or 2) that T cell help and/or other physiological or anatomical requirements may be limiting in situ. Pinkert et al. (10) have shown that only 1 in every 10^5 B cells from these M167 $\mu\kappa$ transgenic mice is capable of responding to PC in the splenic fragment assay, where presumably every B cell should be provided with maximum T cell help. However, as shown in Fig. 1 and in reference (11), the B cells from these mice make excellent antibody responses when placed in culture with PC-KLH and KLH-specific T helper cells. PC-specific antibody responses of 2.5 $\mu\text{g/ml}$ were produced with as few as 3×10^3 B cells per well. Due to the low number of B cells used, no anti-PC antibody was produced from TG⁻ B cells in assays performed in 96 well plates (data not shown).

The data in Table 1 and Fig. 1 indicate that the B cells from M167 $\mu\kappa$ TG⁺ mice are capable of responding normally in vivo and in vitro when provided with cognate T cell help. However, when spleen cells from TG⁺ and TG⁻ mice were cultured with optimally stimulatory concentrations of soluble anti- μ , or Sepharose-conjugated anti- μ , anti- μ^a , anti- μ^b , anti-id antibodies, or PC and analyzed for proliferation by ³H-TdR uptake, spleen cells from TG⁺ mice were unresponsive to soluble goat anti- μ while the normal TG⁻ cultures were stimulated ~30-fold over the medium control. In contrast, the TG⁺ spleen cells gave a 15-fold higher response than the medium control after stimulation with the same preparation of goat anti- μ conjugated onto Sepharose beads. TG⁺ cultures also responded to Sepharose conjugated anti-id, anti- μ^a -allotype, and PC and to LPS (Table 2). Overall, these results suggest that the transgene-encoded sIgM receptor is capable of transducing mitogenic signals when stimulated by Sepharose conjugated anti-Ig, but not soluble anti-Ig.

The unresponsiveness of TG⁺ B cells to soluble anti-Ig could be due to: 1) FcR-mediated inhibition; 2) T-cell suppression; 3) developmental arrest of these B cells; 4) anti- μ induced receptor modulation; or 5) receptor-induced cell death. It has been demonstrated that anti- μ -induced activation can be inhibited by the Fc portion of the antibody molecule acting through the B cell FcR (12). To investigate FcR-mediated inhibition as a possible cause of the unresponsiveness in TG⁺ B cells, the spleen cells from TG⁺ and TG⁻ mice were: 1) cultured with soluble goat anti- μ in the presence of purified

Table 1. Primary in vivo immune response of M167 $\mu\kappa$ transgenic mice to phosphocholine^a

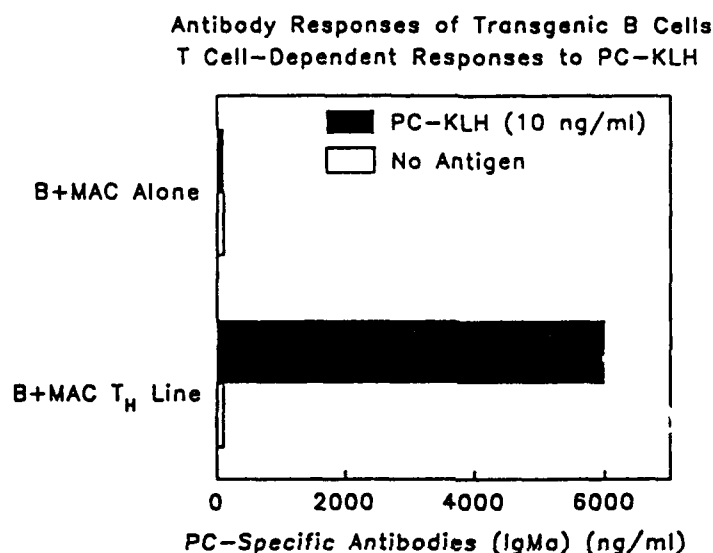
Mouse Phenotype	Plaque Forming Cells Per Spleen			
	Total IgM	% V _H 1-id	% T15-id	% M167-id
Transgene Positive	260,008	99	0	100
Transgene Negative	92,537	100	95	0

a) Mice were immunized I.P. with 100 μg of PC-KLH in CFA. Direct PFC were assayed on PC-SRBC 5 days after immunization.

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Microtiter wells were set up in triplicate with 10^4 anti-Thy1 + C' treated M167 $\mu\kappa$ spleen cells (8 + MAC) with or without 10^6 B10 KLH-specific T_H-cells. Media was changed at day 4 and supernatants assayed on PC-BSA-coated microtiter plates (9) at day 10.

