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Reference: Contract N00014-89-C-0305, Transfection of Murine and Human Hematopoietic Progenitious with Rearranged Immunologlobulin Genes -- Contractor -- Program Resources, Inc.

In accordance with the appropriate contract requirements, attached is the annual report covering the period October 1990 through October 1991 for the referenced contract.

If you have any questions or require further information, please do not hesitate to contact me.

Sincerely,

PROGRAM RESOURCES, INC.

Donna L. Dawson

Donna L. Dawson
Manager, Contracts

TWH:DLD/ekw
Attachment

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Statement A per telecon
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Title: Transfection of murine and human hematopoietic progenitors with rearranged immunoglobulin genes

Principal Investigator: James J. Kenny, Ph.D.

Contract number: N00014-89-C0305

Reporting Period: October 1990-1991; annual report

Work Summary: There are three main objectives of the research conducted under the above contract. First to isolate hematopoietic stem cells in numbers adequate for transfection with rearranged immunoglobulin genes. Second, to develop techniques which will allow B cells expressing the transfected Ig-gene product to be activated by anti-idiotypic antibodies so that high levels of serum antibody are produced. Finally, to develop a human-mouse chimera that will allow us to transfect rearranged Ig-genes into human hematopoietic progenitors and then grow and activate those cells in an animal model.

Section 1: Isolation and characterization of early hematopoietic progenitor cells

Introduction

In the mouse, studies have shown that the stem cell can be identified within a population of cells which do not express mature lineage cell surface markers for T cells, B cells, granulocytes or monocytes. Thus, the stem cell is contained within a population of cells described as lineage-negative (lin-). Furthermore, the stem cell has been shown to express Thy-1 antigen and an antigenic marker called Stem Cell Antigen-1 (SCA-1). The stem cell is also quiescent, i.e. it is not sensitive to killing by cell cycle-active drugs such as 5-fluorouracil. These characteristics have been exploited in the isolation and study of stem cells. The frequency of the stem cell in a normal mouse is however very low, 0.05-0.2% of total bone marrow or approximately 20,000 cells/femur. The low percentage of stem cells in normal marrow makes isolation and manipulation of these cells difficult. However, it should be noted that these cells are greatly enriched in their ability to repopulate lethally irradiated animals, in that, injection of only 30 cells would result in 50% survival 30 days after lethal irradiation.

Section 1A: Murine Progenitor Cell Isolation.

Shown in Figure 1 is a FACS profile of normal bone marrow stained with FITC-conjugated anti-SCA-1 and a cocktail of biotinylated antibodies to mature lineage markers. The gate in Figure 1 shows the percentage of cells expressing SCA-1 and low levels of lineage markers. This gated population would contain the SCA-1,lin- population of cells described in the literature. Indeed, the use of normal mice and the double staining of bulk populations of cells in preparation for fluorescent activated cell sorting is technically labor intensive and would require the use of large numbers of mice. We sought to exploit other criteria of stem cells as a way of enriching for this population of cells. One method which we investigated is to enrich for stem

cells in vivo based on the fact that stem cells are quiescent and therefore escape the cell killing by cycle active drugs. We used the cycle active drug 5-fluorouracil (5FU) to eliminate most cycling progenitors. Previous studies have shown that 5FU-treatment enriches for one early progenitor with high proliferative potential in vitro and therefore we chose to examine the relationship of this high proliferative state with that of SCA-1 positive cell expansion. Table 1 shows a time course of SCA-1 positive cell expression various times post-5FU treatment. Table 1 clearly shows that 5FU treatment can increase the percentage of SCA-1-positive cells to 6-7% by day 7 post-5FU treatment. The level of SCA-1 expression falls to approximately 1% of total nucleated bone marrow cells by day 11. Figure 2 shows the FACS profile of day 4 5FU-treated bone marrow cells which was used for subsequent cell sorting of SCA-positive and SCA-negative cell populations.

In addition to the use of FACS for cell sorting of populations, we are also experimenting with alternate methods of purifying SCA-positive cells. One such mechanism involves the use of specially coated tissue culture flasks which allow you to covalently-couple specific antibodies to the surface and thus allow for positive selection of a given cell population. We are currently experimenting with activated flasks from Applied Immune Science, to which we have coupled the anti-SCA-1 antibody, clone D7. We have compared the cell yield of SCA-1-positive cells obtained from these flasks versus the percentage of cells present in the initial preparation as determined by flow cytometry. Our preliminary data suggests that we can obtain approximately the same percentage of SCA-1-positive cells by positive selection using the antibody-coated flasks as was reported in the preparation by FACS analysis (approximately 16% recovered from flasks versus 12% reported by FACS analysis of bulk bone marrow preparations). This new technique requires further study for optimizing staining and recovery and may provide a more efficient, rapid, and cost effective method for isolation of SCA-1-positive cells. This method is also beneficial because it minimizes the amount of time needed to isolate the cells (1.5 - 2 hr) compared to the several hours required to sort by FACS (4 - 5 hr) and is suitable for immediate injection into mice (some methods of positive selection require that the anti-SCA-1 antibody be attached to immunomagnetic beads thus requiring that the beads be injected into the mice).

Section 1B: Characterization of human hematopoietic stem cells.

In the human, the isolation and characterization of the stem cell is far behind that of the mouse. This is primarily due to the lack of adequate techniques for assessing human stem cell function. We do not have at our disposal the strict criteria of long-term hematopoietic reconstitution that is afforded in the mouse model. At best, we can examine the ability of progenitor cells to maintain clonogenic progeny in vitro. This cell is termed the Long-Term Culture-Initiating Cell (LTC-IC). Studies have identified a small population of cells in human bone marrow as expressing the antigen CD34 and also exhibiting the LTC-IC phenotype. Several studies have also shown that injection of CD34 cells into hematopoietically compromised baboons and humans have resulted in hematopoietic recovery of these animals. However, these studies do not address whether the CD34 cells are providing short-term hematopoietic reconstitution, i.e. survival until the host endogenous hematopoietic progenitors are expanded, or whether the CD34 cells are

responsible for the complete long-term reconstitution of the hematopoietic system.

Through our collaboration with Dr. Steven Kessler (Naval Medical Research Institute, Bethesda, MD) we are examining the positive and negative growth requirements for the CD34-positive cell. Dr. Kessler uses a single step, positive selection technique in which cadaveric bone marrow cells are incubated with magnetic beads which have been previously coupled with an anti-CD34 antibody. Cells and antibody-coated beads are allowed to interact, the beads and any attached cells are removed with a magnet and then the cells are chemically removed from the beads. This procedure yields a preparation of cells which is approximately 99% CD34-positive and does not alter other cell surface antigens. We have studied these cells with respect to their requirements for optimal cell growth in response to known growth factors as well as their response to negative regulators such as Transforming Growth Factor-beta (TGF-B). By understanding the proliferative and antiproliferative responses of this cell population, protocols for culturing these cells for subsequent gene transfer procedures would be established. Since several of the growth factors required for human progenitor cell growth are species specific, the identity of requisite cytokines for growth would suggest that in establishing human-mouse chimeric animals, one would have to supply the necessary human cytokines in vivo for successful engraftment. Our in vitro studies using CD34-positive cells would provide information as to the minimum requirements for human cytokines in these situations. To determine growth factor requirements of cell populations, progenitor cells are cultured in vitro in semi-solid medium containing growth factors. Each responsive progenitor cell will proliferate in culture and form a localized foci of daughter cells, thus producing a colony. By altering the growth factors in the colony forming assay, one can determine the number of responsive progenitor cells present in the preparation.

Several growth factors, also called cytokines, have been shown to stimulate bone marrow cell growth. One cytokine shown previously to stimulate multi-potential early progenitors is Interleukin-3 (IL-3). IL-3 has been shown to stimulate granulocyte, monocyte, megakaryocyte, and erythroid lineages. In contrast to IL-3, there are more "lineage restricted" cytokines such as Granulocyte Colony Stimulating Factor (G-CSF; growth stimulation results in the production of predominantly mature granulocytes) or Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF; growth results in differentiation to mature monocytes and granulocytes). Originally, these latter two CSFs were thought to act on later, more lineage-restricted progenitors. Recently, however, several studies have shown that these CSFs can play a role in stimulation of more primitive progenitors when used in combination with other growth factors. Within the past several months, a new growth factor has been cloned and binds to the proto-oncogene c-kit. This growth factor has been called c-kit ligand, mast cell growth factor, Steel factor, or stem cell factor based on the functional assay used to determine its identity. Stem cell factor (SCF) has little proliferative effect on progenitor cells by itself. However, it is one of the most potent synergistic factors identified to date for stimulating colony formation in the presence of

a single additional cytokine. It is important to note that the more primitive a progenitor cell (the closer it is to a stem cell) the more difficult it is to stimulate its growth in vitro.

Stem cells have been shown to require combinations of as many as 3 - 5 individual cytokines for optimal in vitro growth. Table 2 illustrates the point that individual cytokines do not always provide the optimal growth environment for early progenitors. Using CD34 expression as a criteria for isolating early human progenitors, we examined the cytokine requirements for positive and negative growth in vitro. When plating limiting numbers of cells (1500 per plate), substantial colony formation was only detected in cultures treated with G-CSF. However, if SCF was added to the cultures, there was a dramatic increase in the number of colonies formed. These data indicate that a substantial number of CD34-positive cells require at least two factors for growth, SCF being one of them. In two separate experiments using two different donors, there was a 3-4-fold increase in IL-3-responsive progenitors, 7-8-fold-increase in GM-CSF-responsive progenitors, and a 2-4-fold increase in G-CSF-responsive progenitors when cultured in the presence of SCF. Furthermore, Table 2 also illustrates that cytokine-induced proliferation of CD34-positive cells was inhibited by either TGF-B1 or TGF-B3. Both IL-3- and GM-CSF-responsive growth was inhibited 80-100% by either TGF-B1 or TGF-B3. Similar levels of inhibition were observed in the presence of SCF as well. In contrast to the substantial inhibition observed in IL-3- or GM-CSF-stimulated cultures, G-CSF-induced proliferation was inhibited to a lesser degree by TGF-B.

Section 2: Engraftment of human cells in SCID mice

Introduction

In order to determine the feasibility of expanding functional, antigen specific human B cell within a host, we need to have a model system in which we can introduce antigen specific B cell clones, activate these clones and measure the degree of functional antigen-specific antibody response generated. The emphasis of our contract is to use rearranged immunoglobulin genes specific for the hapten, phosphocholine, in the generation of antigen-specific (phosphocholine-specific) human B cells. It is our intent to transfect human bone marrow progenitors with the rearranged immunoglobulin genes and reconstitute SCID mice with these cells. We can then establish mechanisms for specifically activating these B cells in the mice through various immunization regimens. Initial success of this project depends on the ability to establish long-term engraftment of human cells in the SCID mouse. Since the SCID mouse is defective in rearranging its own immunoglobulin and T cell receptor genes, these mice are deficient in functional T and B cells. This inherent deficiency in murine B cells may provide a niche in which human B cells may expand. To date, several investigators have had limited success establishing long-term human cell engraftment. Several investigators have established the presence of human cells in these mice through injection of human peripheral blood cells, but the predominant human cell type which engrafts is the human T cell. Little success in engrafting SCID mice has been documented using human bone marrow as the source of human cells. Furthermore, in several studies, the engraftment of human B cells is lacking.

Section 2A: Experimental protocols for engrafting human bone marrow cells in SCID mice.

Several experiments were tried which dealt with varying irradiation doses from 150-250 rads prior to injection of human bone marrow cells (total bone marrow or lineage negative human bone marrow). We were unable to detect any human cells in either the murine bone marrow, spleen or thymus. No human immunoglobulin was detected in the serum. One preliminary experiment was tried in which neonatal SCID mice were injected with human bone marrow 24 hours after birth. We were still unable to detect any human cells in the organs mentioned above and no human immunoglobulin was detected in this case either.

Section 2B: Experimental protocols for engrafting human peripheral blood cells in SCID mice.

Several investigators have reported the successful engraftment of human cells in SCID mice when injecting human peripheral blood cells. We have taken advantage of this procedure to determine if prior engraftment of human T cells would provide a more conducive microenvironment for subsequent engraftment of human bone marrow cells. We have instituted a protocol which requires that a specific human donor supply peripheral blood and then 6-8 weeks later provide bone marrow cells. Initially, mice are treated with the antibody against the surface determinant asialo-GM1 which is present on natural killer cells. This pretreatment of mice enhances the long-term engraftment of human peripheral blood cells. Several experiments are underway to determine the effectiveness of low dose irradiation prior to bone marrow injection as a means of enhancing subsequent bone marrow engraftment. Since activated T cells have been shown to be a source of several growth factors such as IL-3 and GM-CSF (both of which are lineage specific), we are experimenting with various ways of activating the engrafted T cells in vivo as a means of generating human growth factors during the bone marrow engraftment period. Studies have focused on concanavalin A, insulin-like growth factor, and in vivo treatment of mice with the anti-CD3 antibody as mechanisms of possible activation. To date, we have been able to detect human immunoglobulin in the serum of mice following peripheral blood injection, however, the experiments are still underway and no data is available as to the level of engraftment observed in these mice with peripheral blood versus peripheral blood plus bone marrow.

Dr. William Murphy, a postdoctoral fellow in the Biological Response Modifiers Program has been reconstituting SCID mice with human PBL or bone marrow followed by treatment of the mice with human growth hormone (see manuscript in appendix). The treatment with growth hormone results in a 10 fold increase in human T cells in lymph nodes, and stable engraftment of human T cells into the mouse thymus. This is the first report to show that human T cells can home to the mouse thymus. Studies are in progress to determine whether human T cells are developing and differentiating within the mouse thymus. We hope that this new model will provide an appropriate environment within the SCID mouse for the subsequent growth of human bone marrow cells and CD34 progenitors.

Section 3: Transfection of human bone marrow with foreign DNA

Introduction

The study of gene transfer in human cells has received a great deal of attention recently. Several reports have had success using high titer retroviral vectors as vehicles for carrying the targeted DNA into the cells. Several reports have focused on the culturing techniques required for optimal infection efficiency. One thing that appears to be mandatory is that the target cell population be actively dividing at the time of retroviral infection to allow for the integration of the retroviral genome. One limitation to the use of retroviral vectors is the size of foreign DNA which can be packaged into the virion. This limitation requires that the size of your DNA be approximately 8 kilobases. If your DNA is larger, one must investigate alternative means of introducing your DNA into your cells. In our case, we are transfecting a plasmid containing a 19 kb DNA fragment. Thus, retroviral techniques are not suitable. We have chosen to use a liposome method called lipofection because we are working with low numbers of cells and thus require a high efficiency of gene transfer. DNA is mixed with a lipid component and incubated with the target cells. The lipid-DNA mixture fuses with the cell membrane, thus introducing the foreign DNA into the cell. The stable integration of DNA is still dependent on the proliferation of the cell, however, unlike retroviral vectors, there is no selective pressure or specific mechanism for integrating the DNA into the chromosome. Lipofection relies on random integration only.

Section 3A: Establishment of culture conditions for optimal transfection of DNA by lipofection.

To determine several preliminary culture conditions for transfection of DNA into human cells, we used the neomycin-resistance gene as our target DNA. The neomycin-resistance gene expression will allow us to follow the expression of the gene by examining the growth of cells in the presence of the drug G418. Proliferating cells which did not incorporate the neo gene will die. Initial experiments were performed to examine the sensitivity of human bone marrow cells to various concentrations of G418. In two separate experiments, concentrations of 1000 and 4000 micrograms of G418 was toxic to normal, untransfected bone marrow (Table 3). Studies are currently underway to transfect the neo gene into these cells and follow the expression of the gene in both liquid culture and colony assay in the presence and absence of 1000 and 4000 micrograms of G418.

Section 3B: Electroporation of mouse Ig-genes into human cells.

Rearranged human Ig-genes have been injected into mouse embryos to produce transgenic mice whose B cells produce and secrete human IgG, however, mouse Ig-genes have not been put into human cells. It is important to show that the genes that we intend to put in human stem cells will be expressed when these cells differentiate into B cells. To test this we have transfected a human B cell lymphoma line (DB) with rearranged mouse H & L chain genes. The drug selectable marker was carried by the light chain gene. The transfected cells were selected in G418 and three lines established. PCR

analysis of DNA and RNA (figure 6, appendix) shows that two of these lines (C4 & B5) express the H chain while the C3 line does not. However, none of the lines appears to be transcribing the DNA into mRNA. These results could represent a potential problem for our studies, however, additional transfections into a second human B cell line are being conducted to determine if this finding can be generalized.

Section 3C: Role of Interleukin-7 in stimulating pre-B cells in mice.

Interleukin 7 has been shown to be a potent stimulator of pre-B cells in vitro and in vivo. The responsive cell is positive for the CD45 B cell-specific antigen B220 and is negative for surface IgM expression. In vivo treatment of mice with IL-7 for 7 days (10 micrograms, twice daily) results in a massive expansion of B220+/sIgM- cells in the bone marrow and spleen (see Figure 7). Almost 80% of the bone marrow and 50% of the spleen is composed of these B cell progenitors. Treatment with IL-7 essentially turns the spleen into a hematopoietic organ for B cells (compare panel B with panel C in Figure 7). Since it has been so difficult to obtain adequate numbers of stem cells for gene transfection studies, we will attempt to electroporate or lipofect our Ig-genes into these IL-7 expanded B cell progenitors. The majority of these cells appear to die following removal of IL-7. Thus, the insertion of rearranged Ig-genes and the subsequent expression of their product as an IgM receptor on the surface of these cells should allow the transfected cells to survive and develop into functional B cells which can then be activated by anti-idiotypic antibodies.

Section 4: Anti-idiotype activation of B cells expressing antigen specific receptors of rearranged Ig-genes

The 207-4 $\mu\kappa$ anti-PC transgenic mouse line produced by Drs. U. Storb and R. Brenster expresses the transgene encoded M167-idiotype (id) on greater than 97% of its B-cells. These B cells respond normally both in vivo and in vitro to the thymus dependent antigen, PC-KLH, and thus represent a unique source of "naive" antigen-specific, id^+ B cells in which to analyze the mechanisms of antigen-driven or anti-id-driven differentiation. We have recently shown that the B cells in these mice are clonally deleted in an antigen-specific, receptor-mediated manner when the M167 $\mu\kappa$ anti-PC transgenes are coexpressed in the presence of the xid gene (see J. Immunol. manuscript in appendix). Furthermore, in M167 μ-only transgenic mice, M167-id^+ B cells, which arise by association of the M167 μ-transgene with an endogenous $V_\kappa 24$ light (L) chain, are expanded 100 to 500 fold over the number expected from random expression of L-chain genes (see J. Exp. Med. manuscript in appendix). This selective expansion of M167-id^+ B cells also appears to be an antigen-driven, receptor-mediated process. Thus, B cells having the same antigen specificity appear to be either clonally deleted or clonally expanded depending on the X-chromosome phenotype of the mouse in which they develop.

Section 4A: In vitro stimulation of B cells with anti-idiotypic antibodies.

The B cells that develop in the $\mu\kappa$ 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 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 5, appendix). 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 form the 207-4 transgenic mice was not observed in the B cells from the $\mu\kappa$ anti-TNP Sp6 transgenic mouse line (Table 7, appendix). 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.

Section 4B: Receptor mediated selection and amplification of antigen-specific, idiotype positive B cells

B-lymphocyte development in the mouse is a complex and dynamic process in which the adult bone marrow produces approximately 16 x 10^6 new B cells each day. However, very few of these B cells appear to enter the stable, long-lived peripheral B-cell pool where the half-life of a B cell, as measured by BrdUrd incorporation, is 3 months or longer. It is of interest to understand how the few B cells that enter this long-lived pool are chosen from the millions of B cells produced each day. This is important because we want to be able to selectively expand and activate the B cells that express the Ig-genes that we transfer into B cell progenitors.

To further address the problem of B-cell selection during ontogeny, we have analyzed the development of PC-specific and M167-id^+ B cells in a series of μ, κ, and $\mu\kappa$ transgenic mice. Storb and her collaborators found that all strains of M167 μ-only and some strains of M167-κ-only transgenic mice expressed high levels of endogenous V_κM167 and V_H1 mRNA, respectively. Many of these mice also exhibited elevated levels of circulating anti-PC antibodies. They suggested that this was due to activation of PC-specific B cells by environmental PC-containing antigens.

In the μ-only transgenic mice, the V_H1-id will be expressed on all B cells expressing the μ^a-transgene product, while the 28-6-20 and 28-5-15 combinatorial idiotopes will be expressed only on those B cells in which the μ^a-transgene product has associated with an endogenous germ-line M167 (V_κ24)

light chain. Since there is only a single copy of the M167 $V_\kappa 24$ L-chain gene in the mouse genome, and this V_κ-gene must rearrange to a $J_\kappa 5$-gene segment to produce the M167 κ L-chain, these M167 combinatorial idiotopes should occur in approximately 0.1% (1/800) of the μ^{a+}-B cells. This estimate is based on the assumption that the 100 to 200 endogenous κ-genes are expressed in a random fashion and randomly rearranged to the 4 functional J_κ-genes during transgenic B-cell development.

Spleen cells from μ-243-4 transgenic mice were stained with FITC-conjugated goat-anti-μ or anti-μ^a and biotin-conjugated anti-$V_H 1$-id, anti-T15-id or anti-M167-id followed by PE-Streptavidin. Fig. 3 (panel A, J.Exp. Med. manuscript in appendix) shows that 14% of the spleen cells or 28% of the B cells in this mouse expressed the transgene-encoded H-chain. The data in panel B show that none of the B cells in these M167 μ-only transgenic mice express the T15-id. Formation of a T15-id$^+$ B cell would require that the $V_H 1$ transgene associate with an endogenous $V_\kappa 22$ L-chain, and this should occur at the same frequency (~0.1%) as the generation of M167-id$^+$ B cells. By contrast, approximately 20% of the total B cells in this mouse express both the 28-5-15 binding-site-specific and the cross-reactive 28-6-20 M167-ids (Fig. 3, panels C & D). When one considers only the $V_H 1^+$ B cells, these $V_\kappa 24$-dependent M167-ids are expressed on 57 and 71% of the TG$^+$ B cells, respectively. This is significantly higher (500-fold) than the 0.1% M167-id$^+$ B cells expected from random expression of endogenous κ-genes.

To determine why M167-id$^+$ B cells were amplified 100 to 500 fold while T15-id$^+$ B cells were not detected, we electroporated the M167-H-chain gene into the non secreting SP2/0 myeloma line along with either the $\kappa 22$ or $\kappa 24$ light chain genes. The antibodies produced by the resulting cell lines were tested for both idiotype expression and for their ability to bind PC-BSA-coated plates. As shown in Table 2 (J. Exp. Med. manuscript in appendix), the antibody formed by association of the M167-μ H-chain and the $V_\kappa 22$ L-chain expresses the T15-idiotopes detected by the T139.2 monoclonal anti-T15-antibodies, but this antibody is at least 100 times less efficient at binding PC than the T15$^+$ IgM antibody HPCM2, which was used as a control to generate the standard curves in the PC-specific ELISA. On the other hand, the M167-id$^+$ antibody formed by association of the M167-μ-transgene and the $V_\kappa 24$ L-chain was PC-specific and bound PC-BSA to the same extent as the control. These data suggest that the in vivo selection and amplification of M167-id$^+$ B cells in the μ-transgenic mice is an antigen-driven rather than an anti-idiotype-driven event. B cells expressing the normally dominant T15-id$^+$ are not selectively amplified because the antibody product formed by the M167-μ transgene product and the endogenous $V_\kappa 22$ L-chain has little or no affinity for PC.

The above data imply that B cells emerging from the bone marrow are selected into a long lived pool of peripherial B cells via activation by antigen or some other receptor mediated process. Thus, it will be very important for us to determine if anti-idiotypic antibody when used in the in vivo situation will result in positive or negative selection of these B cells. During the next year, we will address this question by transfering bone marrow cells from the $\mu\kappa$ anti-PC transgenic mice into irradiated recipients in the presence and absence of anti-id antibodies.

Section 5: Expression of murine CD45 antigen on human cells

Introduction

CD45 antigen (also called Common Leukocyte Antigen) is expressed on all lympho-hematopoietic cells except for platelets and erythrocytes. It is the product of a single gene, however, as many as eight different mRNA transcripts have been detected due to alternate splicing mechanisms. The gene products range in size from 180,000 - 220,000 daltons. The gene encodes for a protein with tyrosine phosphatase activity. The antibodies which recognize the CD45 antigen are grouped into two families, those which recognize conserved epitopes and can detect all isoforms of CD45 versus those which detect isoform-specific sequences. The latter antibodies are called "restricted" antibodies and are designated as CD45R antibodies. The antibodies described in our studies are KC56, which recognizes the common epitope and is therefore considered a pan-CD45 antibody, and RA3-6B2, which is a CD45R antibody recognizing the 220,000 dalton isoform present on murine B cells.

Several studies have shown that expression of the various isoforms can be developmentally regulated. Studies involving T cell differentiation showed that cortical thymocytes can express only the lowest molecular weight form of CD45 and are positive for both CD4 and CD8. In the medulla, CD4 or CD8 single-positive cells were shown to express the restricted isoforms as well as the lowest molecular weight isoform. Mature T cells have also been shown to exhibit differential expression of the CD45 antigens. Virgin T cells express the restricted 220,000 dalton isoform, while memory T cells express the 180,000 dalton isoform and lack the 220,000 dalton isoform. Similarly, early B cell progenitors express low levels of CD45. This low level of antigen expression increased with B cell differentiation and disappeared at the plasma cell stage. Several functional studies have shown that in T cells, CD45 antigen can regulate signal transduction by increasing the proliferative response to PHA via an IL2/IL2 receptor pathway, can block NK activity, and that CD45 phosphatase activity may be involved in feedback regulation of phosphorylation since CD45 activation is necessary for activation of $pp56^{lck}$ activity.

Expression of CD45 is also regulated during myeloid cell differentiation. The 180,000 molecular weight form is dull on immature cells, however, during differentiation, its expression increases. The high molecular weight form is absent on all stages of normal myeloblasts but is expressed on cells from several acute myelogenous leukemic patients. The relevance of the aberrant CD45R expression on leukemic cells is unknown. However, given that many malignancies have increased tyrosine kinase oncogene expression and that several src-related oncogenes require phosphatase activity for activation (i.e. lck or hck), it is possible that the absence of CD45R expression on normal myelocytes and its expression on leukemic blasts plays a role in the maintenance of the undifferentiated state in leukemia. In addition, CD45 antigen has been shown to be involved in progenitor cell growth. Recent studies have shown that inclusion of anti-CD45 antibodies or antisense oligonucleotides inhibited IL-3-, GM-CSF- or GM-CSF/SCF-induced progenitor cell growth but not growth induced by G-CSF or M-CSF. CD45 antigen has also

been shown to affect mature effector cell function. Neutrophil chemotaxis in response to leukotrienes or complement components was inhibited by the pan-CD45 antibody, KC56, but not by a second pan-CD45 antibody (HLe-1). Thus, certain CD45 epitopes can interfere with neutrophil chemotaxis to several but not all chemoattractants.

Section 5A: Expression of B220 on primary human cells.

Following reconstitution of SCID mice with human cells, it is desirable to be able to distinguish human and mouse B cell progenitors. In addition to the human HLA-ABC markers, we felt that this would also be possible based on expression of different epitopes of CD45 which were anticipated to be unique to either the mouse or human cells. However, when human peripheral blood lymphocytes were analyzed by FACS for the expression of the CD45R epitope detected by rat anti-mouse B220, we found that approximately 20% of human peripheral blood cells expressed this epitope (Figure 3A). This antibody has been shown previously to recognize the B220 isoform of murine CD45 found principally on murine B cells. Figure 3B shows the percentage of human peripheral blood cells staining positive with the RA3-6B2 antibody. Approximately 4% of the total 31% CD8-positive cells (anti-T8 antibody) also stained with RA3-6B2. In the case of B cells, approximately 50% of the CD20-positive (anti-B1 antibody) B cells are also B220-positive. Lastly, expression of HLA-DR antigen (human MHC Class II) showed that again, approximately 50% of the lymphoid size cells which are HLA-DR-positive are also B220-positive.

Examination of human bone marrow cells also showed low levels of detectable staining with the antibody RA3-6B2 (Figure 4A). Approximately 16% of the small cells contained in the lymphoid and progenitor cell gate (as determined by forward versus side scatter) were RA3-6B2 positive. In the bone marrow, a portion of CD19-positive B cells were positive, while CD4 and CD8 T cells, as well as macrophages, were negative (Figure 4B). Furthermore, co-ordinate expression of human CD45 (KC56 antibody) and murine B220 (RA3-6B2) shows that all B220-positive cells also express KC56 antigen (Figure 5A). In addition, there appears to be a correlation between the level of expression of these two antigens. Cells that express intermediate levels of antigen density for B220 also express intermediate levels of KC56 expression, while those cells which express high levels of B220 antigen also express high levels of the human CD45. The detection of the murine CD45 epitope is restricted to that recognized by RA3-6B2 since use of an antibody that recognizes a conserved murine CD45 epitope was negative (Figure 5B) as well as other antibody clones shown to react with the murine B220 molecule (Data not shown).

Section 5B: Expression of B220 on human progenitor cell lines.

Examination of several human progenitor cell lines showed that B220 expression was present on the human acute myelogenous leukemic cell line, KG-1. KG-1 cells expressed low levels of antigen detected by RA3-6B2. The specific staining was block by the addition of unlabeled RA3-6B2 antibody, but not by an irrelevant antibody (data not shown). Furthermore, treatment of KG-1 cells with PMA for 5 days resulted in increased B220 staining, with the greatest increase observed in the number of cells positive for both B220 and

the early progenitor antigen CD34 (Table 4). Preliminary experiments to characterize, by Western blot, the antigen recognized by RA3-6B2 on KG-1 cells showed that RA3-6B2 recognized a protein of approximately 220,000 daltons. Preliminary experiments using the glycoprotein inhibitor tunicamycin indicate that the detection by RA3-6B2 may be carbohydrate-dependent (data not shown).

MAJOR ACCOMPLISHMENTS ABSTRACT

Two published papers and one manuscripts are included in the appendix. The two publications demonstrate that many B cells develop in the bone marrow but very few of these B cells are selected into the long-lived B cell pool which exist in the peripherial lymphoid organs. The selection of B cells into this stable pool of cells occurs via a receptor mediated mechanism. In the 207-4 $\mu\kappa$ anti-PC transgenic mice, environmental or autologous PC-antigen may play a key role in selection of these cells. In mice with an x-linked immune defect, these PC-specific cells appear to be clonally deleted by antigen while in normal mice they are expanded. In anti-TNP xid transgenic mice the B cells are not deleted; thus, the PC-specificity of the Ig-receptor in 207-4 mice is determining the fate of the B cells.

The manuscript submitted for publication demonstrates our success in developing a human-mouse chimera. Treatment of SCID mice with human growth hormone results in stable development of human T cells in all mouse lymphoid tissues including the mouse thymus. We hope to use this model to first establish human T cells in the mouse and then transfer in syngeneic bone marrow plus human IL-3, GM-CSF and G-CSF. Using enriched CD34-positive human progenitor cells, we have characterized growth requirements for optimal positive and negative growth regulation. The use of stem cell factor plus either GM-CSF or IL-3 enhanced the cloning efficiency of these cells. The CD34-positive cells were subject to negative regulation by transforming growth factor β. The manipulation of the CD34 cells in vitro through the use of positive regulators will provide a basis for optimizing growth conditions during gene transfer experiments.

Large numbers of stem cells have been difficult to isolate for the purpose of gene transfer studies; however, by using the in vivo cell cycle active drug, 5FU, we have enriched the murine bone marrow for cells expressing the stem cell antigen (SCA-1). This enrichment allows for greater cell sorting efficiency and will hopefully provide us with the number of stem cells needed for transfection of rearranged Ig-genes. We have further demonstrated that large numbers of B cell progenitors develop in IL-7 treated mice, and we will attempt to use these as the target cell for transfer of rearranged Ig-genes; however, short term reconstitution and protection rather than long term reconstitution will occur in this situation as compared to transfected stem cells. In many situations short term antibody protection may be all that is required.

The ability to follow human B cell development in the human-SCID chimera has been complicated by our observation that some human cells express a CD45 restricted determinant that cross-reacts with the rat anti-mouse B220 determinant detected by antibody RA3-6B2. We are currently characterizing the biochemical nature of this determinant and the possible biological reason for its expression on restricted populations of human cells.

TECHNICAL PROBLEMS

- The major difficulty encounter in this project has been the ability to obtain large numbers of highly pure stem cells. Large numbers of cells are required in order to electroporate or lipofect rearranged Ig-genes. Using IL-7 to produce large numbers of B220 progenitor cells may led to a satisfactory solution; however, the end result will be only a temporary production of the desired antibody as opposed to the permanent production, which should result from a transfected stem cell.

- Most gene transfer studies use retroviral vectors. However, at present, our genes are too large to use these vectors, therefore different modes of introduction of genetic material must be tried. Since electroporation destroys approximately 90 % of the cells, we are working out the optimum conditions for lipofection of the DNA.

- Expression of B220 on human cells is at low antigen density. While most of the characterization of the epitope recognized by this antibody can be done using the KG-1 cell line, more detailed functional analysis of its expression on primary cells will be difficult. Sorting and comparing the function of B220-positive and negative B or T cells using primary human cells will be difficult due to the low percentages of each with which we must work.

FUTURE WORK

- Determine whether the B6D2F1 bone marrow can reconstitute the SCID mouse. If so, demonstrate that our 5FU-treatment and SCA-1 sorted cells can reconstitute lethally irradiated mice.
- Determine whether Stem Cell Factor will enrich the reconstitution ability of SCA-1-positive cells in vivo and whether Stem Cell Factor can be used in vitro to aid in our gene transfer studies.
- In the human/SCID chimera studies, determine whether in vivo delivery of IL-3, GM-CSF, plus Stem Cell Factor will aid in the engraftment of human bone marrow cells or CD34-positive cells.
- Characterize transformed human bone marrow stromal cell lines. Determine the feasibility of using these cell lines to precondition the SCID mouse prior to injection of human bone marrow or CD34-positive cells. Determine whether the stromal cell lines will support the growth of human bone marrow cells during gene transfer studies.
- Explore additional techniques for gene transfer which are not retroviral-mediated. In particular, lipofection requirements. Determine whether protocols used to generate increased retroviral gene transfer will apply for lipofection. Use the neo-resistance gene as a selection marker to study gene transfer efficiency. Use the various cytokines and stromal cell lines mentioned above during these studies. Attempt to transfer genes into SCA-1-positive murine cells and look for subsequent in vivo reconstitution of mice with transfected cells. Explore requirements for transfection into CD34-positive cells and examine efficiency of transfer using in vitro selection techniques.

- Attempt transfection of rearranged immunoglobulin genes into murine pre-B cells and reconstitution of mice with these cells. Attempt to stimulate expansion of transfected B cell pool through in vivo immunization using anti-idiotypic antibodies and phosphocholine antigens.

- Further determine the nature of B220 antigen expression on human cells by Western blot. Using sequential immunoprecipitation, determine whether the B220 antigen is located on the human CD45 antigen or whether they are coordinately regulated. Examine the functional consequences of B220 expression in KG-1, i.e. responsiveness to cytokines using colony assay and adherence to stromal cell lines. Continue to characterize the expression in primary human cells. Examine the effects of B220 expression on human immunoglobulin production and determine whether B220 expression correlates with human T cell subsets and its function within the T cell population.

PUBLICATIONS

1. Kenny, J.J., Stall, A.M., Sieckmann, D.G., Lamers, M.C., Finkelman, F.D., Finch, L. and Longo, D.L. (1991) Receptor-Mediated Elimination of Phosphocholine-Specific B Cells in X-Linked Immune-Deficient Mice. J. Immunol. 146:2568.

2. Kenny, J.J., O'Connell, C., Sieckmann, D.G., Fischer, R.T. and Longo, D.L. (1991) Selection of Antigen-specific, Idiotype-positive B Cells in Transgenic Mice Expressing a Rearranged M167-μ Heavy Chain Gene. J. Exp. Med. 174:1189.

3. Murphy, W.J., Durum, S.K., Longo, D.L. (1991) Human Growth Hormone Promotes Engraftment of Murine or Human T Cells in SCID Mice. Proc. Natl. Acad. Sci. USA, submitted.

APPENDIX

TABLE 1. SCA-1 Expression in 5FU-Treated Bone Marrow[a]

Days Post-5FU	Percent SCA-1 Positive Cells[b]
D3	6%
D4	6%
D5	6.6%
D6	6.9%
D7	5.2%
D11	1.2%

[a]$B6D2F_1$ mice were treated with 160 mg/kg of 5FU i.v. BM were harvested from mice at various times post-5FU treatment.

[b]BM cells were harvested, the erythrocytes lysed by hypotonic lysis and the nucleated cells were incubated with either αSCA-1 ab (Clone D7) Biotin or normal rat isotype for 30' at 4°. Cells were washed and incubated with Streptavidin-PE for 20' 4° and analyzed by flow cytometry using the Coulter 753. % Sca-1 staining represents the level of antigen expression minus that of isotype matched control.

TABLE 2. Growth Factor Responsiveness of $CD34^+$ Human Bone Marrow Cells[a]

	Growth Factor/CSF[b]							
TGF-β	0	IL3	GM	G	SCF	IL3/SCF	GM/CSF	G/SCF
0	0	7.3 ± 3.1	5.3 ± 0.9	23 ± 3.6	0	21.3 ± 5.2	39 ± 5.3	84 ± 3.3
	0	3.7 ± 1.9	2 ± 0.8	17.7 ± 3.9	0	14.3 ± 1.7	16.7 ± 5.4	31 ± 3.3
β_1	0	1.2 ± 0.9	0.7 ± 0.9	8 ± 0.8	0	2.7 ± 0.5	0	25.3 ± 4.5
	0	0.3 ± 0.5	0.3 ± 0.5	9 ± 3.3	0	1.3 ± 1.2	1.7 ± 1.2	20 ± 4.4
β_3	0	0.5 ± 0.5	0	4 ± 0.8	0	0.3 ± 0.5	13 ± 3.3	
	0	0	0	6.7 ± 0.5	0	0	17 ± 4.0	

[a]Human $CD34^+$ cells were isolated from two human cadaveric bone marrows by a single-step positive selection technique using immunomagnetic bead selection.

[b]Fifteen hundred $CD34^+$ cells were plated in methylcellulose medium containing various growth factor. The concentrations of cytokines were 20 ng/ml of G-CSF, GM-CSF, or IL3, 100 ng/ml SCF and 10 ng/ml TGF-β_1 or TGF-β_3.

TABLE 3. % Viability of Human BM Cells in G418[a]

	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7
Experiment 5/31							
Total BM, medium	99	97	91	75	47	40	
1000 ug	78	40	55	0	0	0	
4000 ug	58	28	38	0	0	0	
Experiment 6/27							
Total BM, medium	90	82	80				
100 ug	97	68	47				
400 ug	97	65	50				
1000 ug	100	0	50				
4000 ug	41	0	0				

[a]Ficol-hypaque purified human bone marrow cells were cultured in Iscove's 10% FCS medium containing 5637 conditioned medium and IL7. Cultures were incubated with various concentrations of the drug G418. Cell viability was assessed weekly by trypan blue dye exclusion.

TABLE 4. B220 Expression on KG-1 Cells in The Absence or Presence of PMA[a]

ANTIGEN EXPRESSION[b]	PERCENT POSITIVE CELLS[c]	
	Medium-Treated	PMA-Treated
Total B220	7	41
CD34 Only	75	48
B220 Only	0	5
CD34 and B220	9	42

[a]The level of B220 expression on the acute promyelocytic cell line, KG-1, was analyzed by flow cytometry.

[b]One x 10^6 cells were stained with control ab, RA3-6B2 (anti-B220 specific) or anti CD34.

[c]KG-1 cells were cultured for 5 days in the presence or absence of 10 ng/ml PMA. Percent positive cells were determined for total expression of B220, for those cells expressing only B220 or CD34 versus those co-expressing both CD34 and B220. Percent positive cells represent specific staining minus background. Untreated and PMA-treated cells were 98% viable at the end of the 5 day culture period.

TABLE 5. In Vitro Activation of Spleen Cells from M167 $\mu\kappa$ Transgenic Mice

	Transgene +	Transgene -
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
Goat-IgG-Seph.	6,055	3,399
LPS	51,409	73,242

Spleen cells from 207-4 anti-PC transgenic mice were stimulated for 48 hr. with the reagents listed in the left hand column. The cultures were then pulsed with ^{3}H-Thymidine for 18 hr. The data represent the CPM of thymidine incorporated into DNA and demonstrate that soluble anti-μ fails to induce proliferation in the T^+ B cells while this same antibody conjugated to Sepharose beads induces good proliferation. Other reagents such as anti-idiotype, anti-allotype or antigen when conjugated to beads also induce proliferation.

TABLE 6. Inability of T^+ splenic B cells to regenerate surface IgM after capping with goat anti-μ

Treatment		Total Cells				
		T^+			T^-	
		μ^- $B220^+$	μ^+ $B220^+$	μ^{a+} $B220^+$	μ^- $B220^+$	μ^+ $B220^+$
Day 1	Untreated	0.4	15.6	15.7	0.4	37.0
	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	Untreated	0.4	16.6	15.7	1.5	53.7
	Goat IgG	0.4	18.3	16.5	1.8	52.5
	Goat anti-μ	1.3	6.4	6.3	3.1	43.3

Spleen cells from Transgene positive (T^+) and transgene negative (T^-) mice were treated for 1 hr. at 37C with 100 μg/ml of goat anti-μ antibody or normal goat IgG. The cells were washed and part of the cells put in culture overnight. Cells from day 1 and day 2 cultured cells were stained with FITC-conjugated anti-μ and biotin-conjugated anti-B220 plus PE-Streptavidin and then analyzed by flow cytometry. The data represent the % cells staining with these reagents. Greater than 60 % of the T^+ B cells die following treatment with anti-μ while the B cells from T^- mice regrow their sIgM receptors.

TABLE 7. In Vitro Activation of Spleen Cells from Sp6 $\mu\kappa$ Anti-TNP Transgenic Mice

	Transgene +		Transgene -	
Media	2,490	1,783	3,688	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
ConA	228,965	253,187	257,928	251,084

Spleen cells from Sp6 anti-TNP transgenic mice were stimulated for 48 hr. with the reagents listed in the left hand column. The cultures were then pulsed with ^{3}H-Thymidine for 18 hr. The data represent the CPM of thymidine incorporated into DNA, and demonstrate that soluble anti-μ induces good proliferation in both the T^{+} and T^{-} B cells. This is in marked contrast to the results shown in table 6 for the B cells from 207-4 anti-PC transgenic mice.

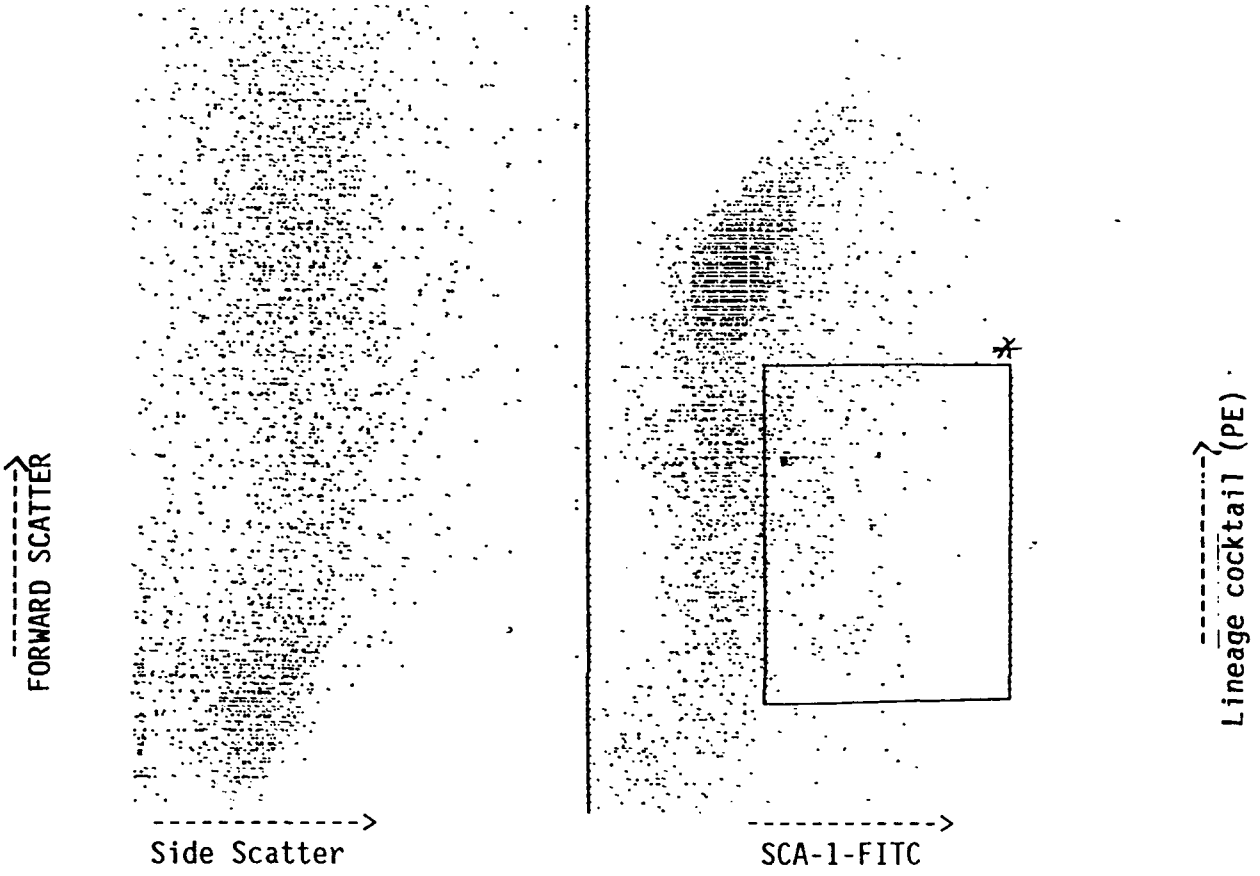


FIGURE 1. SCA-1 Antigen Expression on Normal Murine Bone Marrow Cells. Normal murine bone marrow cells were double-stained with anti-SCA-1-FITC antibody and lineage specific-biotinylated antibodies. Cells were subsequently stained with streptavidin-PE and analyzed by FACS. The boxed area represent the proportion of SCA-1-positive/lin-cells which contain early murine stem cells.

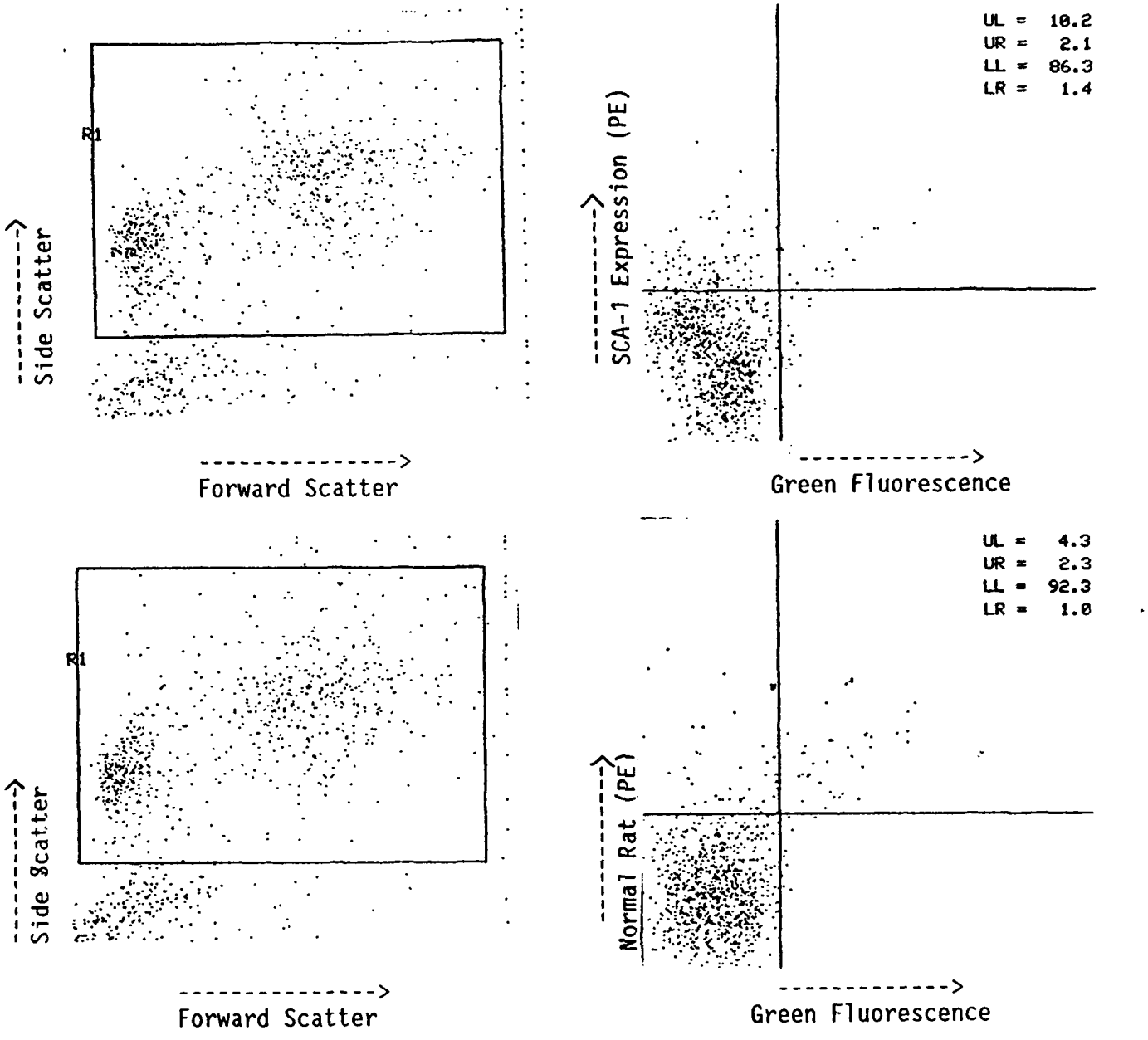
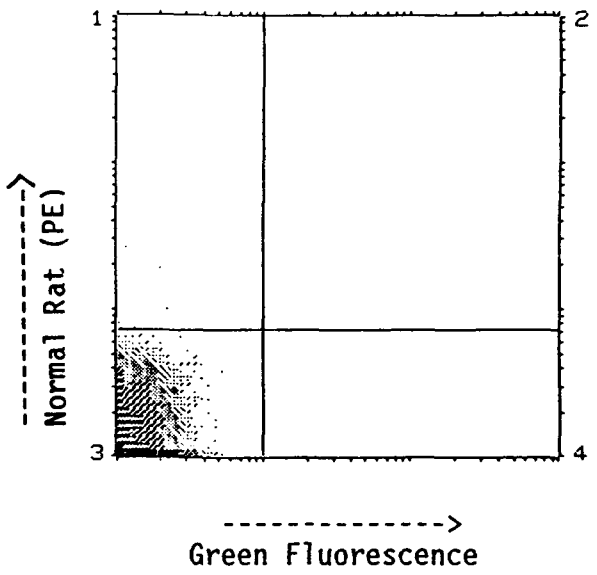
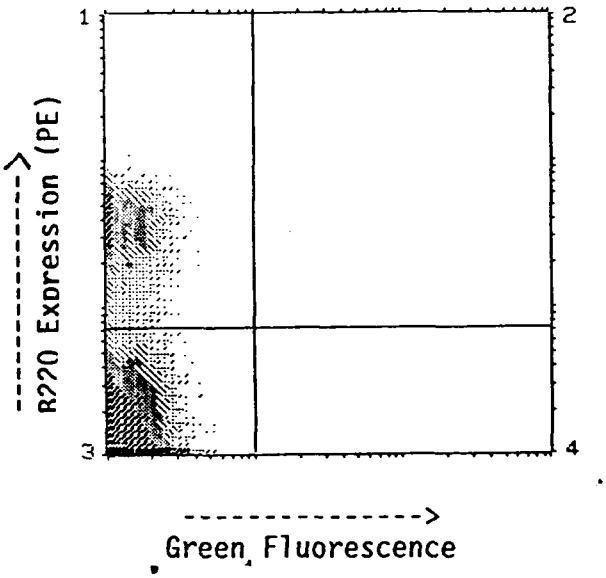


FIGURE 2. SCA-1 Antigen Expression in Day 4 Post-5FU Treated Bone Marrow. Three x 10^7 bone marrow cells were stained with either biotin-conjugated anti-SCA-1 antibody (top panel) or biotin-conjugated normal rat isotype control antibody (bottom panel). Positive cells were detected following incubation with Streptavidin-PE.