Table 2. In vitro activation of spleen cells from M167 $\mu\kappa$ transgenic mice

	Transgene +	Transgene -
	CPM/Culture	
Media	4,372	5,281
Anti- μ	4,453	155,331
Anti- μ -Seph.	62,078	153,550
Anti-V _H -Id-Seph.	221,022	6,882
Anti-IgM ^a -Seph.	36,120	5,868
Anti-IgM ^b -Seph.	2,970	79,641
PC-Seph.	184,871	4,987
MOPC-21-Seph.	3,081	5,406
Rat-IgG-Seph.	8,055	5,441
LPS	51,409	73,242

- a) Spleen cells (3×10^5) from individual T⁺ and T⁻ 207-4 mice were cultured in the presence of soluble goat anti- μ (100 μ g/ml), goat anti- μ -Sephadex (1:150), anti- μ ^a-Sephadex (DS1-Sephadex, 1:100), anti- μ ^b-Sephadex (AF6-78.25-Sephadex, (1:100) for 48 hr. prior to pulsing with ³H-thymidine for 16 hrs.
- b) Data represent the geometric mean of triplicate cultures. The standard error for all groups exhibiting proliferation above that of the medium control was less than 5 % of the mean.

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monoclonal 2.4G2 anti-FcR antibody (13); and, 2) stimulated with goat F(ab')₂ anti- μ . The data in Table 3 demonstrate that anti-FcR antibody had no effect on the anti- μ response of the TG⁺ spleen cells. Treatment of TG⁺ and TG⁻ spleen cells with F(ab')₂ anti- μ resulted in a 3-fold increase in proliferation of the TG⁺ spleen cells, while the proliferation of C57BL/6 and TG⁻ spleen cells was enhanced an average of 1.6-fold over the responses obtained with an equal molar concentration of intact anti- μ -antibody (Table 4). The low response obtained with the F(ab')₂ anti- μ was still 3 times lower than the response of the same cells to anti- μ -conjugated Sepharose and 10 times lower than the response to anti-V_H1-id-conjugated beads. From these experiments, it is evident that FcR mediated inhibition is not the primary reason for the lack of anti- μ induced responses in TG⁺ B cells.

The removal of T cells by treatment with anti-Thy 1.2 + C' also had no effect on the ability of the TG⁺ B cells to respond to anti- μ , and the coculture of TG⁺ and TG⁻ spleen cells in the presence of anti- μ did not suppress the TG⁻ spleen cell response (data not shown). Since the anti-Thy + C' treatment completely eliminated the proliferative response to Con A, the presence of an active anti- μ -specific or non-specific suppressor T cell or suppressor factors in the TG⁺ spleen can be dismissed.

Anti- μ Induced Killing of B Cells from 207-4 Transgenic Mice.

We have previously shown that the B cells in M167 $\mu\kappa$ transgenic mice express high levels of sIgM and do not express sIgD even though ~20% of these B cells coexpress endogenous sIgM (9). In as much as this cell surface phenotype is similar to that of immature B cells that have recently emerged from the bone marrow (14), the restricted anti- μ stimulation defect observed in the B cells from 207-4 transgenic mice could be: 1) due to arrest of these B cells at a stage of development which is easily tolerizable, i.e. sIgM⁺IgD⁻; 2) the

Table 3. Anti- μ stimulation of Transgene Positive and Transgene Negative Spleen Cells in the Presence of Anti-Fc Receptor Antibody^a

Mitogens	Anti-FcR	C57BL/6	Transgene Positive	Transgene Negative
CPM/Culture				
Medium	-	4,456	424	1,352
	+	7,750	327	3,659
Goat anti- μ	-	86,203	861	20,066
	+	86,464	795	67,444
Goat anti- μ -Seph.	-	129,239	15,567	63,649
LPS	-	76,393	24,320	61,309

a) Spleen cells (3×10^6) of individual T⁺ and T⁻ mice were cultured in triplicate wells in the presence of soluble goat anti- μ (100 μ g/ml) and 2.4G2 monoclonal anti-FcR antibody, or Sepharose conjugated goat anti- μ (1:200) or LPS (50 μ g/ml) for 48 hr. prior to pulsing with ³H-thymidine for 16 hrs. Result are represented as a geometric mean of the CPM/culture, and standard errors of the mean were less than 5 % in activated cultures.