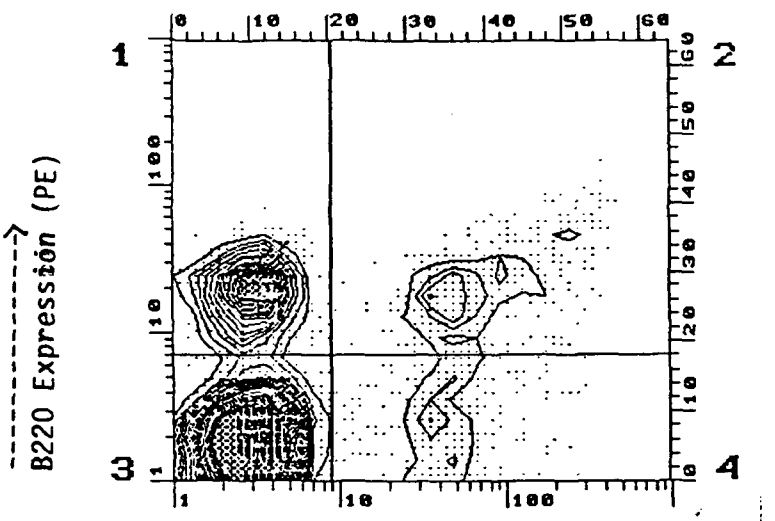


QUAD	PERCENT	PEAK POS	PEAK HT	AREA
1	0.04	0, 19	1	4
2	0.	22, 19	0	0
3	99.96	0, 0	910	9996
4	0.	22, 0	0	0

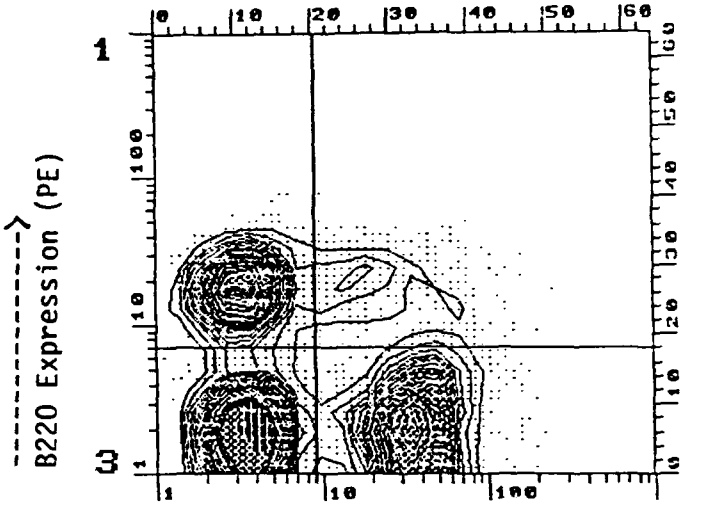


QUAD	PERCENT	PEAK POS	PEAK HT	AREA
1	19.83	0, 35	75	1983
2	0.	22, 19	0	0
3	80.17	0, 0	846	8017
4	0.	22, 0	0	0

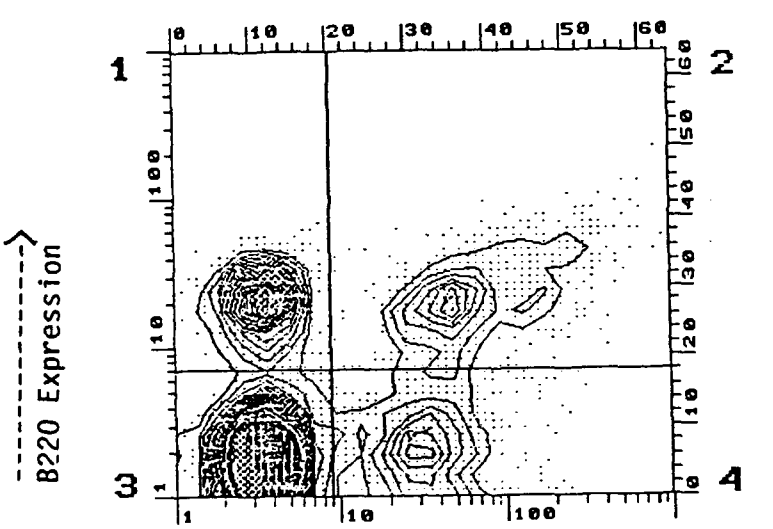
FIGURE 3A. B220 Expression on Human Peripheral Blood Mononuclear Cells. Human peripheral blood mononuclear cells were stained with biotinylated anti-B220 antibody (RA3-6B2) or isotype control antibody and developed with Streptavidin-PE (panel A).



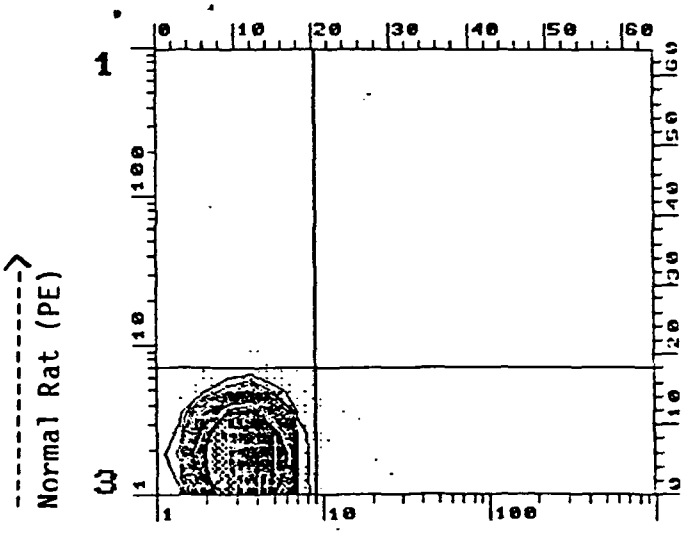
QUAD	PCNT	PK X	PK Y	PK HT	AREA	MEAN X	MEAN Y
1	12.93	13	29	22	1383	10.97	28.21
2	4.78	36	25	8	511	40.37	28.56
3	78.91	11	5	123	8440	10.79	6.55
4	3.38	37	4	6	362	35.29	8.76



QUAD	PCNT	PK X	PK Y	PK HT	AREA	MEAN X	M
1	21.27	13	28	45	2263	11.82	
2	4.29	24	28	7	456	30.13	
3	47.21	12	7	89	5024	12.38	
4	27.23	33	0	32	2898	33.71	



QUAD	PCNT	PK X	PK Y	PK HT	AREA	MEAN X	MEAN Y
1	11.94	13	29	29	1284	12.22	29.17
2	8.43	35	28	11	907	39.77	29.23
3	71.14	13	8	147	7651	12.20	6.57
4	8.49	32	9	12	913	32.83	7.94



QUAD	PCNT	PK X	PK Y	PK HT	AREA	MEAN X	M
1	0.03	12	20	1	3	10.67	
2	0.00	0	0	0	0	0.00	
3	99.87	12	6	198	10578	12.05	
4	0.10	21	0	1	11	23.27	

FIGURE 3B. B220 Expression on Human Peripheral Mononuclear Cells. Human peripheral mononuclear cells were also stained for B cell, T cell and MHC Class II expression versus B220 expression (panel B). An isotype control stain is also shown.

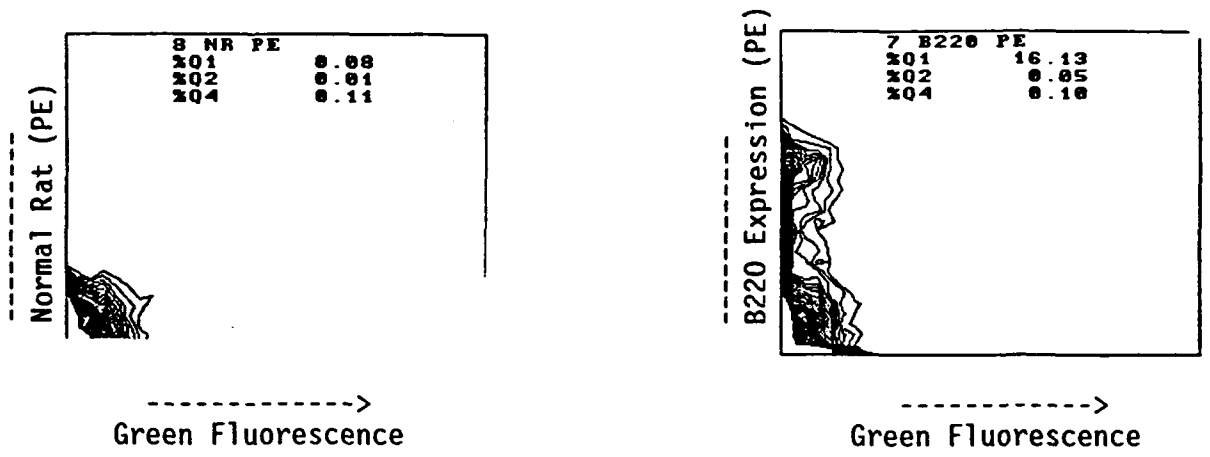


FIGURE 4A. B220 Expression on Human Bone Marrow Cells. Human bone marrow cells were stained with biotinylated anti-B220 antibody (RA3-6B2) or isotype control antibody and developed with Streptavidin-PE (panel A).

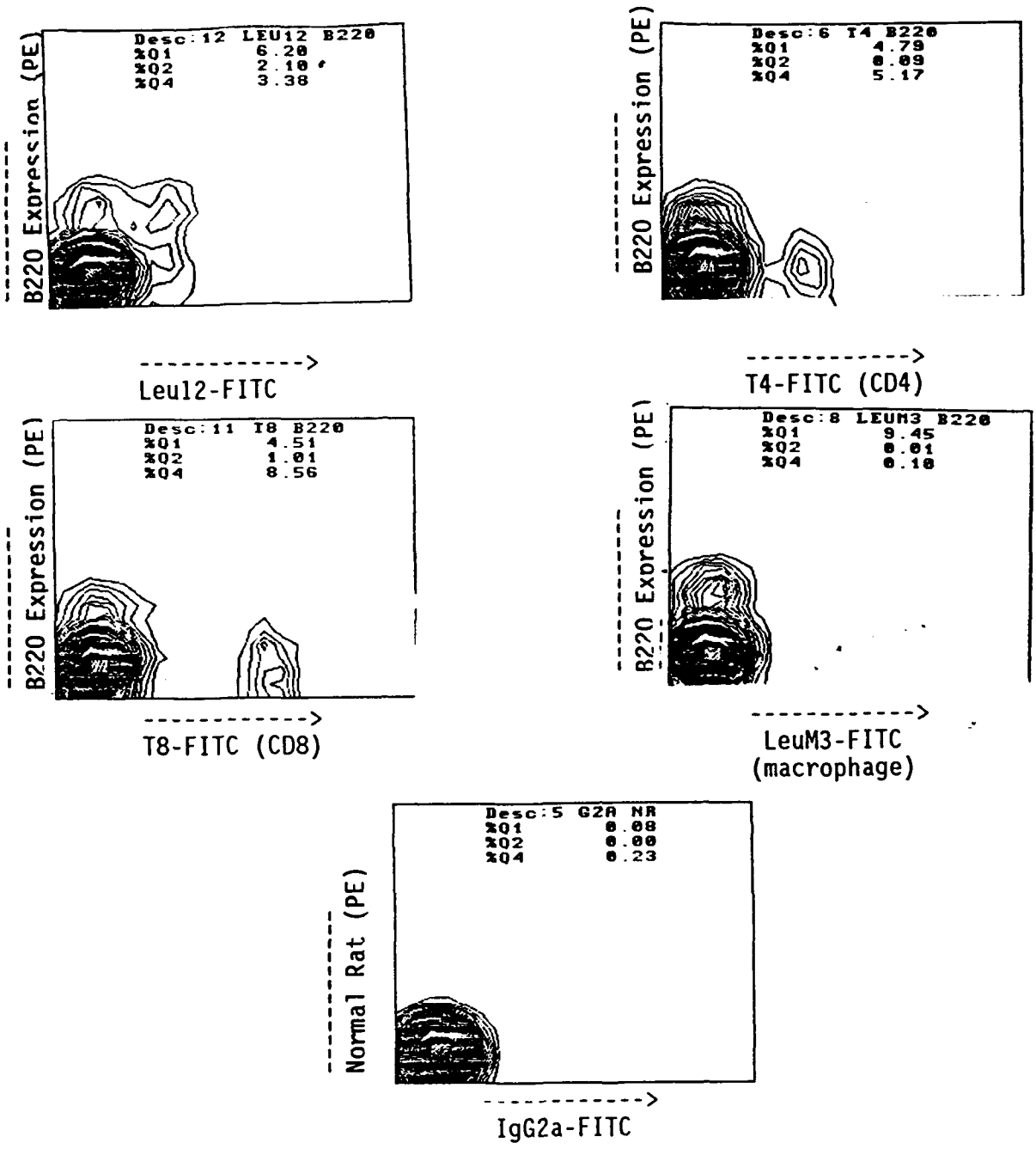


FIGURE 4B. B220 Expression on Human Bone Marrow Cells. Human bone marrow cells were stained for B cell, T cell and MHC Class II expression versus B220 expression (panel B).

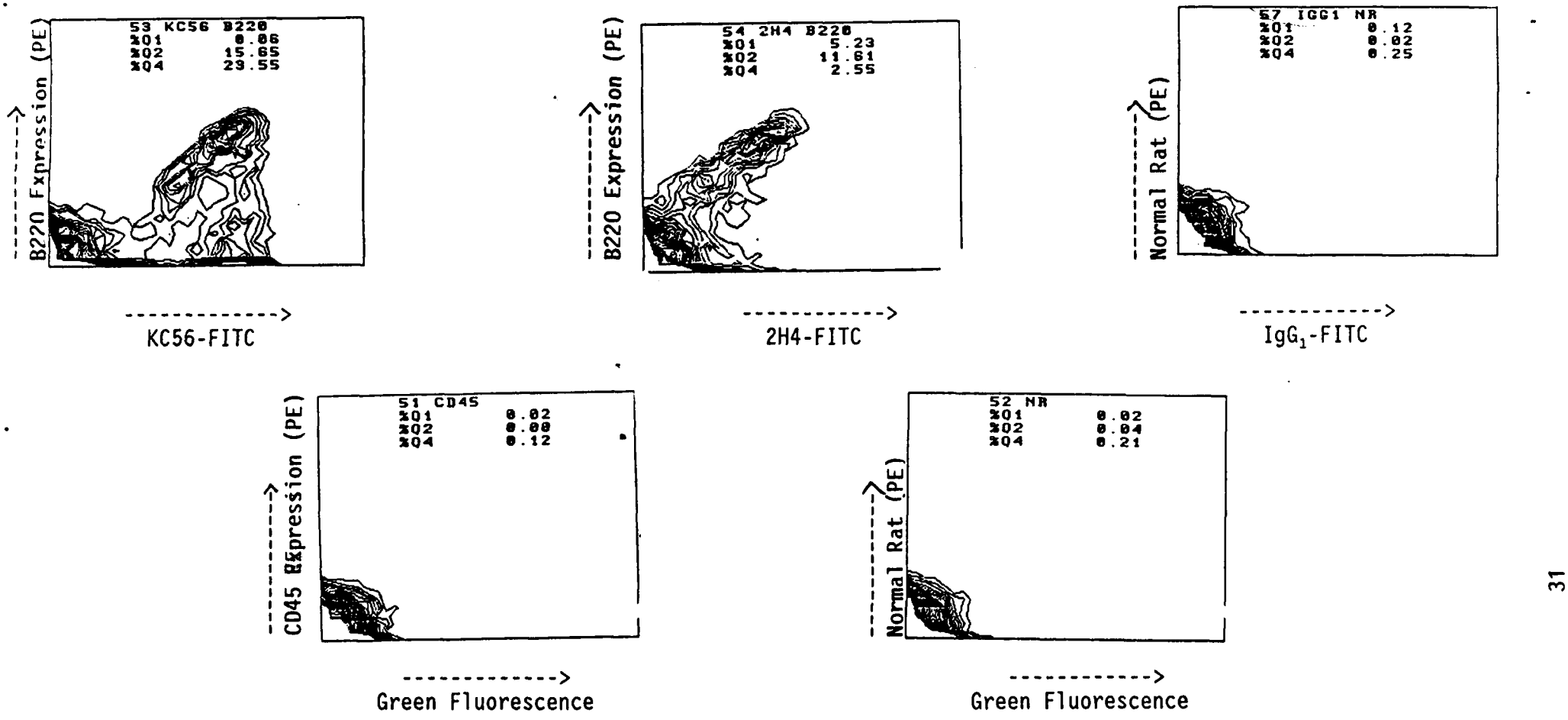
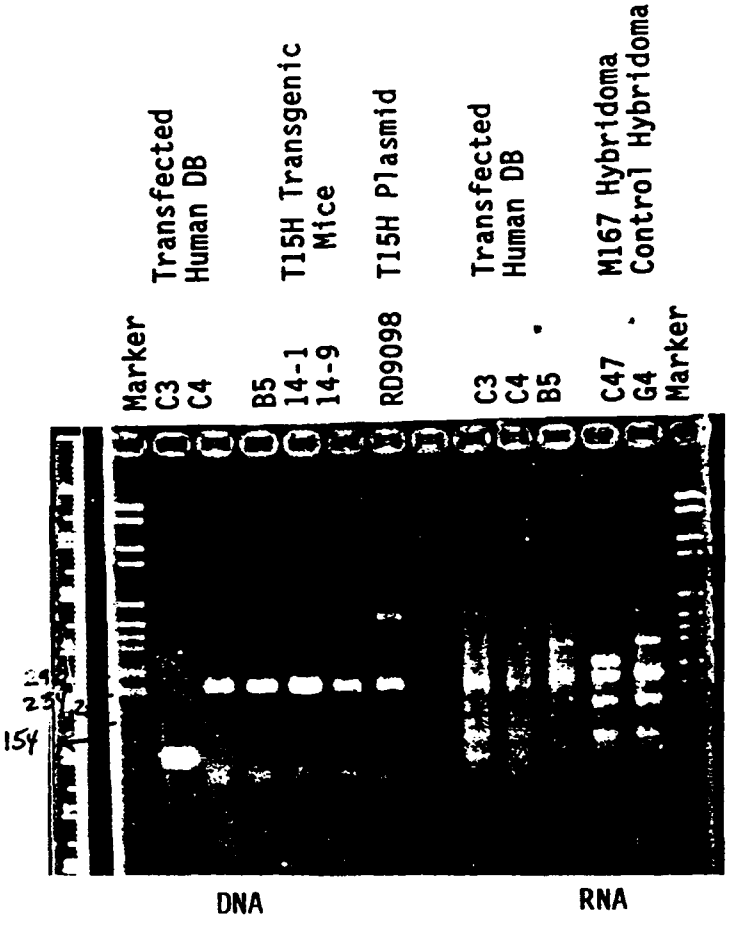


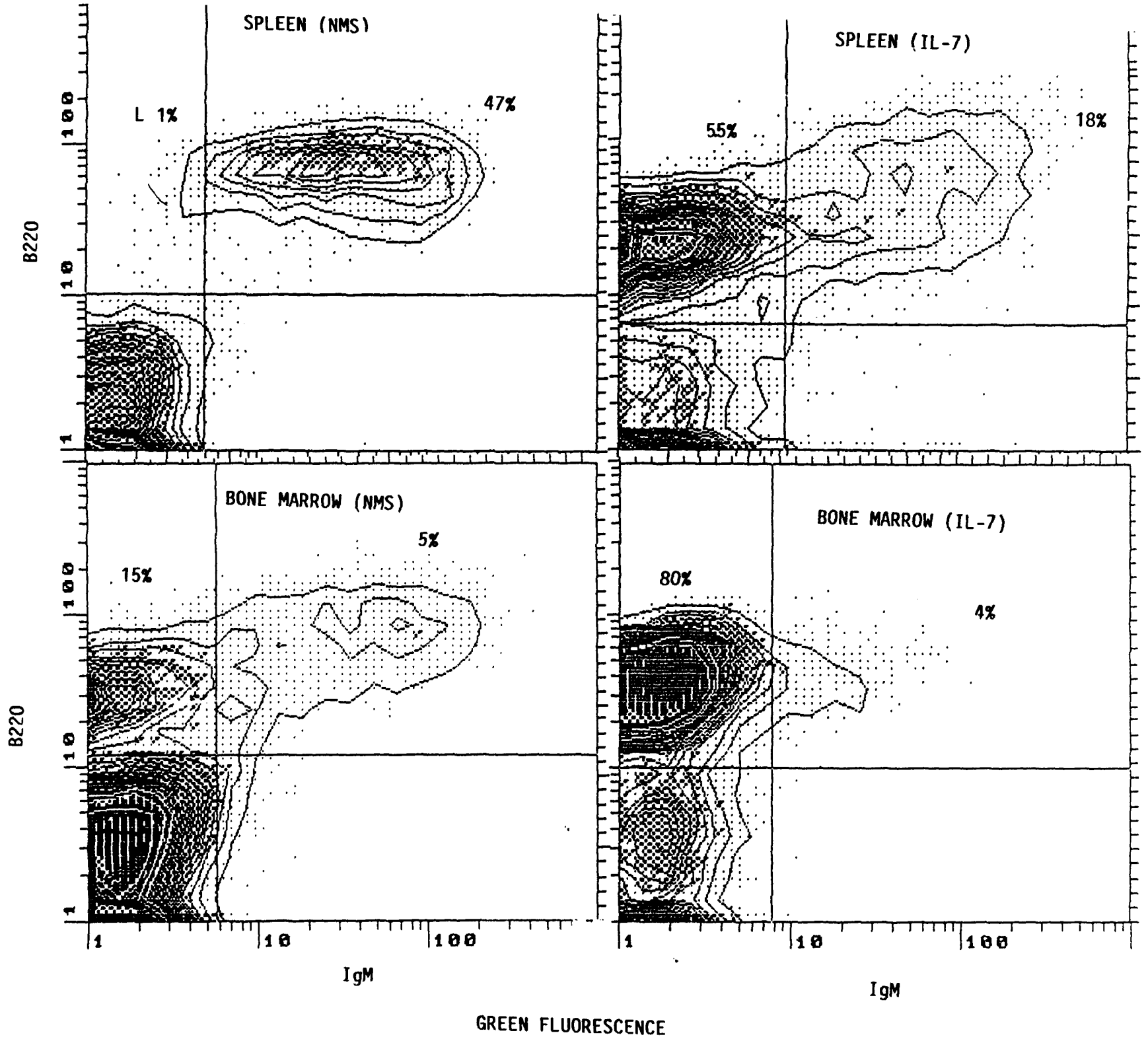
FIGURE 5. Expression of CD45 Isoforms on Human Bone Marrow Cells. Human bone marrow cells were stained with anti-B220 antibody (RA3-6B2) and anti-human CD45 antibody KC56 or anti-human CD45R antibody 2H4 (panel A). Biotinylated antibodies were developed with Streptavidin-PE. Cell were stained with a pan-murine CD45 antibody or isotype matched control antibody and developed with Streptavidin-PE (panel B).

FIGURE 6



DNA from human DB cells transfected with rearranged T15 H & L chain genes was PCR amplified using 5'V_H-region and 3'DJ region primers and RNA from these lines was amplified using the same 5'V_H-region primer and a 3'Cμ primer. T15 H control DNA was run from two T15 H-chain transgenic mice (14-1 & 14-9) and DNA from the plasmid (RD9098) used to transfect these cells. A single band at 234 bp is seen in all the samples except the C3 cell line. This line apparently only integrated the light carrying the drug selectable marker. Analysis of the RNA shows that a PCR product of the right size (marked in red) is obtained only from the positive control hybridoma line C47. The transfected human lines C4 and B5, which clearly have H-chain DNA are not transcribing this into RNA.

FIGURE 7


SPLEEN (NMS)
L 1%
47%
SPLEEN (IL-7)
55%
18%
BONE MARROW (NMS)
5%
15%
BONE MARROW (IL-7)
80%
4%
B220
100
10
1
1
10
100
IgM
YELLOW FLUORESCENCE
GREEN FLUORESCENCE

Legend for figure 7

Spleen cells and bone marrow cells from IL-7 treated (10 μg i.p. 2x/day) and normal mouse serum (NMS) treated controls were stained with FITC-conjugated anti-μ antibody and biotin-conjugated anti-B220 plus PE-Streptavidin. Cells were analyzed on a Coulter 753 EPICS flow cytometer. Panel B shows that the spleen of an IL-7 treated mouse looks like the bone marrow of the normal control in panel C, whereas the IL-7 treated bone marrow is greater than 80 % $B220^{+}\mu^{-}$ pre-B cells. These pre-B cells should make good targets for transfection with rearranged Ig-genes.

September 27, 1991

Human Growth Hormone Promotes Engraftment of Murine or Human T Cells in SCID Mice

(neuroendocrine immune effects/immune reconstitution/AIDS animal model)

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Abbreviations: GH, growth hormone, rhGH, recombinant human growth hormone; SCID, severe combined immune deficiency; huPBL, human peripheral blood lymphocytes; TIL, tumor infiltrating lymphocytes; LAK, lymphokine activated killer

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ABSTRACT

Growth Hormone (GH) has previously been implicated in T-cell development, and here we test its efficacy in promoting T-lymphocyte engraftment. Treatment of SCID mice with rhGH resulted in significantly better T-cell engraftment in the lymph nodes of mice receiving murine thymocytes. Human T-cell engraftment was also strongly promoted by rhGH in SCID recipients receiving huPBL. Although mature human T cells have not been previously described as capable of entering the murine thymus, human T cells were detected in the SCID thymus after treatment with rhGH. Thus, rhGH can be used to optimize long-term peripheral T-cell engraftment in these human-mouse chimeras and may also be useful clinically in the treatment of T-cell deficiencies.

Positive effects of GH on the immune system, particularly on T-cell development, have been previously suggested based on studies in GH-deficient mice (1), use of antibodies to GH in vivo (2), and the implantation of a GH-secreting tumor in aged rats (3). We have investigated the possible therapeutic use of recombinant human GH (rhGH) on T-cell transplantation using two in vivo model systems: 1) transfer of murine T lymphocytes into mice with severe combined immune deficiency (SCID), and 2) transfer of human T cells into SCID mice.

SCID mice lack T or B cells due to a defect in their recombinase system which prevents the productive rearrangement of their immune receptor genes (4). The lymphoid organs in SCID mice, while devoid of mature lymphocytes, can be successfully reconstituted with the transfer of normal murine lymphoid progenitors (5) or with human peripheral blood lymphocytes (huPBL) (8). In the first model, in which murine thymocytes are transferred into SCID mice, the effects of the exogenous rhGH would augment endogenously produced murine GH, since murine cells respond to both human and murine GH. In the second model, where huPBL are transferred into SCID mice, the transferred human cells would be unresponsive to the endogenous murine GH and would respond only to the exogenous rhGH (6). In both SCID models, significant and sustained effects on mouse or human T-cell reconstitution were observed after treatment of SCID mice with rhGH.

MATERIALS AND METHODS

Animals. C.B-17 scid/scid (SCID) mice were bred in our colony at NCI-FCRDC, Frederick, MD. BALB/c mice were obtained from the Animal Production Area, NCI-FCRDC, Frederick, MD. Mice were used at 8-12 weeks of age and were kept under specific-pathogen-free conditions. SCID mice were housed in

microisolator cages and all food, water, and bedding were autoclaved prior to use. SCID mice received 40 mg trimethoprim and 200 mg sulfamethoxazole per 320 ml drinking water.

Transfer of cells into SCID mice and treatment with rhGH. Thymocytes (9×10^7) from H-2 identical BALB/c mice were injected intraperitoneally into SCID mice. When human cells were to be transplanted into SCID recipients, huPBL were obtained from healthy donors in leukopacks. All donors were screened for HIV-1 and hepatitis before donation and provided informed consent. The huPBL were then purified by counter-current elutriation and the lymphocyte fractions, containing >90% lymphocytes were obtained. The huPBL ($1x\ 10^8$) were injected intraperitoneally into recipient SCID mice. One day before injection of huPBL, all mice received 20 μl of anti-asialo GM1 (Wako Chemicals, Dallas, TX) intravenously, a procedure which we have found to improve engraftment of huPBL in SCID mice (manuscript submitted). Some SCID recipients received 5 μg recombinant human growth hormone (rhGH) (provided by Genentech, South San Fransisco, CA) in 0.2 ml HBSS intraperitoneally for three days on day 0,2, and 4 after cell transfer.

Antibodies and immunofluorescence studies. For determining the extent of engraftment of murine thymocytes in SCID recipients, the following anti-murine mAbs were used: anti-CD4(L3T4-FITC) and anti-CD8(Lyt-2-biotinylated) were purchased from Becton-Dickinson, Mountain View, CA; anti-CD3(2C11-FITC) was a kind gift from Dr. James J. Kenny, Program Resources, Inc./DynCorp, Frederick, MD. The following anti-human mAbs were used: HLA-ABC-FITC was purchased from Olympus, Lake Success, NY. CD4 (Leu3a-FITC), CD8 (Leu2a-biotinylated), and CD3 (Leu4-biotinylated) were purchased from Becton-Dickinson, Mountain View, CA. Antibodies were used to stain single cell suspensions of thymocytes, lymph

node cells (containing axillary, brachial, inguinal, and mesenteric nodes), and peritoneal cavity cells. Staining was performed in the presence of 2% human AB serum (Gibco BRL, Grand Islands, NY) to saturate human and mouse Fc receptors. After primary antibody incubation, cells were analyzed using an EPICS flow cytometer. Experiments were performed at least four times with 2-3 mice per group, and a representative individual being shown.

RESULTS

rhGH Promotes Murine T-Cell Reconstitution in the Lymph Nodes of SCID Mice. H-2 identical BALB/C thymocytes were transferred into SCID mice and the recipients were analyzed at various time points to determine extent of peripheral lymphocytic reconstitution. Treatment of the recipients with only three 5 μg injections of rhGH resulted in accelerated and more extensive engraftment in the lymph nodes as shown in Fig. 1. The cell yields from the lymph nodes of rhGH-treated recipients were two-fold greater than mice receiving thymocytes alone. SCID mice normally have no detectable $CD4^+$, $CD8^+$, or $CD3^+$ cells (5). In the recipients receiving thymocytes, the major cell type affected by rhGH treatment was the $CD4^+$ cell population, which within 2 wk after transfer, comprised 51.2% of the lymph node cells (LNC) in rhGH-treated mice compared to the controls, which contained only 6.2% $CD4^+$ cells (Fig. 1a,b). Normal levels of the T-cell antigen receptor complex (CD3) were expressed on the T cells from all the engrafted recipients (Fig. 1c). The engraftment of $CD8^+$ cells also was augmented by rhGH, increasing from 1.0 to 6.6% of the LNC. The preferential engraftment of $CD4^+$ cells over $CD8^+$ cells may not be due entirely to rhGH; a difference in the ability of the two cell types to engraft in SCID mice has recently been reported (7) but the mechanism is unknown.

The effect of rhGH was evident for weeks after administration. Three weeks following the last rhGH treatment, the level of T-cell engraftment in the lymph nodes persisted to a much greater extent than in controls (Fig. 1d,e). $CD4^+$ cells comprised 17.7% of the LNC of the rhGH-treated mice compared to 2.8% in mice receiving thymocytes alone. Interestingly, at this later time point, the CD4/CD8 ratio had become more normal due to the late appearance of $CD8^+$,$CD3^+$ cells. Thus, rhGH appears to significantly affect the kinetics and extent of peripheral T-cell reconstitution of SCID mice by donor murine thymocytes.

rhGH Promotes Peripheral Human T-Cell Engraftment in SCID Mice. Human peripheral blood lymphocytes (huPBL) have previously been shown to engraft in SCID mice (8); however, it has been difficult to obtain long-term reproducible human cell engraftment in these chimeras (9,10). Other studies have attempted to improve human cell engraftment in mice through the addition of various human cytokines, since many murine cytokines do not act on human cells; however, the use of human cytokines has so far yielded negative results (11). Since there were significant effects of rhGH on murine T-cell engraftment in SCID mice, we postulated that the human lymphocytes may require the presence of human neuroendocrine hormones (e.g. human GH) for successful localization and long-term engraftment in mice. Therefore, rhGH was tested for its effects on human T-cell engraftment in SCID mice. In a representative experiment, there were 33.5% $HLA\text{-}ABC^+$ cells in the peritoneal cavity of the SCID mice receiving huPBL alone, while treatment with rhGH reduced this to 12.9% (Fig. 2a). The total number of cells recovered from the peritoneal cavity between the two groups was not significantly different. It appears that treatment with rhGH allowed more of the human lymphocytes to exit the peritoneal cavity

and enter peripheral lymphoid organs since the percentage of human $CD4^+$ T cells in the lymph nodes of control SCID recipients was 4.1%, compared to 47.5% in mice receiving injections of rhGH (Fig. 2b). The cell yields from the LNC of the chimeras also remained the same regardless of whether they received rhGH. Human $CD8^+$ cells in the lymph nodes also increased from 1.8% to 15.5% under the influence of rhGH. The CD4:CD8 ratio of engrafted human T cells in these chimeras tends to be variable depending on the huPBL donor. However, regardless of the donor, we observed that treatment with rhGH resulted in better engraftment of both cell-types in all of the 30 chimeric mice analysed when compared with mice receiving huPBL alone.

Novel effects of rhGH treatment were noted on the entry of human cells into the SCID thymus. HuPBL have not been previously observed in the murine thymus of these xenogeneic lymphoid chimeras (8,10-12). We observed no detectable human cells in the thymus of SCID mice that received huPBL but no rhGH (Fig. 2c). However, in SCID recipients treated with rhGH, human cells were detected in the thymus, of which 21.5% were human $CD4^+$ and 3.2% were human $CD8^+$. Additionally, there was also a small percentage of HLA^+ $CD3^-$ cells in the thymus. These cells were presumably monocytes since they were HLA-DR^+ yet $CD3^-$ (data not shown). Although the thymus is the site of T-cell differentiation, these human T cells had already differentiated before entering the mouse thymus, based on the paucity of immature thymocyte forms ($CD4^+CD8^+$). A small percentage of $CD4^+CD8^+$ human T cells was sometimes found (3.2% in the thymus shown). The thymus increased in size to 5×10^6 cells (a SCID thymus normally contains approximately 2×10^6 cells). Treatment with rhGH appears to make the murine thymus the primary site for human T-cell localization in these chimeras: in 6 out of 30 rhGH-treated xenogeneic

chimeras, human T cells were detected only in the thymus, and were missing from other peripheral lymphoid organs. It will be of particular interest to determine if the localization of these cells to the murine thymus alters their functional capabilities.

In the huPBL-SCID chimeras treated with rhGH, engraftment was long-lasting (at least 4 months). All of the chimeras appeared healthy, although they exhibited marked splenomegaly suggesting that a xenogeneic graft-versus-host-reaction was occurring (manuscript submitted). Histological examination demonstrated an expansion and repopulation of the splenic white pulp (data not shown). There was no significant human B-cell engraftment in the lymphoid organs of the rhGH-treated mice as the majority of HLA^+ cells were also $CD3^+$, although a significant percentage (90%) of the chimeras had circulating human immunoglobulin in their serum.

DISCUSSION

We have shown that rhGH promotes the peripheral engraftment of murine or human T cells in SCID mice. The mechanism(s) behind these effects could involve proliferation, cellular half-life, and localization of the donor T cells. Proliferative effects may partly account for some of the results seen: T-cell proliferation was somewhat augmented by rhGH in vitro in that we detected a 1.5 to 2-fold increase in ^{3}H-TdR incorporation by antigen-primed T cells (not shown). Effects on the localization of lymphocytes could be another mechanism of rhGH action since there were significant effects concerning the ability of human lymphocytes to enter the murine thymus. The thymus differs from other lymphoid organs in that it manifests more restricted cell entry and it has been suggested that human T cells cannot traffic to the murine thymus due to a lack of appropriate adhesion molecules (12). Treatment

with rhGH may induce these molecules on human T cells or the murine thymic cells allowing for successful entry into the murine thymus. These results suggest the GH may play a pivotal role in normal T-cell development by allowing thymic entry of progenitor cells and promoting peripheral localization of the mature T cells as they exit the thymus.

These findings indicate potential clinical applications for rhGH as a therapy to accelerate T-cell reconstitution in AIDS or after bone marrow transplantation where it would be advantageous to accelerate the expansion of T cells. Treatment with rhGH may also have potential to increase the efficacy and localization of tumor infiltrating lymphocytes (TIL) or lymphokine activated killer (LAK) cells in the treatment of cancer, or to enhance the survival and expansion of transgene-bearing T cells in the experimental treatment of adenosine deaminase deficiency. The data presented here also suggest the existence of important interactions between the neuroendocrine and immune systems.

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REFERENCES

1. Baroni, C. (1967) *Experientia* 23, 282-283.
2. Pierpaoli, W. & Sorkin, E. (1968) *J. Immunol.* 101, 1036-1043.
3. Kelley, K.W., Brief, S., Westly, H.J., Novakofski, J., Bechtel, P.J., Simon, J., & Walker, E.B. (1986) *Proc. Natl. Acad. Sci USA* 83, 5663-5667.
4. Schuler, W., Weiler, I.J., Schuler, A., Phillips, R.A., Rosenberg, N., Mak, T.W., Karney, J.F., Perry, R.P., & Bosma, M.J. (1984) *Cell* 46, 963-972.
5. Bosma, G.C., Custer, R.P., & Bosma, M.J. (1983) *Nature* 301, 527-530.
6. Golde, D.W., Bersch, N., & Li, C.H. (1976) *Science* 196, 1112-1113.
7. Rudolphi, A., Spieβ, S., Conradt, P., Claesson, M.H., & Reimann, J. (1991) *Eur. J. Immunol.* 21, 523-533.
8. Mosier, D.E., Gulizia, R.J., Baird, S.M., & Wilson, D.B. (1988) *Nature* 335, 256-259.
9. Mosier, D.E., Gulizia, R.J., Baird, S.M., & Wilson, D.B. (1989) *Nature* 338, 211.
10. Pfeffer, K., Heeg, K., Bubeck, R., Conradt, P., & Wagner, H. (1989) *Curr. Top. Microbiol. Immunol.* 152, 211-217.
11. Kamel-Reid, S. & Dick, J.E. (1988) *Science* 242, 1706-1709.
12. McCune, J.M., Namikawa, R., Kaneshima, H., Shultz, L.D., Lieberman, M., & Weissman, I.L. (1988) *Science* 241, 1632-1639.
13. Agus, D.B., Surh, C.D., & Sprent, J. (1991) *J. Exp. Med.* 173, 1039-1046.
14. Mercola, K.E., Cline, M.J., & Golde, D.W. (1981) *Blood* 58, 337-340.
15. Arrenbrecht, S. (1974) *Nature* 252, 255-256.
16. Kiess, W. & Butenandt, O. (1985) *J. Clin. Endocrinol. Metab.* 60, 740-746.

17. D'Ercole, A.J., Stiles, A.D., & Underwood, L.E. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 935-939.

18. Berczi, I., Nagy, E., Kovacs, K., & Horvath, E. (1981) *Acta Endocrinol.* **98**, 506-513.

FIGURE LEGENDS

FIG. 1. rhGH and murine T-cell engraftment: LNC staining profile following thymocyte reconstitution of SCID mice.

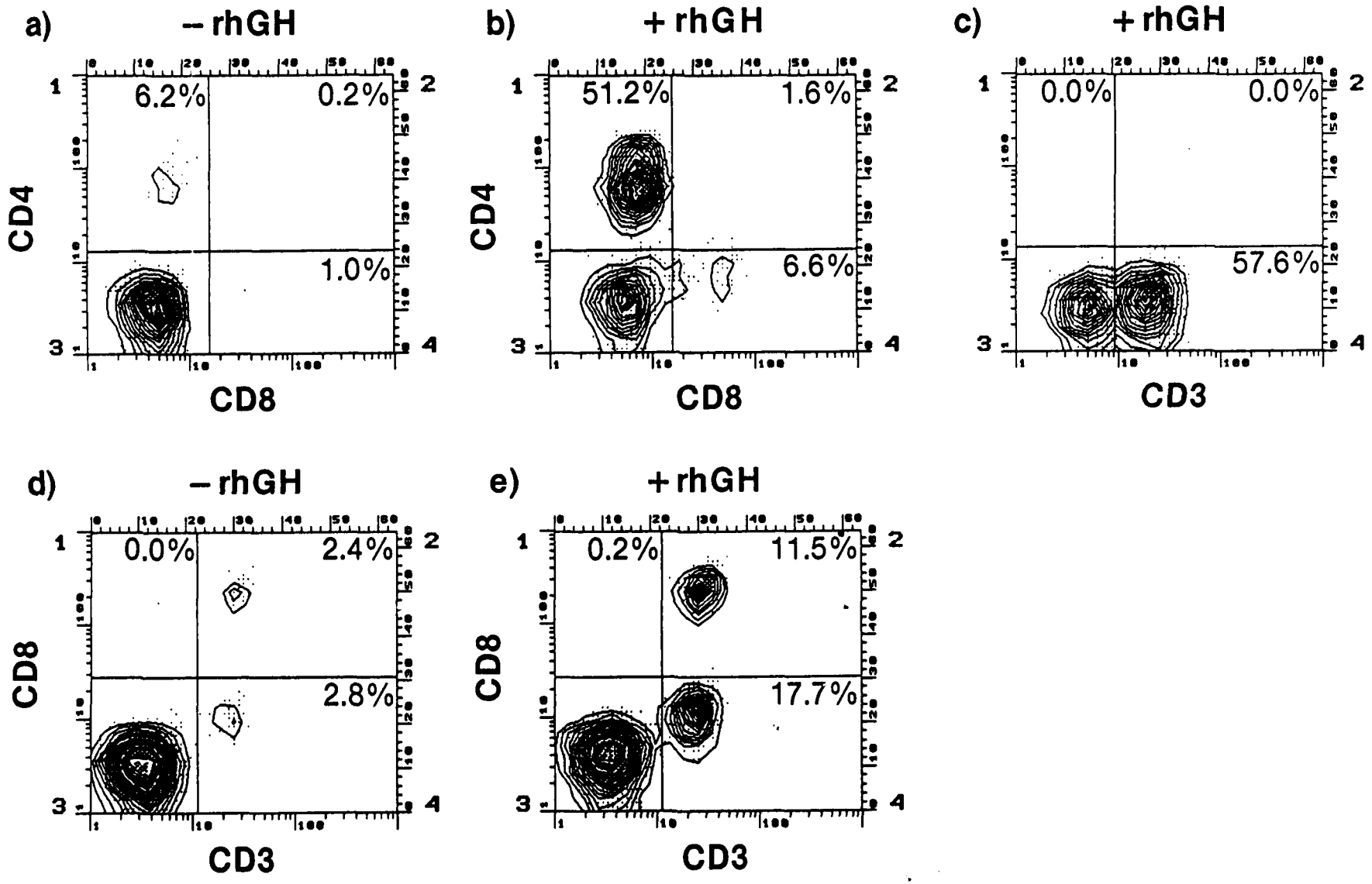
SCID mice were injected intraperitoneally with H-2 identical BALB/c thymocytes. rhGH was injected intraperitoneally, 5 μg/mouse, on day 0, 2 and 4 in 0.2 ml PBS. a) Two weeks after thymocyte transfer, lymph node cell (LNC) suspensions from mesenteric, axillary, inguinal, and brachial lymph nodes, were pooled and analyzed for donor T cells by staining for CD4 and CD8. b) LNC of SCID mouse receiving BALB/c thymocytes and rhGH. SCID mice receiving rhGH without thymocytes did not contain cells positive for either CD4, CD8, or CD3 in their thymus, spleen, or LNC (5,data not shown). c) LNC of SCID receiving thymocytes with rhGH two weeks after transfer stained with anti-CD3 only. d) SCID mice injected with BALB/c thymocytes i.p. examined 4 weeks after transfer. LNC were stained with anti-CD3 and anti-CD8. e) LNC of SCID mice receiving thymocytes and rhGH. All data shown are representative of 3 experiments containing 2-3 mice per group.

FIG. 2. rhGH and huPBL engraftment: FACS profile of lymphoid organs 4 weeks following huPBL transfer in SCID mice.

SCID mice were injected intraperitoneally with huPBL. rhGH was given to some mice for three days at 5 μg in 0.2ml PBS intraperitoneally on day 0,2, and 4. One month following lymphocyte transfer, various lymphoid organs (peritoneal cells, LNC, and thymus) were analyzed for human cells. Cells were stained with directly labeled mAbs for the human antigens CD4, CD8, CD3, and HLA-ABC. Cells from various lymphoid organs of untreated SCID mice or SCID mice treated with rhGH did not stain with antibodies for CD3, CD4, CD8 or HLA-ABC (data not shown). All data shown are representative of 6 experiments with

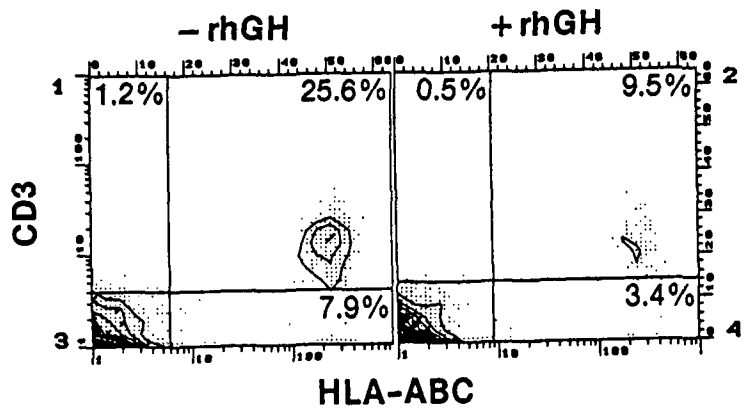
2 mice per group. a) Cells present in the peritoneal cavity of mice. b) Cells present in the lymph nodes of SCID recipients. The cell yield from the LNC of these chimeras treated with rhGH was similar to that of SCID mice receiving huPBL only. c) Cells present in the thymus of SCID mice 4 weeks after huPBL transfer.