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Table 4. F(ab')₂ Anti- μ Stimulation of Transgene Positive and Transgene Negative Spleen Cells^a

Stimulating Agent	Transgene Positive	Transgene Negative
	CPM/Culture ^b	
Media	3,599	5,356
LPS	43,750	88,404
Goat anti- μ -Seph	39,475	77,671
Goat anti- μ 100 μ g/ml	6,100	69,157
F(ab') ₂ anti- μ 61 μ g/ml	12,354	207,095

a,b) Spleen cells were cultured and data analyzed as described in table 2.

consequence of a previous encounter with autologous or environmental PC which has resulted in a selective inactivation of certain biochemical activation pathways; or, 3) an activation defect common to all $\mu\kappa$ transgenic mice, which results from transgene-induced alteration in B cell development. This latter possibility was addressed by anti- μ stimulation of spleen cells from the $\mu\kappa$ anti-TNP Sp6 transgenic mouse line (15). The data in Table 5 show that both soluble anti- μ and anti- μ Sepharose beads induced significant proliferation in both TG⁺ and TG⁻ B cell populations; thus, the defect in B cells from M167 $\mu\kappa$ transgenic mice is not simply the result of $\mu\kappa$ transgene expression but may be related to the changes induced in these B cells following antigen-induced selection or to an arrest in their development. It is known that the TG⁺ TNP-specific B cells that coexpress endogenous sIgM also express sIgD (16). This suggests that the B cells in the TNP-transgenic mice have fully matured, whereas, those in the PC-transgenic mice may be immature.

It has been shown that anti- μ treatment of neonatal B cells, which express predominantly sIgM-only, results in either down modulation and failure to reexpress sIgM receptors (17) or the induction of apoptosis (Chang, T.L., submitted). On the other hand, mature sIgM:sIgD positive B cells will reexpress their receptors within 18 hr of anti-Ig stripping (17) and will then proliferate. It was therefore possible that soluble anti- μ caused either receptor down modulation on the IgM-only TG⁺ B cells and thus, no response would be seen because multiple rounds of anti- μ signaling are required to get effective induction of proliferation (18). Alternatively, the anti- μ stimulation could result in the death of these cells. To test both of these possibilities, TG⁺ and TG⁻ spleen cells were incubated with soluble goat anti- μ antibody or control goat IgG for one hr at 37°C to allow binding and capping of the sIgM on the B cells. The cell suspensions were then washed and a portion of the cells stained with biotin conjugated anti-B220 antibody plus either FITC conjugated goat anti- μ , anti- μ^a , or rabbit anti-goat-IgG. Only low levels of goat antibody remained after the stripping and wash procedure (not shown). The remainder of the cells were incubated overnight in RPMI-1640 + 10% FCS to allow regeneration of the membrane IgM and were then stained for B220, IgM and IgM^a-allotype. The results of one

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Table 5. Anti- μ Induced Activation of Spleen Cells from Sp6 $\mu\kappa$ Anti-TNP Transgenic Mice^a

Stimulating Agent	Transgene Positive		Transgene Negative	
	Mouse 1	Mouse 2	Mouse 1	Mouse 2
	CPM/Culture			
Media	2,490	1,783	3,680	5,321
Anti- μ	42,922	51,591	63,561	81,565
Anti- μ -Seph.	81,536	82,656	139,872	119,206
LPS	61,555	54,780	94,014	97,602

a) Spleen cells from two TG⁺ and TG⁻ mice were cultured and data calculated as described in table 2.

representative experiment are shown in Table 6. The TG⁺ and TG⁻ spleen cell populations initially had 13.6 and 36.5% B cells respectively. After 1 hr incubation with soluble anti- μ , staining of sIgM was reduced to less than 1%; thus, sIgM was efficiently capped and removed from both cell types. In both the TG⁺ and TG⁻ populations, the loss of B220⁺IgM⁺ cells was balanced by an increase in the number of B220⁺IgM⁻ cells. Twenty four hours after treatment with goat anti- μ , 48.3% of the TG⁻ spleen cells reexpressed sIgM as compared with 52.5% in the control. In marked contrast, only 6.4% of the TG⁺ cells reexpressed their surface IgM and very few B220⁺IgM⁻ cells remain in these cultures. These results indicate that approximately 2/3 of the TG⁺ B cells actually die within the first 24 hr following anti- μ stimulation. The B cells that remain do not appear to down modulate their receptors. These findings suggest that the failure of TG⁺ B cells to proliferate following anti- μ stimulation is due to preferential killing of these sIgM-only B cells.