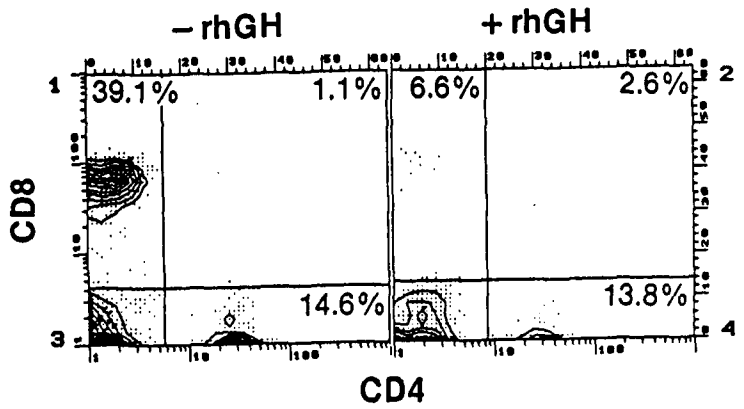
BALB/c Thymocytes → SCID



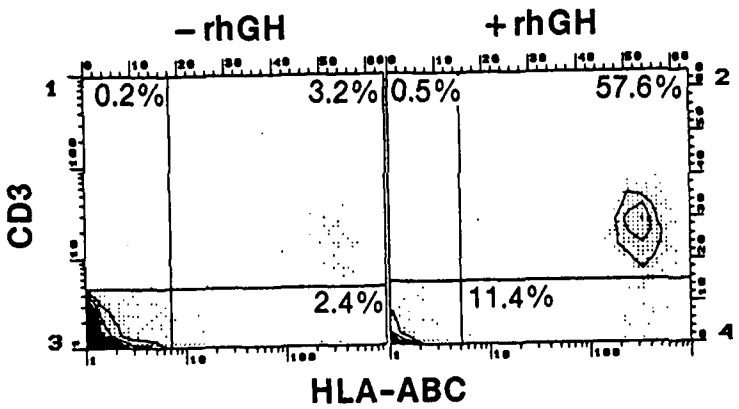
HuPBL → SCID

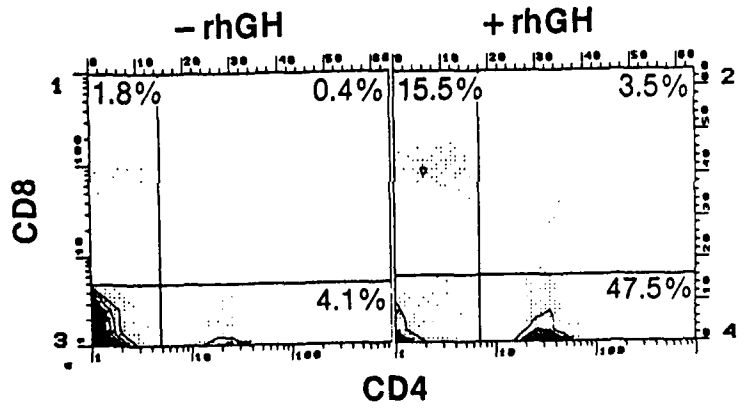
a) Peritoneal cavity



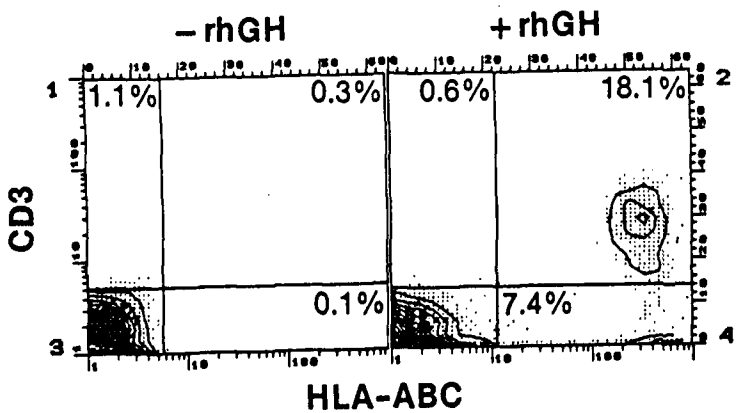


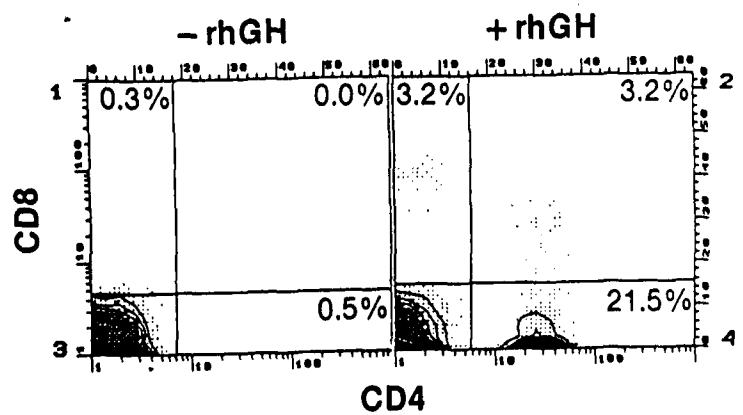
b) Lymph nodes





c) Thymus





Selection of Antigen-specific, Idiotype-positive B Cells in Transgenic Mice Expressing a Rearranged M167-μ Heavy Chain Gene

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Summary

Flow cytometric analysis of antigen-specific, idiotype-positive (id$^+$), B cell development in transgenic mice expressing a rearranged M167-μ gene shows that large numbers of phosphocholine (PC)-specific, M167-id$^+$ B cells develop in the spleen and bone marrow of these mice. Random rearrangement of endogenous V_κ genes, in the absence of a subsequent receptor-driven selection, should give rise to equal numbers of T15- and M167-id$^+$ B cells. The observed 100–500-fold amplification of M167-id$^+$ B cells expressing an endogenous encoded $V_\kappa 24J_\kappa 5$ light chain in association with the M167 $V_H 1$-id transgene product appears to be an antigen driven, receptor-mediated process, since no amplification of non-PC-binding M167 $V_H 1/V_\kappa 22$, T15-id$^+$ B cells occurs in these μ-only transgenic mice. The selection and amplification of antigen-specific, M167-id$^+$ B cells requires surface expression of the μ transgene product; thus, no enhancement of M167-id$^+$ B cells occurs in the M167 $\mu\Delta$mem-transgenic mice, which cannot insert the μ transgene product into the B cell membrane. Surprisingly, no selection of PC-specific B cells occurs in M167-κ-transgenic mice although large numbers of B cells expressing a crossreactive M167-id are present in the spleen and bone marrow of these mice. The failure to develop detectable numbers of M167-id$^+$, PC-specific B cells in M167-κ-transgenic mice may be due to a very low frequency of M167-V_H-region formation during endogenous rearrangement of $V_H 1$ to D-J_H segments. The somatic generation of the M167 version of a rearranged $V_H 1$ gene may occur in less than one of every 10^5 bone marrow B cells, and a 500-fold amplification of this M167-Id$^+$ B cell would not be detectable by flow cytometry even though the anti-PC antibody produced by these B cells is detectable in the serum of M167-κ-transgenic mice after immunization with PC.

B lymphocyte development in the mouse is a complex and dynamic process in which the adult bone marrow produces $\sim 6 \times 10^7$ new B cells each day (1). However, very few of these B cells appear to enter the stable, long-lived peripheral B cell pool where the half-life of a B cell, as measured by bromodeoxyuridine (BrdUrd) incorporation, is 3 mo or longer (2, 3). It is of interest to understand how the few B cells that enter this long-lived pool are chosen from the millions of B cells produced each day.

Analysis of the adult peripheral B cell V_H repertoire (4–9) suggests that it is randomly generated in as much as it reflects the complexity of the V_H gene families expressed in the mouse genome (8, 10); however, several studies (11–17) have also suggested that the peripheral B cell pool is selected and does not simply reflect the repertoire that emerges from the bone marrow. Yancopoulos et al. (11) found that the preferential utilization of J_H proximal V_H genes in Abelson virus–transformed pre-B cells, which persists in the bone marrow of adult BALB/c mice (9, 12), is not mirrored in the B cell repertoire of the adult spleen. Freitas et al. (9) have also found that local environmental factors can lead to increased representation of the V_H-J558 family in lymph nodes, while the V_H-X24 family is overexpressed in Peyer's patches. The selection of individual idiotypes or H/L chain combina-

(μ^{a+}/id$^+$) B cells (see Fig. 3 *C*) were then sorted using an EPICS 753 flow cytometer (Coulter Electronics).

RNA Isolation and PCR Reactions. RNA from 2 × 10^6 μ^a/M167-id$^+$ spleen cells or M167-id$^+$ ($V_H1/V_\kappa24$) hybridoma cells from 207-4 transgenic mice were isolated using the guanidine thiocyanate-CsCl centrifugation method (30). CsCl was obtained from Bethesda Research Laboratories (Gaithersburg, MD) and guanidine thiocyanate from Fluka Biochemicals (Ronkonkoma, NY). For cDNA synthesis from RNA, 1.0 μg of total RNA was added to PCR reaction buffer (Perkin-Elmer Corp., Norwalk, CT) with final concentrations of: 5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl, pH 8.3. 1 mM dATP, dCTP, dGTP, and dTTP was added to the mixture, as well as 1.0 μM 3′ primer specific for C_κ and 1 U of RNase inhibitor (Perkin-Elmer Corp.). The reaction mixture was heated to 65°C for 10 min, then placed on ice. M-MLV reverse transcriptase (Bethesda Research Laboratories) was added to a final concentration of 200 U/reaction and transcription allowed to proceed for 30 min at 37°C. After cDNA synthesis, the reactions were incubated at 99°C for 5 min and placed on ice. A PCR reaction was set up according to the instructions (GeneAmp RNA PCR kit; Perkin-Elmer Corp.). The PCR reactions contained cDNA, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 U Taq Polymerase, 1.0 mM DTT, 0.2 mM dNTPs, and 0.2 μM 5′ and 3′ primers. A thermocycler (Perkin-Elmer Corp.) was used for the amplifications. The reactions were allowed to proceed for 30 cycles consisting of 90 s at 94°C, 90 s at 55°C, and 150 s at 72°C. A soak cycle at the end of the 30 cycles at 72°C for 6 min terminated the reaction.

Analysis of PCR Products. 20 μl of the 100 μl amplified products was separated on 3% agarose gels containing 2.25% Nu-sieve GTG agarose (FMC Bioproducts, Rockland, ME) and 0.75% regular agarose (Bethesda Research Laboratories), then stained with ethidium bromide and photographed. Only products of the size predicted by the locations of the primers within the κM167 and MOPC-21 sequences were present. κM167 PCR products were sequenced using the Sequenase 2.0 sequencing kit (U.S. Biochemicals Corp., Cleveland, OH) with the following modifications (31): 80 μl out of 100 μl of the PCR product was extracted once with an equal volume of phenol/chloroform (1:1), then primers were removed by passage through a Sepharose CL-6B spin column (Boehringer-Mannheim Biochemicals, Indianapolis, IN). The final volume from the spin column was 80 μl. NP-40 was added to the sequencing reaction to a final concentration of 0.4%, 7 μl of PCR product and 20 pmol of C_κ primer were used. The reaction components were denatured by boiling for 3 min, then snap-cooled in powdered dry ice. The dGTP labeling mix was diluted 1:20, the sequenase enzyme 1:8. The labeling reaction was incubated at room temperature for 2 min, and the termination reaction proceeded for 4 min at 37°C.

Oligodeoxynucleotide Primers. Unmodified oligodeoxynucleotides (ODNs) were prepared by the Nucleic Acid Synthesis Laboratory (Program Resources, Inc./DynCorp, NCI-FCRDC, Frederick, MD) using B-phosphoramidite chemicals on an automated DNA synthesizer (8750; Biosearch, Millipore, Milford, MA). The ODNs were purified on denaturing polyacrylamide gels, electro-eluted, and ethanol precipitated. Concentrations were established by densitometry (A_{260} of 1 = 20 μg).

A V_κ 167-specific 5′ primer, MOPC-21-specific 5′ primer, and C_κ 3′ primer were synthesized using the published sequences (32–35) obtained through computer databank searches. Primers were selected from these sequences through the use of the PCR Primer Selection Program (Henry M. Jackson Foundation for the Advancement of Military Medicine, Rockville, MD): V_κM167 5′ primer: 5′-GCT-CCT-GAT-CTC-TTT-GAT-GTC-C-3′; V_κM-21 5′ primer: 5′-AAT-GAC-CCA-ATC-TCC-CAA-ATC-C-3′; 3′ C_κ primer: 5′-GCC-ATT-TTG-TCG-TTC-ACT-GCC-3′.

Transfection of V_κ Genes into a V_H-M167 Cell Line. The J558L-M167μ cell line was obtained from Dr. Carol Sibley (University of Washington, Seattle, WA). This cell line, which was produced by transfection of the J558L cell line with the p167μ plasmid constructed by Storb et al. (21), produces an IgMλ antibody that bears the V_H1-id but does not bind PC (see Table 2). This cell line was then transfected by electroporation (capacitance of 960 μFD at 180 V) with 10 μg of DNA from either the pSV2-Neo-S107-κ plasmid (provided by Dr. Phil Tucker, University of Texas, Dallas, TX) or the p167κ-plasmid (21) (provided by Dr. Ursula Storb, University of Chicago, Chicago, IL) plus pSV2-Neo, and the resulting lines were selected in the presence of Geneticin (microbiological potency of 400 μg/ml) (Gibco Laboratories, Grand Island, NY). The antibodies produced by these cell lines were tested for the presence of T15-id and M167-id using the antiidiotypic antibodies described above, and they were also tested in ELISA for their ability to bind PC-BSA (36).

Results

Idiotype Analysis of Spleen Cells from M167 μ/κ Transgenic Mice. We have previously shown that phenotypically normal M167 μ/κ transgenic mice express the IgM^a (μ^a) transgene-encoded anti-PC antibody on 97% of their splenic B cells (36). When the spleen cells of these μ/κ TG$^+$ mice were stained with FITC-anti-μ plus biotin-conjugated antiidiotypic antibodies, as shown in Fig. 1, >95% of the B cells stained with the H chain–specific anti-V_H1-id (*A*), and also with the H + L combinatorial anti-M167-ids 28-5-15 (*C*) and 28-6-20 (*D*). None of the B cells from these M167 μ/κ transgenic mice stained with anti-T15-id antibody (*B*), and <1% of spleen cells from TG$^-$ littermates stained with any of the anti-id antibodies (data not shown). Fig. 2 shows the staining of spleen cells from these same M167 μ/κ transgenic mice with a combination of FITC-anti-μ^a plus anti-id in the absence (*A–C*) and presence (*D–F*) of 5 × 10^{-3} M PC. As previously shown by Desaymard et al. (23), the binding of the T68.3 anti-V_H1-id antibody to its idiotope is not blocked by PC (*A* vs. *D*). The binding of the crossreactive anti-M167-id 28-6-20 is also unaffected by the presence of PC (*C* vs. *F*), indicating that its idiotope is not in or near the binding site. On the other hand, the binding of the anti-M167-id antibody from clone 28-5-15 to the M167-id$^+$ spleen cells is inhibited by >90% in the presence of PC (*B* vs. *E*).

Idiotype Analysis of B Cells from M167 μ-only Transgenic Mice. The combined use of these anti-id antibodies allows us to follow the independent development of both H chain and H + L chain id$^+$ B cells and to quickly determine the frequency of those B cells that are also antigen specific. Using these anti-Id reagents, we have analyzed B cell development in transgenic mice carrying either the M167 μ-only or κ-only transgenes. In the μ-only transgenic mice, the V_H1-id will be expressed on all B cells expressing the μ^a transgene product, while the 28-6-20 and 28-5-15 combinatorial idiotopes will be expressed only on those B cells in which the μ^a transgene product has associated with an endogenous

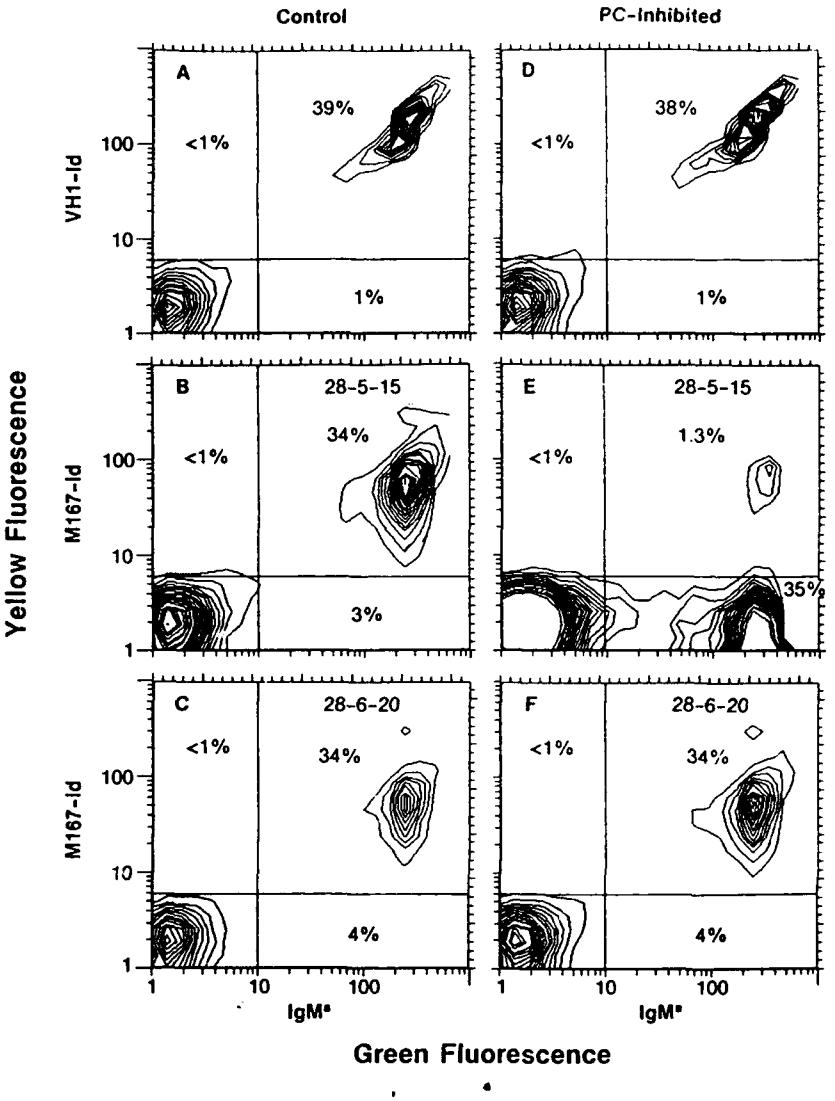

Figure 2. PC inhibition of antiidiotypic antibodies. Spleen cells from 207-4 transgenic mice were stained with 1 μg each of FITC-conjugated anti-μ^a allotype and biotin-conjugated anti-id plus PE-streptavidin in the presence and absence of 5×10^{-3} M PC as described Materials and Methods and then analyzed as in Fig. 1.

compared to the bone marrow of the same mouse, but the M167-id levels in the bone marrow are still 24-fold higher than expected if the H and L chains were associating randomly. However, it is possible that many of these M167-id$^+$ bone marrow B cells could have reentered the bone marrow from the periphery. Forster et al. (2) have recently shown that the vast majority of δ^+ bone marrow B cells do not appear to arise from the rapidly dividing pre-B cells, but appear to represent long-lived, nondividing B cells that circulate through the bone marrow. When the bone marrow of μ-only 243-4 mice was stained with FITC-anti-δ and biotin-anti-V_H1-id plus PE/streptavidin, ~50% of the V_H1^+ B cells also expressed δ (data not shown). In transgenic mice, this may represent a minimal estimate of recirculating TG$^+$ B cells, since the B cells that express the V_H1 transgene product in the absence of endogenous μ or δ may also be cycling back to the bone marrow. Thus, the elevated numbers of M167-id$^+$ B cells in the bone marrow are probably due to recirculation of mature peripheral B cells back to the marrow.

Selective Amplification of B Cells Bearing the M167-id Is Dependent on Cell Surface Expression of the μ Transgene Product. Storb et al. (21) had observed that M167 κ-mRNA was elevated in all the M167 μ-only transgenic mouse lines they produced, whereas, mRNA for this L chain was not detected in M167 $\mu\Delta$mem transgenic mice where the transgene product could not be inserted into the B cell membrane. We have confirmed this observation by staining the spleen cells from M167 $\mu\Delta$mem 254-3 transgenic mice with FITC-anti-μ plus biotin-anti-M167-id. As shown in Fig. 4, none of the μ^+ B cells from these mice stained with either the binding site–specific 28-5-15 or crossreactive 28-6-20 anti-M167-ids. The selective amplification of M167-id$^+$ B cells in the 243-4 μ-only mice would therefore appear to be a receptor-mediated event and possibly antigen driven as suggested by Storb et al. (21).

M167-id$^+$ B Cells in μ-only Transgenic Mice Are PC Specific. To determine if the M167-id$^+$ B cells in the μ-only transgenic mice were indeed antigen specific, spleen cells from TG$^+$ and TG$^-$ mice were stained with the binding

Table 1. *PC-specific ABC in M167 Transgenic Mice*

Mouse strain	Transgene(s) present	Percent ABC*‡	
		TG+	TG-
243-4	μ	5.05	0.05
254-3	$\mu\Delta$mem	0.04	0.03
234-4	κ	0.05	0.05
233-8	κ	2.40	0.45
207-4	$\mu\kappa$	46.0	0.04

* Spleen cells from various strains of M167 transgenic mice were adjusted to 10^7 per ml; 200 μl of spleen cells was rosetted with 100 μl of 2% PC-SRBC as previously described (15, 36). ABC were counted on a hemocytometer and the ABC data expressed as a percent of the total number of spleen cells. The μ/κ 207-4 mice were used as a positive control since >95% of their B cells have been shown to bind PC-SRBC (36).
‡ The PC specificity of ABC was tested by rosetting in the presence of 5×10^{-3} M PC. Greater than 90% of the ABC in the 243-4-μ-transgenic mice were inhibited by PC, while none of the ABC in the 233-8-κ-transgenic mice were PC inhibitable.

Antibodies Produced by the Association of the M167 μ Chain and the κ22 L Chain Are T15-id+ but exhibit Low Affinity for PC. At least three L chains (V_κ8, V_κ22, and V_κ24) are known to associate with a V_H1 gene product to form PC-specific antibodies (41). Yet only the $V_H1/V_\kappa24$ H/L combination has been selectively amplified in the M167 μ-transgenic mice, while T15-id+ ($V_H1/V_\kappa22$) B cells, if present, are below the level of detection (Fig. 3). To determine whether or not the M167-μ transgene product would form a PC-specific antibody when associated with a κ22 L chain, we electroporated rearranged V_κ22 and V_κ24 L chain genes into a cell line containing the M167 μ gene. The antibodies produced by these cell lines were tested for both id expression and for their ability to bind PC-BSA-coated plates. As shown in Table 2, the antibody formed by association of the M167-μ H chain and the V_κ22 L chain expresses the T15-ids detected by both the T139.2 monoclonal and rabbit polyclonal anti-T15 antibodies, but this antibody is at least 100 times less efficient at binding PC than the T15+ IgM antibody HPCM2, which was used as a control to generate the standard curves in the PC-specific ELISA. On the other hand, the M167-id+ antibody formed by association of the M167-μ transgene and the V_κ24 L chain was PC specific and bound PC-BSA to the same extent as the control. Yet antibodies formed by a V_κ24 L chain plus either an M603 or a T15 H chain were M167-id+ but not capable of binding PC (data not shown). These data suggest that the in vivo selection and amplification of M167-id+ B cells in the μ-transgenic mice is an antigen-driven rather than an antiidiotype-driven event. B cells expressing the normally dominant T15-id+ are not selectively amplified because the antibody product formed by the M167-μ transgene product and the endogenous V_κ22 L chain has little or no affinity for PC.

Analysis of the Endogenous κ L Chain Expressed in the M167-id+ B Cells of μ-transgenic Mice. To demonstrate that the endogenous L chain expressed in the M167-id+, PC-specific B cells of μ-transgenic mice was the product of an endogenous $V_\kappa24$-$J_\kappa5$ gene rearrangement, the μ^{a+}/id+ B cells shown in the upper right quadrant in Fig. 3 *C* were isolated by sorting them on the flow cytometer. RNA from these double-positive B cells was PCR amplified using a V_κM167 5′ primer and a C_κ 3′ primer. RNA from the double-negative cells (lower left quadrant of Fig. 3 *C*), the MOPC-21 myeloma, two M167-id+ hybridomas from μ/κ 207-4-transgenic mice, and a cell line expressing J558λ/μ-M167 were also PCR amplified using the M167 5′ primer (Fig. 5 *A*). PCRs were carried out after

Table 2. *The IgM T15-id+ Antibody Formed by M167-μ and V_κ22 Does Not Exhibit Good Binding to PC-BSA*

Cell line*	Transfected V genes‡	κ§	λ	IgM+	V_H1-id	T15-id		M167-id		PC-BSA
						T139.2	RdT15‖	28-5-15	28-4-3	
J558L +	V167μ	−	+	+	+	−	−	−	−	−
J558L +	V167μ	+	+	+	+	+	+	−	−	−
+	V_κ22									
J558L +	V167μ	+	+	+	+	−	−	+	+	+
+	V_κ24									

* The J558L-V167μ cell line produces a V_H1-id+, IgM+λ antibody that lacks the V_κ22- and V_κ24-dependent T15 and M167 idiotypes and does not bind PC.
‡ When this cell line is transfected with either a V_κ22 or V_κ24 L chain gene, the resulting cell lines produce antibodies that express κ and the appropriate T15 or M167 idotypes, respectively.
§ The total amount of antibody bearing each of the above markers was determined in a capture ELISA in which plates coated with goat anti-μ were developed by addition of biotin-conjugated antibodies specific for the indicated isotype, allotype, or idiotype as described in Materials and Methods. The same biotin conjugates were used to develop PC-BSA-coated plates. Standard curves were generated in all assays using either the IgM T15-id+ hybridoma HPCM2 or the M167-id+ IgM hybridoma HPCM27 (24).
‖ Polyclonal rabbit anti-T15id antiserum (25).

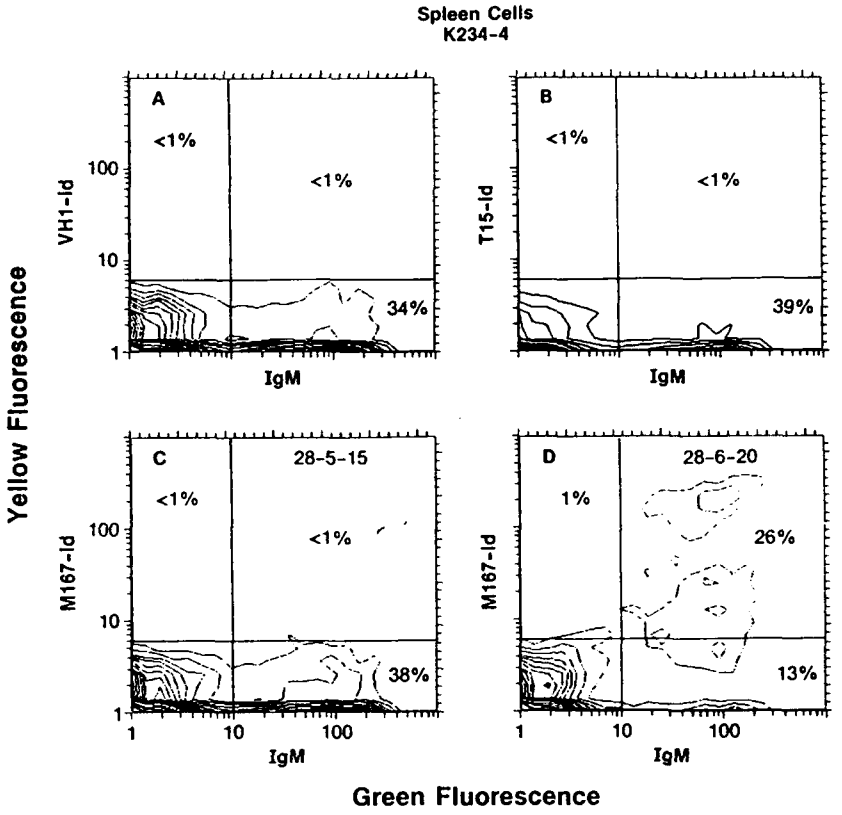

Figure 6. Idiotype analysis of spleen cells from M167-κ 234-4-transgenic mice. Spleen cells from κ 234-4-transgenic mice were stained and analyzed as described in Fig. 1.

face, while 23% of the splenic B cells from κ-233-8 mice expressed this idiotype (data not shown). The IgM$^+$ B cells in the bone marrow of these mice also exhibited high levels of the crossreactive 28-6-20 id; 86% of the total IgM$^+$ cells in the single 234-4 mouse analyzed, and 16 and 22% of the total IgM$^+$ cells in the two 233-8 TG$^+$ mice analyzed. TG$^-$ mice always exhibited <1% M167-id$^+$ B cells in their bone marrow. These data suggest that the κ transgene product is being expressed in association with endogenous μ chains in a large number of the B cells from these M167 κ mice, but very few of these spleen cells express an endogenous M167-μ H chain, which is required to produce a PC-specific antibody.

Two populations of 28-6-20 M167-id$^+$ B cells are present in the κ-only transgenic mice, one with high levels of id and one with low levels of id (Fig. 6 *D*). This difference in M167-id expression is not due to a difference in density of IgM expression on the B cells but might be due to differences in IgD expression, or it could result from the coexpression of endogenous L chains, which would lower the intensity of M167-id staining.

Discussion

In this paper, we have presented data suggesting that there is a preferential selection and expansion of M167-id$^+$, PC-specific B cells in transgenic mice that express a rearranged M167-H chain gene, and that a similar amplification of M167-id$^+$, PC-specific B cells does not occur in mice expressing a rearranged M167 κ transgene. In the M167-μ-transgenic mice, 20–75% of the splenic B cells expressing the μ^a transgene product also coexpress an endogenous V_κ24-J_κ5 L chain. The frequency of expression of this H/L chain pair is 100–500-fold higher than the 0.1% frequency expected from a random expression and association of this or any other endogenous κ L chain gene product with the M167-μ transgene product. The selective expansion of M167-id$^+$, PC-specific B cells in these μ-transgenic mice appears to be the result an antigen-driven, receptor-mediated process; hence, it is dependent on the expression of the transgene product on the surface of the B cell, and it only occurs when the transgene-encoded H chain pairs with a light chain that confers PC binding specificity. Thus, there is no selection of PC-specific B cells in the M167-μΔmem-transgenic mice, which cannot insert the transgene-encoded antibody into their B cell membranes (22; Fig. 4 and Table 1), and furthermore, there is no selection for T15-id$^+$ B cells even though the M167-μ chain can associate with a V_κ22 L chain to form T15-id$^+$ antibodies. The failure to selectively expand these T15-id$^+$ M167V_H/V_κ22 B cells is probably due to the fact that they have little or no affinity for PC, as was demonstrated in gene tr...sfection studies (Table 2). On the other hand, the PC-specific V_H1/V_κ24-expressing B cells are likely amplified in vivo via encounter with autologous or environmental PC in these μ-transgenic mice.

The observations presented in this paper may provide important insights into how B cells in general are selected by antigen- or other receptor-mediated interactions from the short-lived pool of rapidly renewing bone marrow B cells

seen in TG$^-$ controls. However, Storb et al. (21) were able to detect elevated levels of the secretory form of V_H-167 mRNA in some of the M167-κ-transgenic mice using a S107 V_H family–specific probe. Thus, V_HT15 family B cells are amplified and activated in the κ-transgenic mice but not to a level detectable by flow cytometry.

In conclusion, our data suggest that the expression of the M167-μ H chain in the B cells of transgenic mice results in an antigen-specific skewing of the B cell repertoire. A disproportionate number of the B cells of such animals express the M167-id and bind to PC. By contrast, when the same transgene product cannot be inserted into the cell membrane, the B cell repertoire is unaffected; there are no M167-id$^+$ cells detected by flow cytometry, and the PC response in the $\mu\Delta$mem TG$^+$ animals is dominated by T15-id$^+$ B cells. The skewing of the repertoire in μ-only transgenic mice appears to result from an antigen-driven rather than an antiidiotype-driven process. The M167-μ transgene product can form a T15-id$^+$ antibody by associating with an endogenous V_κ22 L chain, but such an antibody does not bind to PC, and cells expressing such antibodies are not expanded in these mice. If the T15-id domination of the PC response in normal animals were based primarily upon id selection, we should have seen many PC-nonbinding T15-id$^+$ B cells in the μ-only transgenic mice. Data from the M167 κ-only mice also support the conclusion that the repertoire selection is antigen driven. The V_κ24 L chain associates with the μ H chain of many V_H genes to generate a M167-crossreactive id, but forms a PC-binding antibody only in the rare event that it associates with a particular alternatively spliced V_H1-rearranged gene product that generates an alanine residue at position 96.

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References

1. Osmond, D.G. 1990. B cell development in the bone marrow. *Semin. Immunol.* 2:173.
2. Forster, I., P. Vieira, and K. Rajewsky. 1989. Flow cytometric analysis of cell proliferation dynamics in the B cell compartment of the mouse. *Int. Immunol.* 1:321.
3. Forster, I., and K. Rajewsky. 1990. The bulk of the peripheral B-cell pool in mice is stable and not rapidly renewed from the bone marrow. *Proc. Natl. Acad. Sci. USA.* 87:4781.
4. Dildrop, R., U. Krawinkel, E. Winter, and K. Rajewsky. 1985. V_H-gene expression in murine lipopolysaccharide blasts distributes over the nine known V_H-gene groups and may be random. *Eur. J. Immunol.* 15:1154.
5. Schulze, D.H., and G. Kelsoe. 1987. Genotypic analysis of B cell colonies by in situ hybridization. Stoichiometric expression of three V_H families in adult C57BL/6 and BALB/c mice. *J. Exp. Med.* 166:163.
6. Yancopoulos, G.D., B.A. Malynn, and F.W. Alt. 1988. Developmentally regulated and strain-specific expression of murine V_H gene families. *J. Exp. Med.* 168:417.
7. Jeong, H.D., and J.M. Teale. 1988. Comparison of the fetal and adult functional B cell repertoires by analysis of V_H gene family expression. *J. Exp. Med.* 168:589.
8. Sheehan, K.M., and P.H. Brodeur. 1989. Molecular cloning of the primary IgH repertoire: a quantitative analysis of V_H gene usage in adult mice. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2313.
9. Freitas, A.A., M.-P. Lembezat, and A. Coutinho. 1989. Expression of antibody V-region is genetically and developmen-

NK cells in immune responses to T cell regulated antigens type 1 and 2. *Immunol. Rev.* 99:105.
46. Metcalf, E.S., I. Scher, and N.R. Klinman. 1980. Susceptibility to in vitro tolerance induction of adult B cells from mice with an X-linked B-cell defect. *J. Exp. Med.* 151:486.
47. Cosenza, H., and H. Kohler. 1972. Specific inhibition of plaque formation to phosphorylcholine by antibody against antibody. *Science (Wash. DC).* 176:1027.
48. Sher, A., and M. Cohn. 1972. Inheritance of an idiotype associated with the immune response of inbred mice to phosphorylcholine. *Eur. J. Immunol.* 2:319.
49. Lieberman, R., M. Potter, E.B. Mushinski, W. Humphrey, Jr., and S. Rudikoff. 1972. Genetics of a new IgV_H (T15 idiotype) marker in the mouse regulating natural antibody to phosphorylcholine. *J. Exp. Med.* 139:983.
50. Claflin, J.L., R. Lieberman, and J. Davie. 1974. Clonal nature of the immune responses to phosphorylcholine. I. Specificity, class, and idiotype of phosophorylcholine-binding receptors on lymphoid cells. *J. Exp. Med.* 139:58.
51. Lee, W., H. Cosenza, and H. Kohler. 1974. Clonal restriction of the immune response to phosphorylcholine. *Nature (Lond.).* 247:55.
52. Feeney, A.J., S.H. Clarke, and D.E. Mosier. 1988. Specific H chain junctional diversity may be required for non-T15 antibodies to bind phosphorylcholine. *J. Immunol.* 141:1267.
53. Feeney, A.J., and D.J. Thuerauf. 1989. Sequence and fine specificity analysis of primary 511 anti-phosphorylcholine antibodies. *J. Immunol.* 143:4061.
54. Feeney, A.J. 1990. Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. *J. Exp. Med.* 172:1377.
55. Decker, D.J., N.E. Boyle, J.A. Koziol, and N.R. Klinman. 1991. The expression of the Ig H chain repertoire in developing bone marrow B lineage cells. *J. Immunol.* 146:350.
56. Stenzel-Poore, M.P., and M.B. Rittenberg. 1989. Clonal diversity, somatic mutation, and immune memory to phosphocholine-keyhole limpet hemocyanin. *J. Immunol.* 143:4123.

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RECEPTOR-MEDIATED ELIMINATION OF PHOSPHOCHOLINE-SPECIFIC B CELLS IN X-LINKED IMMUNE-DEFICIENT MICE[1]

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The combined expression of the M167 μ/κ anti-phosphocholine (PC) transgenes with the x-linked immunodeficiency gene, *xid*, results in an almost total failure to develop B cells in the peripheral lymphoid organs of such mice. Although there is no significant difference between the normal transgene positive (TG^+) female offspring and the immunodeficient TG^+ *xid* males with respect to the number of $B220^+$ pre-B cells and IgM^+B220^+ B cells that develop in their bone marrow, the hemizygous *xid* males have 85% fewer B cells in their spleens than the phenotypically normal heterozygous F1 females. In *xid* M167-μ-transgenic mice, PC-specific B cells also fail to develop in the spleen; however, numerous B cells bearing the $\mu^{a+}V_H1^+$-transgene product associated with endogenous κ L chains that do not give rise PC-specific antibodies are present. In the phenotypically normal TG^+ (B6.CBA/N × μ 243-4)F1 female mice, PC-specific B cells represent almost 10% of the total B cell population, and these B cells express an M167-Id that has been produced by association of the V_H1 transgene product with an endogenous Vκ24 L chain. B cells expressing the normally dominant T15-Id are not detectable in the spleens of these M167 μ-transgenic mice. Furthermore, M167-Id^+ B cells are present at a fivefold lower level in the bone marrow of μ-TG^+ normal mice than in their spleens. These data suggest that the PC-specific B cells that develop in TG^+ *xid* mice are either clonally deleted via some "IgR-directed" mechanism or they fail to receive the appropriate signals to exit the bone marrow or to enter the peripheral lymphoid tissues. This hypothesis is supported by the finding that TNP-specific B cells develop normally and do not undergo clonal deletion in *xid* mice carrying the Sp6 μ/κ anti-TNP transgenes.

The CBA/N mouse carries an X-linked immune defect (*xid*) that results in a profound inability to respond to type 2 thymus-independent Ag such as TNP-Ficoll, type III pneumococcal polysaccharide, and dextran (the CBA/N defect is reviewed in reference 1). However, mice expressing the *xid* gene also fail to mount a primary immune response to the hapten, PC[2] on either type 2 thymus-independent or thymus-dependent carriers such as *Streptococcus pneumoniae* or keyhole limpet hemocyanin, respectively (2, 3). The alterations in the immune response of *xid* mice to both TI-2 antigens and to PC can be accounted for by the absence of a subset of B cells that expresses Lyb-3 (4), Lyb-5 (5), and Lyb-7 (6) membrane Ag and develops late in ontogeny (5). A second subset of B cells, which expresses the Ly-1 alloantigen, also appears to be absent in the peripheral lymphoid tissue of most *xid* mice (7). The absence of Lyb-5 and Ly-1 B cells from the spleen and lymph nodes of *xid* mice appears to be due to a block in their maturation and subsequent migration from the bone marrow. Although CBA/N (*xid*) mice generate the same absolute number of $B220^+$ pre-B cells (8, 9), Abelson murine leukemia virus target cells (10), and sIg^+ B cells (8) in their bone marrow as normal mice, they have less than one-third the normal number of mature B cells in their spleen, lymph nodes, and thoracic duct (11). The B cells that develop in *xid* mice are, however, normal with respect to their half-life and their ability to recirculate (8, 11).

The mechanism responsible for the loss of two-thirds of the B cells that develop in CBA/N mice and the specific elimination of the $Lyb\text{-}5^+$ and $Ly\text{-}1^+$ B cell subsets is still unclear. De La Hera et al. (12) have shown that CBA/N mice can develop Ly-1 B cells after treatment with a combination of irradiation and cyclosporin A. This sug-

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[2] Abbreviations used in this paper: PC, phosphocholine; ABC, Ag binding cell; sIg, surface Ig; PC-KLH, PC conjugated keyhole limpet hemocyanin; TG, transgenic mice; T^+, transgene positive; T^-, transgene negative; μ^a, IgM bearing the a-allotype; μ^b, IgM bearing the b-allotype; Xn, normal X chromosome; HEL, hen egg white lysozyme

gests that the development of this B cell subset may be actively suppressed in *xid* mice. However, Klinman and Stone (13) proposed that the PC-specific precursors in *xid* mice, which are largely restricted to the Lyb-5$^+$ B cell subset in normal mice (14–16), are clonally aborted after sIgR binding of environmental Ag in the absence of T cell help. To test this latter hypothesis, male mice from the M167 μ + κ anti-PC transgenic mouse line 207-4 (Tg(Igh + Igk)Bri12) and M167 μ-only mouse line 243-4 (Tg(Igh)Bri35) developed by Storb et al. (17) were crossed with *xid* immune defective B6.CBA/N females. We have previously shown (18) that the phenotypically normal TG$^+$ F1 female mice derived from the $\mu\kappa$ 207-4 cross develop large numbers of splenic B cells, 97% of which express the PC-specific transgene product as an antigen-specific, cell surface receptor; however, the B220$^+$, sIg$^-$, pre-B cells in the bone marrow of these transgenic mice are markedly depressed. The depression in pre-B cells is presumably related to the earlier than normal expression of the rearranged μ/κ transgenes during B cell ontogeny. In this report, we demonstrate that the combination of the *xid* gene and the PC-specific M167 $\mu\kappa$ transgenes results in the elimination of most B cells from the peripheral lymphoid tissues of these *xid* mice, whereas only the PC-specific B cells are eliminated from the *xid* M167 μ-only mice. This apparent Ag-specific, IgR-directed elimination of B cells does not occur in *xid* transgenic mice carrying μ/κ anti-TNP transgenes. The blockade in PC-specific B cell development in the *xid* transgenic mice appears to occur during the exodus of B cells from the bone marrow and their subsequent migration to the peripheral lymphoid tissues.

MATERIALS AND METHODS

Animals. The transgenic mice carrying the MOPC-167 μ + κ transgenes (line 207-4, designation Tg(Igh + Igk)Bri12) and the MOPC-167 μ transgene (line 243-4, designation Tg(Igh)Bri35) were obtained from Dr. U. Storb (Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL) through Dr. R. L. Brinster (School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA) and have been described previously (17). These lines are maintained in our breeding colony by back-crossing transgene positive (TG$^+$) males to C57BL/6 female mice. The progeny are typed for the presence of the transgene by ELISA analysis of antibodies bearing the IgM^a allotype (μ^a). TG$^+$ males were crossed with B6.CBA/N female mice (Igh^b) and the phenotypically normal, heterozygous (Xn,Xid), F1 female and hemizygous (Xid,Y), immune defective, F1 male mice were used in the studies presented below. All mice were more than 8 wk of age and less than 24 wk of age when analyzed. The consomic *xid* B6.CBA/N mouse strain was obtained from Dr. Carl Hansen, Division of Veterinary Medicine, National Institutes of Health, Bethesda, MD and was derived as previously described (19). The μ/κ anti-TNP (Sp6) transgenic mice developed by Rusconi and Kohler (20) were back-crossed to BALB/c mice under specific pathogen-free conditions at the Max-Planck-Institute for Immunobiology and TG$^+$ males were then bred to CBA/N females to produce TG$^+$-immunodeficient *xid* male mice.

Antibodies and serum analysis. Preparation of the antibodies, Ag, the ELISA procedures used for detection of serum antibodies to PC were as previously described (18, 21, 22). The rat-anti-V_H1-Id hybridoma T68.3, which recognizes all antibodies carrying a V_H1 H chain and the anti-T15-Id hybridoma T139.2 (23) were obtained from Dr. M. Scharff, Albert Einstein College of Medicine, Bronx, NY. The rat-anti-M167-Id hybridomas 28-5-15 and 28-6-20 were produced by immunizing with affinity purified MOPC-167 myeloma protein (IgA,λ24) and boosting with HPCM27 (IgM,λ24) (24). The complete characterization of these and other anti-M167-idiotypic antibodies will be presented in detail elsewhere (D. G. Sieckmann, E. Martin, D. L. Longo, and J. J. Kenny, manuscript in preparation). In brief, antibodies from clone 28-5-15 recognize only the V_H1-V,24 H:L chain combination and appears to be binding site specific, whereas antibody 28-6-20 also binds 3 Vλ24 antibodies having specificity for phenylphosphocholine and possessing J558 V_H H chains. Thus, this antibody recognizes a Vλ24-dependent cross-reactive idiotope and it is not binding-site specific.

Flow cytometric analysis. The preparation, staining and flow cytometric analysis of spleen and bone marrow B cells was as previously described (18, 21, 22).

ABC. The number of spleen cells in TG$^+$ and TG$^-$ mice capable of binding PC was determined in a rosette assay (25). SRBC were conjugated with diazophenylphosphocholine as previously described (15) and adjusted to 2% v/v. Spleen cells were adjusted to 10^7/ml and 200 μl of spleen cells and 100 μl of PC-SRBC were placed together in a 12 × 75 tube, spun at 1000 rpm for 10 min in the cold, resuspended and placed on a hemocytometer. The number of ABC was determined by counting the number of white cells having four or more SRBC bound. The specificity of ABC was tested in the presence of 10^{-3} M PC.

RESULTS

We have previously shown that phenotypically normal M167 $\mu\kappa$ transgenic mice express anti-PC antibody on 97% of their splenic B cells (18). If the rearrangement and expression of PC-specific V genes occur normally in the bone marrow of *xid* mice and the resulting V_H1 receptor-positive B cells are eliminated during maturation and migration to the peripheral lymphoid tissues, (as proposed by Klinman and Stone (13)), then one would predict that the spleens of these M167 $\mu\kappa$ anti-PC *xid* transgenic mice would be virtually devoid of B cells. To test this hypothesis, the spleen cells from TG$^+$ and TG$^-$ normal (X$^+$) and *xid* mice were stained with B cell specific reagents and analyzed by flow cytometry.

Flow cytometric analysis of spleen cells from (B6.CBA/N × TG)F1 mice. The flow cytometric analysis of spleen cells from normal F1 female and immune defective F1 male mice stained with FITC-anti-μ and biotin-conjugated anti-B220 plus PE-streptavidin is shown in Figure 1. As previously shown (18), the normal TG$^+$ F1 females develop substantial numbers of bright IgM$^+$-B220$^+$ B cells in their spleens (Fig. 1*B*) (range, 18 to 70% IgM$^+$). In general, the normal F1 female TG$^+$ spleen contains approximately 50% as many B cells as its normal TG$^-$ littermate (Fig. 1*A*). In marked contrast, the vast majority (18/22) of immune defective, TG$^+$-*xid* males (Fig. 1*D*) have less than 5% B cells in their spleens (mean = 3.6 ± 1.4), which is less than 7% of the absolute B cell number present in the TG$^-$ *xid* littermate (mean = 49 ± 2.5) (Fig. 1*C*) and less than 12% of the normal TG$^+$ F1 female mice.