Early Signal Transduction Events in Transgenic B Cells Appear to be Normal

The B cells from these M167 $\mu\kappa$ anti-PC transgenic mice present a unique opportunity for elucidating the difference(s) in the biochemical pathway(s) that lead either to cell proliferation or cell death following signal transduction through the same Ig-receptor. High concentrations of soluble anti- μ have been shown to induce both intracellular and extracellular calcium transport and increased phosphatidylinositol (PI) turnover within minutes of sIgM receptor cross-linking (19). Thus, we analyzed PI turnover in the B cells from M167 $\mu\kappa$ transgenic mice to determine whether or not this early activation event was altered in these cells. The data in Table 7 show that there was no difference in PI turnover in TG⁺ vs TG⁻ B cells following activation with anti- μ , LPS or aluminum fluoride. Preliminary analysis of Ca-flux in these cells also indicates that this activation step is unaltered, although initial unstimulated Ca levels may be higher in the B cells from TG⁺ mice (J. Mond, unpublished data). However, Hornbeck et al. (manuscript in preparation) have demonstrated that phosphomyristin C levels in unstimulated TG⁺ B cells are elevated 5 fold over those of TG⁻ B cells. Since phosphomyristin C is a primary substrate for protein kinase C and it

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Table 6. Goat anti- μ treatment of spleen cells from transgene positive mice induces B-cell death

		Percent of Total Cells ^{b)}				
Treatment ^{a)}		Transgene Positive			Transgene Negative	
		μ B220 ⁺	μ B220 ⁺	μ B220 ⁺	μ B220 ⁺	μ B220 ⁺
Day 1	Goat IgG	0.7	13.6	15.1	0.3	36.5
	Goat anti- μ	13.8	0.3	0.2	32.5	0.4
Day 2	Goat IgG	0.4	18.3	16.5	1.8	52.5
	Goat anti- μ	1.3	6.4	6.3	3.1	48.3

a) Spleen cells (1×10^7 /ml) from T⁺ and T⁺ 207-4 mice were cultured in the presence of soluble goat anti- μ (100 μ g/ml) for 1 hr. at 37°C, washed 3 times and cultured overnight at 37°C.

b) Spleen cells (1×10^6) were stained before and after anti- μ treatment with FITC-conjugated anti- μ and biotin-conjugated anti-B220 plus PE-Streptavidin and analyzed as described in the methods section.

Table 7. Anti- μ induced activation of the phosphatidylinositol cycle in B cells from M167 $\mu\kappa$ transgenic mice^{a)}

Inducing Agent	Ratio: PI/Total Myoinositol $\times 10^{-2}$	
	Transgene Positive	Transgene Negative
None	5.0	5.7
Anti- μ	8.0	8.4
LPS	6.0	6.3
ATF	10.8	11.1

a) Anti-Thy + C' treated spleen cells from M167 $\mu\kappa$ TG⁺ and TG⁻ littermates were cultured for 4 hours in inositol-free RPMI supplemented with 200-400 μ Ci of ³H-myo-inositol (58.5 Ci/mMole). Cells were washed, stimulated with soluble anti- μ for 30 min. and PI turnover measured as previously described (20).

is induced following cross-linking of sIgM, this observation may indicate that a biochemical change has indeed been induced in these cells following their initial in vivo selective encounter with antigen.

The data presented above indicate that the initial biochemical pathways that ultimately lead to either the induction of proliferation or cell death following high dose anti- μ cross-linking of sIgM receptors appear to be shared. However, it is not yet clear whether the cell death pathway has been programmed into the PC-specific B cells as a result of their positive selection by antigen or that it results from a developmental arrest of these cells as sIgM⁺:sIgD⁺ cells which are extremely susceptible to tolerance induction following excessive cross-linking of their IgM receptors (17). It

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will be necessary to analyze several strains of sIgM-only transgenic mice before we can rule out developmental arrest as a possible explanation.

When B cells are stimulated with soluble anti- μ , most sIgM receptors are engaged and modulated from the surface membrane, whereas, anti- μ on a Sepharose bead may engage only a limited number of receptors. It is therefore possible that the extent of receptor cross-linking is responsible for the difference in signaling. Thus, limited receptor cross-linking leads to proliferation of these cells while extensive cross-linking leads to cell death perhaps by apoptosis. Studies are in progress using different conjugation ratios of PC-dextran to elucidate the cross-linking parameters that differentiate between induction of death and proliferation. Brunswick et al. (21) have shown that very low concentrations of dextran-conjugated anti- μ or anti- δ can lead to B cell proliferation in the absence of Ca-flux or PI turnover, while higher doses of this polyclonal activator induce Ca-flux, PI turnover and proliferation. Thus, there appears to be at least 3 separate biochemical pathways that can be activated following cross-linking of the sIgM receptor. Elucidating these pathways and distinguishing what determines which pathway will be utilized should lead to new insights on B cell development and regulation.

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MODULATION OF SIGNAL TRANSDUCTION IN PHOSPHOCHOLINE-SPECIFIC B CELLS FROM $\mu\kappa$ TRANSGENIC MICE

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