To further characterize the B cells in the transgenic *xid* mice, the spleen cells from TG$^+$ and TG$^-$ *xid* mice were stained with FITC-anti-IgM^a allotype plus biotin-conjugated anti-IgM^b allotype followed by PE-streptavidin or Texas red streptavidin. The mice were placed into two groups (see Fig. 2, *row 1* vs *row 3*) based on the pattern of IgM allotype expressed on the surface of their splenic B cells, and mice that had <1% B cells in their spleens were placed into a third group (not shown). The percent of sIgM$^+$ B cells in the spleens of TG$^+$ *xid* mice ranged from 0 to 32 (mean = 3.6 ± 1.4); however, as was seen in the TG$^+$ normal (Xn) females (18), the vast majority of these IgM$^+$ *xid* B cells expressed the transgene-encoded IgM^a allotype and very few B cells expressed the endogenous IgM^b allotype in the absence of the μ^a transgene product (Fig. 2). The expression of endogenous Ig was more variable than that of the transgene encoded Ig. The mice in group 1 (7/22) expressed endogenously encoded IgM^b on more than 50% of their splenic B cells (Fig. 2,

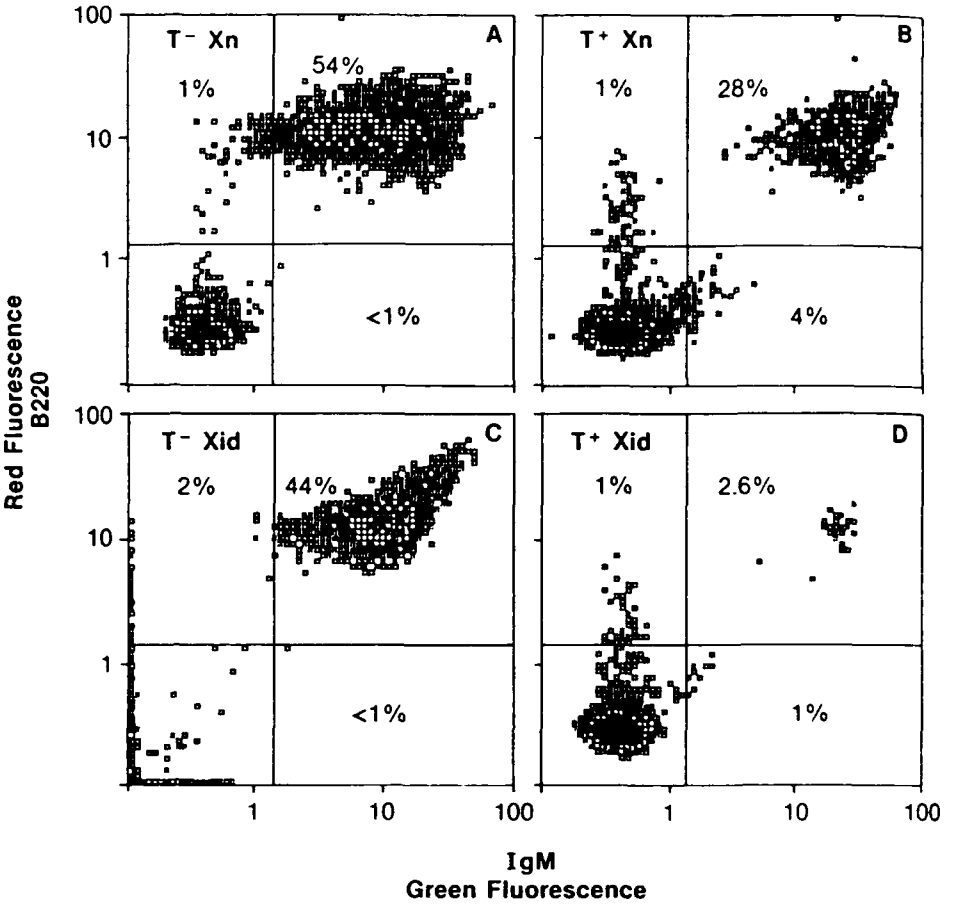

Figure 1. Flow cytometric analysis of IgM⁺ and B220⁺ spleen cells from T⁺ and T⁻ (B6.CBA/N × TG)F1 mice. One × 10^6 spleen cells from TG⁺ and TG⁻ normal (*Xn*) F1 female (*A* and *B*) and immune defective (*xid*) male (*C* and *D*) littermates were stained with 10 μl (1 μg) each of FITC-conjugated goat anti-μ and biotin-conjugated anti-B220 (6B2) plus phycoerythrin-conjugated streptavidin. The stained cells were then analyzed on a Coulter EPICS 753 Cytofluorograph (Coulter Electronics, Hialeah, FL) as previously described (18).

mouse 35); thus, the dominant B cell type in these mice was $\mu^a + \mu^b$ double allotype positive. The B cells in group 2 mice (7/22 mice) expressed predominantly the μ^a allotype (Fig. 2, mouse 91). Overall, the percentage of splenic B cells in group 1 and 2 *xid* TG⁺ mice expressing endogenous IgM^b appears to be much higher (55% ± 6.3, range 12 to 100) than in normal TG⁺ F1 female mice (mean = 18% ± 8, range 4 to 30%) (18); however, because the number of B cells is greatly decreased in TG⁺*xid* male mice, the absolute number of double-allotype positive cells is much greater in the normal TG⁺ mice. Eight of the 22 *xid* TG⁺ positive mice examined had less than 1% B cells in their spleens. Half of these mice expressed no detectable IgM^+B220^+ B cells whereas the other four appeared to have between 0.2 to 0.4% $\mu^{a+}B220^+$ B cells.

The peritoneal cells of the mice shown in Figure 2 were also analyzed for μ^a and μ^b surface expression. The phenotype of these cells followed the same pattern as that seen in the spleen, i.e., mouse 35 had predominantly double-allotype positive B cells and mouse 91 had mainly μ^a-only B cells (data not shown). As expected, there were no $IgM^+Ly\text{-}1^+$ or $IgM^+Mac\text{-}1^+$ B cells detected in either of these TG⁺ *xid* mice; however, a significant number of Ly-1 lineage ($IgM^+Ly\text{-}1^+/Mac\text{-}1^+$) B cells was detected in the TG⁻ *xid* control (mouse 65, Fig. 2, data not shown).

Analysis of B cell development in bone marrow and spleen of normal and xid transgenic mice. The above data are consistent with the hypothesis that most PC-specific B cells in *xid* mice fail to migrate from the bone marrow to the spleen, whereas, the PC-specific B cells that develop in both TG⁻ and TG⁺ normal mice are able to migrate to the peripheral lymphoid tissues. If this interpretation of the data is correct, then one should find that the bone marrow of TG⁺ *xid* F1 male mice is essentially equivalent to the normal TG⁺ F1 female with respect to the development of IgM^-B220^+-pre-B cells. However, it is possible that the transgenes are activated earlier in the B cell progenitors that give rise to *xid* B cells or that this B cell subset is more sensitive to feedback regulation by the transgene product than the B cell progenitors that develop in normal mice. This latter possibility would result in a highly reduced level of pre-B cells and B cells in the TG⁺ *xid* bone marrow compared to the normal TG⁺ bone marrow.

The data in Table I compare the TG⁺ and TG⁻ normal (Xn) and *xid* mice with respect to the total number of nucleated cells, IgM^+B220^+ B cells, and IgM^-B220^+ pre-B cells present in their spleen and bone marrow tissues. A high degree of variability existed in all four groups of mice with respect to both their spleen and bone marrow nucleated cell numbers; however, the only significant difference among these four groups with respect to total cell count was in the greater numbers of spleen cells present in the TG⁻ normal (Xn) mice. When the number of IgM^+ B cells and $B220^+$ pre-B cells were compared between the TG⁻ and TG⁺ mice, both normal and *xid* TG⁻ mice had significantly greater numbers of IgM^+ B cells in their spleen and bone marrow and significantly greater numbers of $B220^+$ pre-B cells in their bone marrow than the TG⁺ normal and *xid* mice, respectively (Table I; Fig. 3). Thus, the presence of the $\mu + \kappa$ M167 transgenes in both normal and *xid* mice results in an 80 to 90% reduction of pre-B cells and a 60 to 80% loss in B cells from the bone marrow of these mice. The loss in IgM^+ and

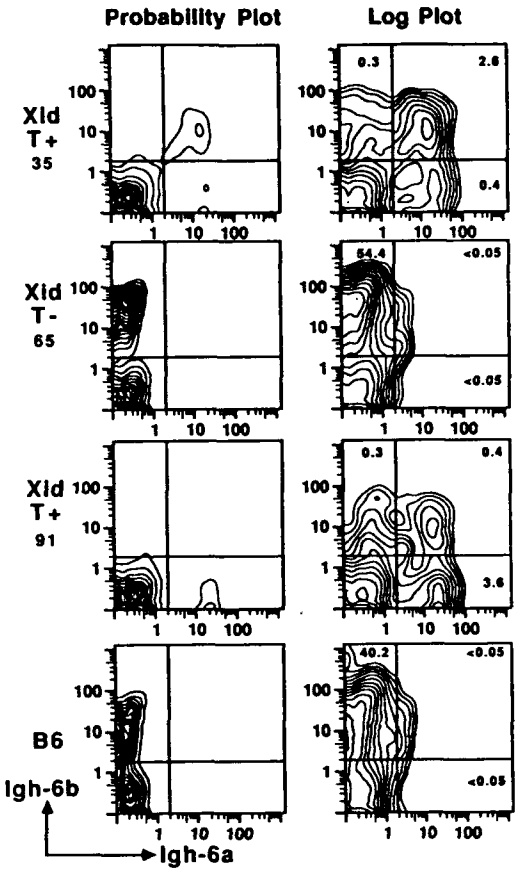

Figure 2. Relative frequency of Igh-6a (μ^a), Igh-6b (μ^b) and double-positive B cells in the spleens of T⁺ and T⁻ *xid* mice. One × 10^6 spleen cells from TG⁺ (*rows 1* and *3*) and TG⁻ (*row 2*) *xid* and a C57BL/6 (B6) control were stained with 10 μl (1 μg) each of FITC-conjugated anti-μ^a allotype (DS1) and biotin-conjugated anti-μ^b allotype (AF6) plus Texas red streptavidin. Flow microfluorimetry analyses were conducted as previously described (21). The analysis in the *left panels* are presented as 5% probability contour plots. Although the major lymphocyte subpopulations are obvious in these plots, minor populations (<2%) are not easily visualized. The identical analyses are presented in the right panels as 50% logarithmic contour plots. This method of contouring emphasizes small numbers of cells, readily revealing the small μ^{a+} population in the T⁺*xid* mice. The probability and logarithmic contour plots were generated according to Moore and Kautz (38). The numbers shown in the *left margin* for each *xid* mouse (i.e., 35, 65, 91,) correspond to the ear tag numbers provided at the time of serum phenotyping for presence or absence of the transgene.

B220⁺ cells in the bone marrow is evident not only in the IgM vs B220 staining profiles (compare *panels 2* and *6* to *panels 10* and *14*, Fig. 3), but also in the reduction of small to medium size cells seen in the forward vs obtuse light scatter patterns shown in *row 1* Figure 3 (compare the areas enclosed in *blocks* within each *panel*). However, there was no significant difference between the pre-B cell numbers ($p = 0.6$) or numbers of IgM⁺ B cells ($p = 0.7$) in the bone marrow of TG⁺Xn and TG⁺*xid* mice (Table I, *p* values in bold face); furthermore, no difference in the total number of cells in the bone marrow or spleens of these two groups was evident. However, there was a highly significant difference ($p < 0.001$) in the total number of IgM⁺ B cells found in their spleens (Table I). An average of 15 × 10^6 B cells was found in the spleens of the TG⁺ normal (Xn) mice whereas <2 × 10^6 B cells were present in the spleens of the TG⁺ *xid* mice (Table I). Thus, despite equivalent B cell development at the level of the bone marrow, the majority of the B cells that develop in the TG⁺ *xid* mice fail to migrate to the spleen. These observations suggest that the B cells in the TG⁺ *xid* mice are being eliminated in the bone marrow or during migration from the bone marrow to the spleen.

When one compares the IgM⁺B220⁺ B cells in the bone marrow of TG⁻ and TG⁺ mice (Fig. 3, *row 2*), it is evident that the majority of B cells in TG⁺ mice have less IgM and B220 on their surface than the B cells in the bone marrow of TG⁻ mice, regardless of their X-chromosome phenotype and that B cells with high sIgM and high B220 do not develop in the TG⁺ mice (compare *shaded areas* in Fig. 3, *row 2*). However, the B cells present in the spleens of these TG⁺ mice express B220 levels that are as bright and IgM levels that are brighter than the B cells in the TG⁻ controls (Fig. 1). The phenotype of the IgM⁺ B cells in the bone marrow and spleens of TG⁺ mice is characteristic of more immature B cells (8); thus, in the bone marrow, low levels of both sIgM and B220 is characteristic of immature B cells, whereas in the spleen, immature B cells exhibit high levels of sIgM (1).

The bone marrow of TG⁺ and TG⁻ normal and *xid* mice were also stained with FITC-conjugated anti-μ^a plus biotin-conjugated anti-μ^b or anti-V_H1 followed by Texas red streptavidin. The data shown in Figure 3, *rows 3* and *4* indicate that both TG⁺*xid* (*panels 15* and *16*) and TG⁺Xn mice (*panels 11* and *12*) have B cells that express only the transgene μ^a product on their surface, and that all these μ^{a+} B cells coexpress the expected V_HT15 idiotope (*panels 12* and *16*). Staining profiles virtually identical to those seen in *panels 12* and *16* were obtained when bone marrow from TG⁺ normal and *xid* mice was stained with FITC-anti-μ and biotin-conjugated anti-M167-id (data not shown). Thus, both the μ and κ transgene products are expressed on all the bone marrow B cells from

TABLE I

Comparison of total lymphoid cell numbers, B cells and pre-B cells in TG⁺ and TG⁻ bone marrow, and spleen of normal and xid mice

Tissue/Cell[a] Type Analyzed (Total No. × 10^{-6})	Mouse Phenotype[b]						
	TG⁻Xn		TG⁺Xn		TG⁺Xid		TG⁻Xid
Spleen cells	66 ± 6.8	($p = 0.003$)[c]	39 ± 4.5	($p = 0.7$)[c]	42 ± 5.3	($p = 0.09$)[c]	55 ± 4.8
sIgM⁺ spleen cells	37 ± 5.6	($p = 0.05$)	15 ± 3.8	($p < 0.001$)	1.8 ± 0.7	($p < 0.001$)	30 ± 4.1
Bone marrow cells	23 ± 3		25 ± 2		26 ± 2		26 ± 4
IgM⁺B220⁺ bone marrow cells	2.4 ± 0.6	($p = 0.002$)	0.8 ± 0.1	($p = 0.7$)	0.7 ± 0.2	($p = 0.02$)	1.7 ± 0.5
IgM⁻B220⁺ pre-B bone marrow cells	6.5 ± 1.4	($p < 0.001$)	0.6 ± 0.3	($p = 0.6$)	0.5 ± 0.1	($p < 0.001$)	3.1 ± 0.7

[a] The spleen and bone marrow cell numbers were determined after $(NH_4)Cl$ lysis of the RBC. Bone marrow numbers are based on the cells obtained from two femurs. The number of IgM⁺ and B220⁺ cells was calculated from the percent positive cells seen in flow cytometric analysis of the whole spleen and bone marrow cells.

[b] The number of animals analyzed of each phenotype and tissue varied from a low of 6 for the TG⁻Xn bone marrow to 24 for the TG⁺Xid spleen. The data shown represent the mean ± SE.

[c] Groups were compared using an unpaired Student's *t*-test. The *p* values obtained are shown between the groups being compared. The comparison of TG⁺ normal and TG⁺Xid is in bold face.

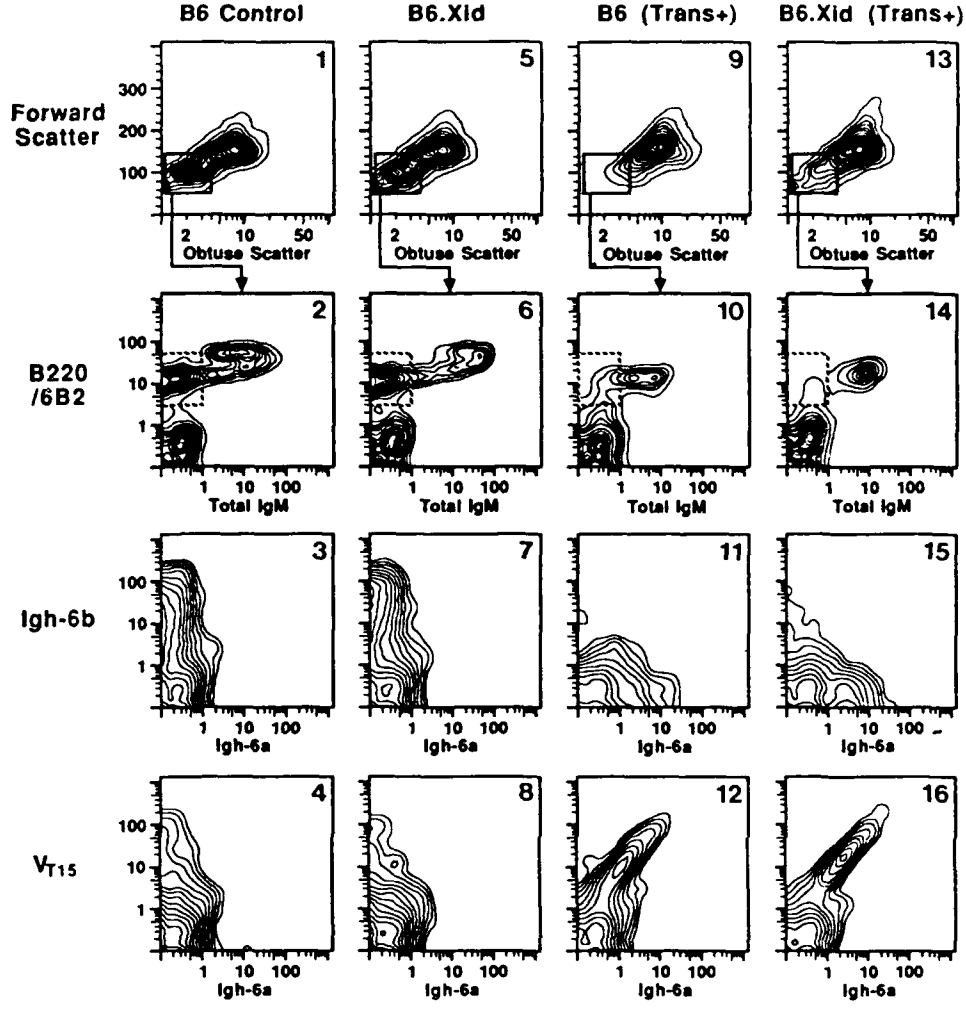

Figure 3. Flow microfluorimetry analysis of bone marrow from TG⁺ and TG⁻ mice. One × 10^6 bone marrow cells from C57BL/6 (*column 1, panels 1* to *4*), TG⁻ *xid* (*column 2, panels 5* to *8*), TG⁺ Xn (*column 3, panels 9* to *12*), and TG⁺ *xid* (*column 4, panels 13* to *16*) mice were stained with a combination of: 1) FITC-anti-IgM and biotin-anti-B220; 2) FITC-anti-μ^a and Biotin-anti-μ^b; or 3) FITC-anti-μ^a and biotin-anti-V_HT15 followed by Texas red-avidin. The *panels* shown in the *first two rows* are presented as 5% probability plots and the *last two rows* as 50% logarithmic plots. Cells were analyzed and contour plots generated as in Figure 2. The *top panels* in *row 1* show the forward scatter vs obtuse scatter of the total bone marrow. The remaining panels in *rows 2* to *4* show the phenotype of the cells within the scatter gates marked by the boxes in the *top panels*. In *row 2*, the pre-B cells (IgM⁻,B220⁺) are marked by *striped boxes* and bright IgM⁺,B220⁺ B cells by a *shaded area*. The cells defined by this area are missing in the TG⁺ mice.

both types of TG⁺ mice. Endogenous μ^b was detected in the bone marrow of only 1 of 22 *xid* mice examined and this was in the single mouse found to express 23% μ^a + μ^b double allotype positive spleen cells. None of the six TG⁺Xn mice examined had endogenous μ^b-allotype expressed at the level of their bone marrow (Fig. 3, *panel 11*) but all of them had double-allotype positive cells present in their spleens (18).

Id analysis of spleen cells in xid μκ transgenic mice. The above data suggest that the majority of PC-specific B cells in M167 μκ TG⁺ *xid* mice are clonally deleted after receptor expression in the bone marrow but before migration to the spleen. However, the approximately 2 × 10^6 B cells present in the *xid* TG⁺ spleens clearly express the μ^a transgene product (Fig. 2) but escape clonal deletion. It was possible that these B cells expressed the constant region allotype marker of the transgene but had lost or somatically mutated the V_H or V_L genes so that they neither bound PC nor expressed the M167 or V_H1-idiotopes. To test this possibility, the spleen cells of these μκ anti-PC TG⁺ mice were stained with FITC-anti-μ plus biotin conjugated anti-idiotypic antibodies as shown in Figure 4. These data clearly demonstrate that the majority of these *xid* B cells express both the V_H1-idiotope (Fig. 4*A*) and M167-idiotopes (*C* and *D*). The 28-5-15 anti-M167-Id is dependent on the expression of both the V_H1 H-chain and the V,24 L chain and is binding site specific, whereas the 28-6-20 anti-M167-idiotope recognizes V,24

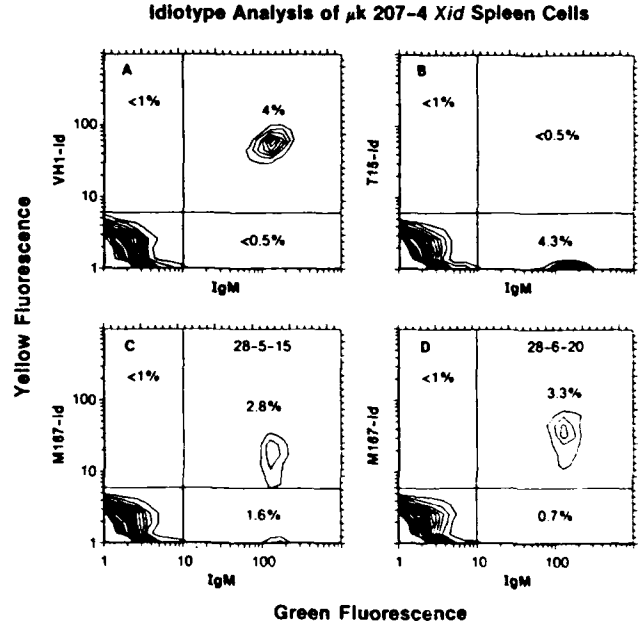

Figure 4. Id analysis of the spleen cells in xid μκ transgenic mice. One × 10^6 spleen cells from (B6 CBA/N × 207-4)F1 *xid* male mice were stained with 10 μl (1 μg) of FITC-anti-μ and 10 μl (1 μg) of biotin conjugated anti-V_H1-id (*A*), anti-T15-Id (*B*), or anti-M167-id (*C* and *D*) followed by PE-streptavidin. Stained cells were then analyzed as in Figure 1.

in association with additional V_H gene products and is not binding site specific (D. G. Sieckmann, et al. manuscript in preparation). There is a minor population of B cells (17 to 36%) in the TG⁺ *xid* mice which is not recognized by these anti-M167-idiotypic antibodies but clearly bear the V_H1-idiotope. The number of PC-specific ABC is approximately equivalent to the number of M167-Id⁺ B cells present in the $\mu\kappa$ TG⁺ *xid* mice (data not shown).

Analysis of (CBA/N × Sp6)F1 anti-TNP transgenic mice. To determine whether the apparent receptor directed clonal deletion of PC-specific B cells in TG⁺ *xid* mice was a consequence of the V_H and V_L genes expressed in these B cells, or resulted from the expression of the *xid* gene independent of the Ig transgenes expressed, the splenic B cells of normal (Xn) and *xid* mice bearing anti-TNP μ/κ transgenes were examined. The normal and xid Sp6 $\mu\kappa$ transgenic mice, like the M167 TG⁺ mice, express the H and L transgene products on the majority of their splenic B cells, and 60 to 70% of the B cells in both the normal and xid Sp6 mice bind TNP-PE (data not shown). The data presented in Figure 5 and Table II show that the number of IgM⁺B220⁺ cells in the TNP-specific TG⁺ *xid* mice is reduced to the same extent as that seen in the TG⁺Xn mice when both TG⁺ mice are compared to their respective TG⁻ control (i.e., 53 vs 64%). This is very similar to the 59% decrease in absolute B cells seen in the spleen of the anti-PC, M167 $\mu\kappa$, TG⁺ normal (Xn) mice (Table I), whereas, the TG⁺ *xid* anti-PC transgenics exhibit a 94% decrease in their splenic B cell population relative to the TG⁻ *xid* controls. These data support the idea that the clonal deletion of PC-specific B cells in the M167 *xid* transgenic mice is mediated via the Ag-specific receptor expressed on these B cells.

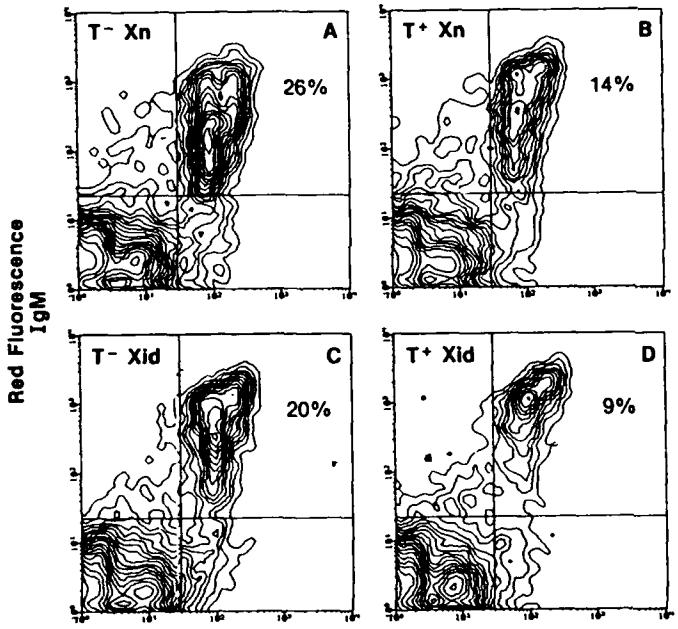

Figure 5. Flow cytometric analysis of spleen cells from (CBA/N × Sp6)F1 anti-TNP T⁺ and T⁻ normal (Xn) and *xid* mice. Spleen cells were stained with FITC-conjugated anti-B220 (14.8) and Biotin-conjugated anti-μ plus PE-streptavidin and analyzed as described in the legends of Figures 1 to 3. Data are shown as 5% probability plots.

Analysis of B cell development in M167 μ-only transgenic mice. Inasmuch as *xid* mice fail to develop substantial numbers of either Ly-1 B cells (8) or Lyb-5⁺ B cells (1), it was necessary to demonstrate that the loss of PC-specific B cells in *xid* mice was due an Ig-receptor mediated clonal deletion rather than restricted B cell subset expression by the V_H1 gene product. We therefore analyzed B lymphocyte development in normal and *xid* transgenic mice expressing only the M167 μ-H chain gene. If the expression of the V_H1 H chain is restricted to the Ly-1 or Lyb-5⁺ B cell subsets, then *xid* mice carrying the M167 H chain transgene should be similar to the μ/κ *xid* anti-PC transgenic mice and thus lack TG⁺ B cells in their spleen; however, if the failure to develop PC-specific B cells is due to receptor mediated, Ag-specific clonal deletion, the immunodeficient F1 male TG⁺ *xid* mice should express the μ-transgene product on large numbers of their peripheral B cells but should lack PC-specific B cells. The data in Figure 6 show that large numbers of spleen cells from both TG⁺ normal and *xid* mice (*A* and *B*) express the transgene encoded μ^a⁺V_H1⁺ H chain, although fewer TG⁺ B cells exist in the μ-*xid* mice. However, when the spleen cells from the M167 μ-transgenic mice were analyzed for the presence of B cells expressing known PC-specific, H + L chain dependent Id, the phenotypically normal F1 female mice exhibited a clearly defined population of M167-Id⁺ B cells (Fig. 6*G*), whereas these M167-Id⁺ B cells were absent in the spleens of the F1 *xid* male mice (*H*). T15-Id⁺ B cells were not seen in either the normal or *xid* TG⁺ mice. It is of interest that the M167 μ^a-transgene product in the phenotypically normal F1 female mice has associated with the appropriate Vκ24 endogenous L chain in approximately 10% of the B cells rather than with any of the other three or four κ L chains found in PC-specific antibodies. This preferential expansion of M167-Id⁺ B cells in the spleens of normal F1 TG⁺ female mice is probably IgR driven because there is a fivefold lower expression of M167-Id⁺ B cells in the bone marrow of these mice (data not shown).

In light of the above flow cytometric data, it was important to determine whether the decreased expression

TABLE II

Analysis of (CBA/N × Sp6)F1 spleen cells[a]

Mouse Phenotype	Total No. Cells/ Spleen × 10^{-6}	Percent IgM⁺	Total No. B Cell/ Spleen × 10^{-6}	Percent Decrease[b]
TG⁻Xn	85	26	22	
TG⁺Xn	57	14	8	64
TG⁻Xid	34	20	6.8	
TG⁺Xid	36	9	3.2	53

[a] Spleen cells from (CBA/N × Sp6)F1 normal (Xn) females and *xid* males were stained and analyzed as described in Figure 4 and Reference 22. Data shown above represent a single representative experiment.

[b] Percent decrease is calculated with respect to the appropriate X chromosome control. The TG⁻Xid on this (CBA/N × BALB/c)F1 background is reduced by 69% compared to the B cell number in the normal control. This is similar to the spleen of a CBA/N vs a CBA/J (11) but both strains are different from *xid* mice on the B6 background as shown in Table I

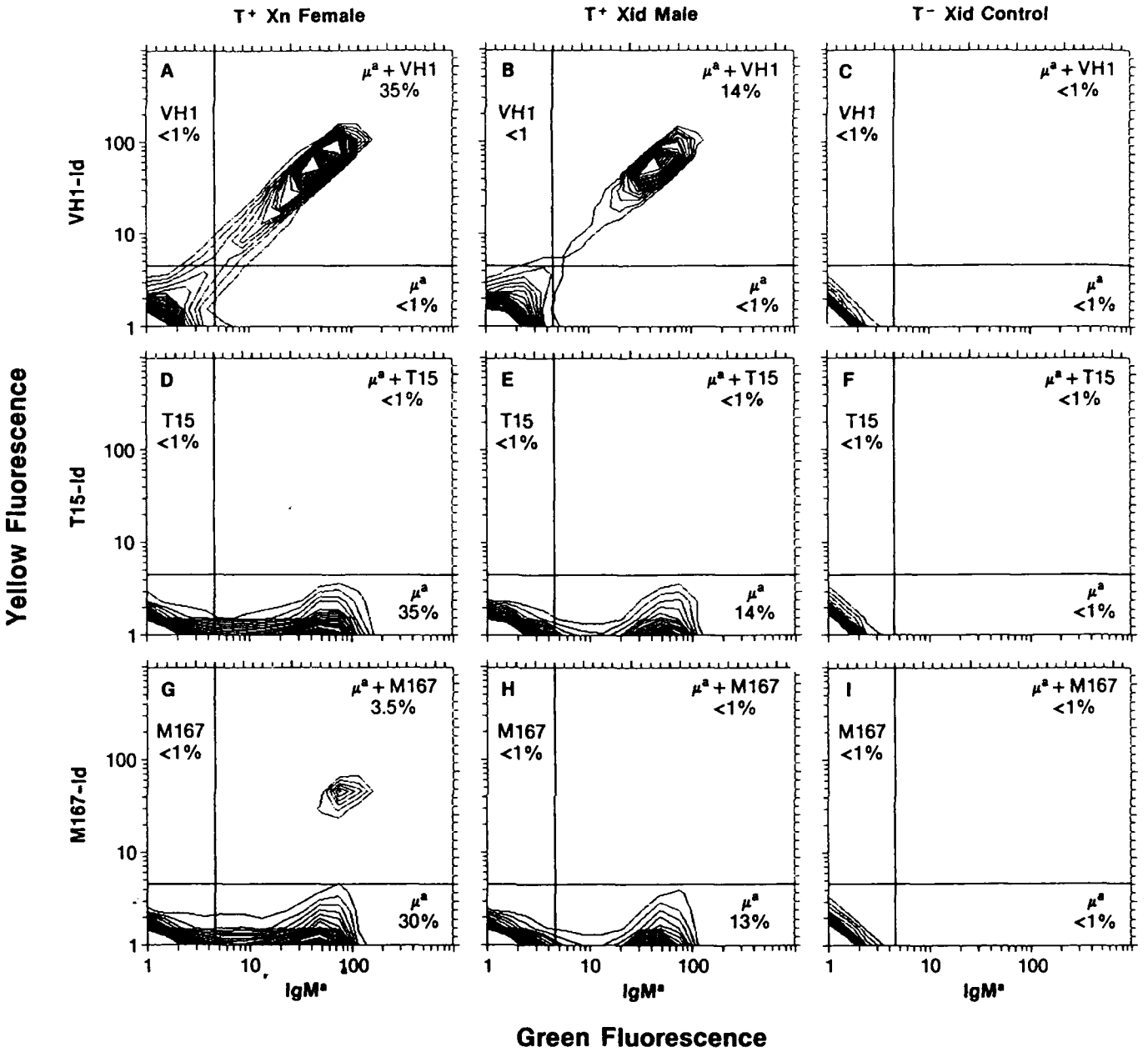

Figure 6. Flow cytometric Id analysis of spleen cells from M167 μ-transgenic mice. One × 10^6 spleen cells from transgene positive (T^+) and transgene negative (T^-) (B6.CBA/N × μ 243-4)F1 normal female and immune defective *xid* male mice were stained with 10 μl (1 μg) each of FITC-conjugated anti-IgM^a-allotype-specific antibody and either biotin-conjugated anti-V_H1-id (*A* to *C*), anti-T15-id (*D* and *F*), or anti-M167-id (*G* to *I*) followed by phycoerythrin-conjugated streptavidin. The stained cells were analyzed on a Coulter EPICS cytofluorograph and the analysis presented as 5% probability contour plots.

of the V_H1 transgene H chain product and the absence of M167-Id$^+$ B cells in the *μ-xid* mice correlated with a concomitant decrease in PC-specific B cells. To elucidate the number of PC-specific ABC in μ-transgenic mice, 10^7 spleen cells from normal (Xn) and *xid* TG$^+$ and TG$^-$ mice were rosetted with a 2% suspension of PC-SRBC and the ABC counted on a hemocytometer. The data in Table III show that 4% of the spleen cells in normal TG$^+$ F1 female mice (11% of the B cells) formed rosettes with the PC-SRBC. Such PC-binding cells were greatly reduced in TG$^+$ *xid* males. In fact, there was no difference in the number of ABC found in the TG$^+$ *xid* males and the TG$^-$ controls. Greater than 90% of the ABC in TG$^+$ normal mice were inhibited with 5 × 10^{-3} M PC, whereas <10% of the ABC in the TG$^+$ *xid* males or the TG$^-$ controls were PC inhibitable. The loss of PC-specific B cells in the TG$^+$ *xid* mice

TABLE III

Phosphocholine-specific Ag-binding cells in M167 μ-transgenic mice[a]

	Phenotype			
	T^-Xn	T^+Xn	T^+Xid	T^-Xid
Percent ABC	0.5	4.0	0.6	0.7
Percent AB B cells	0.9	11.0	2.1	2.3
ABC/Sp × 10^{-5}	4.2	37.0	4.7	6.8

[a] Spleen cells from (B6.CBA/N × μ-243-4)F1 normal female (Xn) and immune defective male (*xid*) mice were adjusted to 1 × 10^7/ml; 200 μl of spleen cells were rosetted with 100 μl of 2% PC-SRBC as previously described (5). ABC were counted on a hemocytometer and the data normalized to the number of B cells present and to the absolute number of spleen cells. The high background observed in the rosette assay is due to non-specific binding, binding of SRBC determinants or nitrophenylphosphocholine-SRBC-determinants because <10% of the ABC in T^- mice were PC inhibitable.

is directly correlated with a sevenfold decrease in μ^{a+} anti-PC antibody relative to the total μ^{a+} antibody in the serum of TG$^+$ *xid* F1 males as compared to the serum of normal TG$^+$ F1 females (Table IV). There is no significant difference ($p = 0.52$) in the μ^a serum levels of TG$^+$ *xid* and TG$^+$ normal mice, whereas, the μ^a anti-PC serum levels are significantly different ($p < 0.001$). The combined data on PC-ABC and serum anti-PC antibody levels strongly suggest that PC-specific B cells are being clonally deleted in these μ-only TG$^+$ *xid* mice whereas these same PC-specific B cells seem to be greatly expanded in the TG$^+$ F1 females.

DISCUSSION

We have analyzed the effect of the X-linked immunodeficiency gene (*xid*) on the development of Ag-specific B lymphocytes in two different sets of transgenic mice carrying rearranged H and L chain genes coding for anti-PC and anti-TNP antibodies, respectively. In the anti-PC transgenic mice, large numbers of TG$^+$, PC-specific B cells are detectable in the spleens of mice bearing a normal X chromosome, whereas B cells are either absent or reduced more than 90% in TG$^+$ *xid* mice. In contrast, the coexpression of the anti-TNP μ/κ transgenes with the *xid* gene does not lead to a greater decrease in absolute splenic B cells than occurs in TG$^+$ mice with a normal X chromosome; thus, a 60% reduction occurs in both types of anti-TNP transgenics compared to their respective TG$^-$ controls. To demonstrate that the loss of PC-specific B cells in the $\mu\kappa$ 207-4 anti-PC transgenic mice was due to an IgR-mediated event rather than a failure of *xid* mice to develop Ly-1$^+$ or Lyb-5$^+$ B cells, we analyzed B cell development in *xid* mice expressing a M167 μ H chain only. The data presented in Figure 4 and in Tables III and IV show that 1) the V_H1-μ^a transgene product is readily expressed in *xid* B cells, and therefore, it is not developmentally restricted to Ly-1$^+$ or Lyb-5$^+$ B cells; 2) M167-id$^+$ PC-specific B cells are clonally deleted in (B6.CBA/N × μ 243-4)F1 *xid* male transgenic mice, whereas, these PC-specific B cells are greatly expanded in the peripheral lymphoid organs of F1 female μ-transgenic mice; 3) the M167 μ H chain transgene product appears to associate preferentially with an endogenous Vκ24 L chain to produce large numbers of M167-Id$^+$ B cells in the spleens of normal TG$^+$ F1 female mice; 4) the M167-Id is restricted to B cells bearing the transgene-encoded μ^a-allotype and is not found on B cells expressing only the endogenous μ^b-allotype, thus, there is no network induced selection of endogenous V_H-Id; and, 5) T15-Id$^+$ B cells (V$_H$1:Vκ22), which normally dominate the PC repertoire, appear to be absent in these M167 μ transgenic mice despite the fact that a rearranged V_H1 transgene is expressed in these mice. These observations are consistent with the hypothesis that most PC-specific B cells are clonally deleted via an IgR-mediated mechanism after their development in the bone marrow of mice expressing the *xid* gene. The mechanism responsible for this IgR-directed clonal deletion of PC-specific B cells does not cause a similar elimination of TNP-specific B cells in the Sp6 (μ/κ) anti-TNP *xid* transgenic mice.

TABLE IV

Loss of anti-PC antibodies from serum of xid μ-transgenic mice

Antibody[a] Epitope Assayed	Mouse Phenotype			
	T$^-$Xn	T$^+$Xn	T$^+$Xid	T$^-$Xid
	μg/ml of serum antibody[b]			
IgM^a	<1	383 ± 79	210 ± 40	<1
IgM^a anti-PC	<1	13 ± 3	1 ± 0.2	<1

[a] The serum from (B6.CBA/N × μ-243-4)F1 mice was assayed in a capture ELISA for total IgM of the a-allotype (IgM^a) and in a direct binding ELISA on PC-BSA coated plates for anti-PC antibodies bearing the IgM^a-allotype.

[b] The data were derived from the pre-immune serum of 10 to 20 eight-wk-old mice of each phenotype and are shown as the mean ± SE. Standard curves for both the capture and direct binding anti-PC assay were generated using the IgM^a anti-PC hybridoma protein HPCM2 (24). TG$^+$ normal females and immune deficient *xid* males were compared using a Student's *t*-test and were not significantly different for total IgM^a antibody ($p = 0.52$) but were significantly different with respect to IgM^a anti-PC antibody ($p < 0.001$).

An extensive analysis of the bone marrow from anti-PC transgenic mice was conducted in an attempt to determine how and where the *xid* gene was acting. Although the total number of bone marrow cells in TG$^+$ normal and *xid* mice is indistinguishable from that in their TG$^-$ littermates (Table I), the flow cytometric profile of the cells present in the bone marrow of TG$^+$ mice was quite different from that of their TG$^-$ littermates. Staining of bone marrow cells with FITC-anti-μ and biotinylated-anti-B220 plus PE-streptavidin revealed three major differences between TG$^+$ and TG$^-$ mice (Fig. 3). First, when one compares the forward versus right angle (obtuse) light scatter of the TG$^+$ and TG$^-$ bone marrow, it is evident that both the normal and *xid* TG$^+$ mice have highly reduced numbers of small nucleated cells. This is similar to the earlier observations in M54 μ-transgenic mice (21, 26), and presumably results from early expression and feedback of the transgenes during B cell development. Second, the IgM$^+$-B220$^+$ B cells in the bone marrow of both types of TG$^+$ mice (Fig. 3; *panels 10* and *14*) stain less intensely for both of these surface markers than do the bone marrow B cells from the TG$^-$ mice (*panels 2* and *6*). This suggests that the IgM$^+$ bone marrow cells in TG$^+$ mice are either less mature B cells than those present in the bone marrow of the normal controls, or they represent a B cell population that has been "tolerized" and subsequently down-regulated the expression of these surface molecules. Although we favor the former explanation, Goodnow et al. (27) have observed the down regulation of sIgM expression in the HEL-specific splenic B cells of transgenic mice that express both a transgene encoding HEL and transgenes (μ/κ) encoding an anti-HEL antibody. Finally, the absolute number of both IgM$^+$-B220$^+$ B cells and IgM$^-$B220$^+$ pre-B cells in the bone marrow of both normal and *xid* TG$^+$ mice is greatly reduced (~80 to 90%) compared to the TG$^-$ controls. However, there is no significant difference between the normal TG$^+$ and *xid* TG$^+$ mice with respect to numbers of bone marrow pre-B or B cells. Thus, the substantial reduction in B cells in the peripheral lymphoid tissue of $\mu\kappa$ TG$^+$ *xid* mice must be due to a failure of migration and/or a clonal deletion of the PC-specific B cells during migration from the bone marrow to the spleen. The few B cells that manage to home to the spleens of $\mu\kappa$ TG$^+$ *xid* mice are indistinguishable from those in the normal TG$^+$ spleen with respect to the intensity of transgene encoded μ^a receptor and the expression of the V_H1 or M167-idiotopes present on the cell surface. These B cells appear to be PC specific and to function normally after immunization with a thymus-dependent Ag, PC-KLH (data not

shown). Why these few PC-specific B cells are not deleted in the $\mu\kappa$ TG$^+$ *xid* mice is not clear. They are not Ly-1$^+$ B cells and do not appear to be Lyb-5$^+$ B cells, since they do not respond to the PC-carbohydrate antigen on *Staphylococcal pneumoniae* (data not shown). However, there appears to be a higher percentage of double-allotype positive B cells (55%) in those TG$^+$ *xid* mice that express endogenous μ^b than in the normal TG$^+$ mice (18%) (18). This simultaneous expression of endogenous IgM^b may reduce the functional number of PC-specific receptors per B cell and thus provide these double-allotype positive B cells with a selective survival advantage. Having a lower number of functional receptors per cell may prevent a TG receptor-directed clonal deletion mechanism from functioning on these double-allotype positive cells. Goodnow et al. (28) have demonstrated that the number of occupied IgR on a B cell is critical in determining whether or not that B cell will be tolerized. However, the fact that some B cells expressing only the M167 transgene product are also surviving in the *xid* mice, and that these B cells appear to function normally when stimulated in vivo with PC-KLH, can not be accounted for by expression of lower numbers of functional receptors per cell.

When there is no detectable difference in B cell development or the expression of the transgene-encoded anti-PC receptor in the bone marrow of TG$^+$ *xid* and normal mice, how can one account for the apparent "IgR-directed" clonal deletion of the B cells in the *xid* transgenic mice when large numbers of TG$^+$ B cells are homing to the spleens of mice carrying the normal X chromosome? One possible explanation might be that TG$^+$ *xid* mice, like all *xid* mice, fail to develop significant numbers of either Lyb-5$^+$ or Ly-1$^+$ B cells, and the Lyb-5$^-$ cells that develop in their bone marrow are tolerized by either autoantigen, an environmental PC-containing Ag, or by anti-idiotypic interactions. We have previously shown that the majority of T15$^+$, anti-PC-specific splenic B cells reside in the Lyb-5$^+$ B cell subset inasmuch as they can be eliminated by treatment with anti-Lyb-5 antiserum and complement (15, 16). Stein et al. (29) similarly demonstrated that anti-dextran-specific B cells resided in the Lyb-5$^+$ B cell subset, and they suggested that the majority of B cells capable of responding to any polysaccharide determinant reside in this subset. Stall et al. (30) and Hayakawa and Hardy (31) also suggested that PC-specific, dextran-specific, and other B cells specific for carbohydrate determinants may be derived almost exclusively from the Ly-1 B cell lineage. The data in Figure 4 demonstrate that the V_H1 H-chain transgene product is expressed in large numbers of B cells in *xid* μ-transgenic mice; however, PC-specific B cells are still clonally eliminated. These data show that the V_H1 H-chain can be expressed in the Lyb-5$^-$ B cells of *xid* mice and it is not developmentally restricted to the Lyb-5$^+$ or Ly-1 lineage of B cells. It is only the V_H1, PC-specific B cells that are functionally restricted to these B cell subsets in normal mice because the PC-specific Lyb-5$^-$ B cells are clonally deleted.

According to our working hypothesis, the exit of Lyb-5$^+$ B cells from the bone marrow and/or the subsequent expansion of this subset of B cells in the peripheral lymphoid organs may depend on the interaction of the anti-PC, transgene-encoded receptor with ligand in the presence of soluble T cell factors but in the absence of cognate T cell help. The interaction of Lyb-5$^+$ or Ly-1$^+$, TG$^+$ B cells with a relevant ligand such as a TI-2-like environmental PC-containing Ag, autologous PC, or anti-Id-bearing B cells in the presence of T cell, macrophage, or stromal cell factors would result in the release of these cells from the bone marrow and their subsequent expansion in peripheral lymphoid organs. Evidence for the in situ expansion of PC-specific B cells in spleens of normal mice was obtained in the μ-transgenic mice. Thus, there was a fivefold increase in the relative number of M167-Id$^+$ B cells in the spleens of these mice compared to the number present in the bone marrow. Inasmuch as *xid* B cells can not proliferate in response to Ag and soluble T cell factors (1, 32), they would undergo tolerance via clonal deletion unless cognate T cell help is provided. We have in fact demonstrated that a PC-specific response can be rescued from *xid* mice when they are immunized with a TD PC-Ag that lacks the diazophenyl-tyrosine rings and thus prevents the induction of the high affinity phenylphosphocholine-specific B cells that dominate the secondary response to PC-KLH (33) (J. J. Kenny, G. Guelde, and D. L. Longo, manuscript in preparation).

Our observation that the PC-specific B cells in normal μ transgenic mice bear the M167-Id rather than the normally dominant T15-Id strongly suggests that the B cells in which the M167 H chain has associated with a Vκ24 L chain make the best PC-specific antibody and are therefore clonally expanded. Inasmuch as B cells having the M167 μ-transgene associated with a Vκ22 (T15) L chain were not observed, we assumed this H:L combination did not give rise to a PC-specific antibody. We have recently tested this hypothesis by cotransfecting the M167 V_H1 μ-H chain gene and the Vκ22 L chain gene and found that a $\mu\kappa$ T15-Id$^+$ antibody is produced by the transfected cells, however, this antibody does not bind PC (J. J. Kenny, C. Moratz, C. O'Connell, M. Beckwith, J. L. Claflin, and D. L. Longo, manuscript in preparation). Furthermore, these H:L gene cotransfection studies show that the only V_H1:VκH:L gene combinations that give rise to PC-specific antibodies are V_H1(T15):Vκ22, V_H1(M167):Vκ24 and V_H1(M603):Vκ8. All other V_H1:Vκ gene combinations tested gave rise to IgM V_H1-Id$^+$ antibodies but these Id$^+$ positive antibodies did not bind PC. Our observations, along with the recent findings of Claflin and collaborators (34, 35) and Feeney and colleagues (36, 37) showing that both M603-like and M167/511-like anti-PC antibodies are generated via somatic mutation or junctional diversification of the V_H1 gene, could provide new insight into the reason for T15-Id dominance in normal mice.

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REFERENCES

1. Scher, I. 1982. CBA/N immune defective mice: evidence for the failure of a B cell subpopulation to be expressed. *Immunol. Rev.* 64:117.
2. Mond, J. J., R. Lieberman, J. K. Inman, D. E. Mosier, and W. E. Paul. 1977. Inability of mice with a defect in B-lymphocyte maturation to respond to phosphorylcholine on immunogenic carriers. *J.*

Exp. Med. 146:1138.
3. **Quintans, J.** 1977. The "patchy" immunodeficiency of CBA/N mice. *Eur. J. Immunol. 7:749.*
4. **Huber, B. R., R. K. Gershon, and H. Cantor.** 1977. Identification of a B cell surface structure involved in antigen-dependent triggering: absence of the structure on B cells from CBA/N mice. *J. Exp. Med. 145:10.*
5. **Ahmed, A., I. Scher, S. O. Sharrow, A. H. Smith, W. E. Paul, D. H. Sachs, and K. W. Sell.** 1977. B lymphocyte heterogeneity. Development and characterization of an alloantiserum which distinguishes B-lymphocyte differentiation alloantigens. *J. Exp. Med. 145:101.*
6. **Subbarao, B., A. Ahmed, W. E. Paul, I. Scher, R. Lieberman, and D. E. Mosier.** 1979. Lyb-7, a new B cell alloantigen controlled by genes linked to the IgC_H locus. *J. Immunol. 122:2279.*
7. **Hayakawa, K., R. R. Hardy, A. M. Stall, and L. A. Herzenberg.** 1983. The "Ly1 B" cell subpopulations in normal, immunodefective and autoimmune mice. *J. Exp. Med. 157:202.*
8. **Reid, G. K., and D. G. Osmond.** 1985. B lymphocyte production in the bone marrow of mice with x-linked immunodeficiency (xid). *J. Immunol. 135:2299.*
9. **Kincade, P. W., H. Jyonouchi, K. S. Landreth, and G. Lee.** 1982. B lymphocyte precursors in immunodeficient, autoimmune and anemic mice. *Immunol. Rev. 64:81.*
10. **Karagogeos, D., N. Rosenberg, and H. Wortis.** 1986. Early arrest of B cell development in nude, X-linked immune-deficient mice. *Eur. J. Immunol. 16:1125.*
11. **Sprent, J., J. Bruce, Y. Ron and S. R. Webb.** 1985. Physiology of B cells in mice with x-linked immunodeficiency. I. Size, migratory properties, and turnover of the B cell pool. *J. Immunol. 134:1442.*
12. **De La Hera, A., M. A. R. Marcos, M. L. Toribio, C. Marquez, M. L. Gaspar, and C. Martinez-A.** 1987. Development of Ly-1 B cells in immunodeficient CBA/N mice. *J. Exp. Med. 166:804.*
13. **Klinman, N. R., and M. R. Stone.** 1983. Role of variable region gene expression and environmental selection in determining the anti-phosphorylcholine B cell repertoire. *J. Exp. Med. 158:1948.*
14. **Wicker, L. S., G. Guelde, I. Scher, and J. J. Kenny.** 1982. Antibodies from the Lyb-5$^-$B cell subset predominate in the secondary IgG response to phosphocholine. *J. Immunol. 129:950.*
15. **Wicker, L. S., G. Guelde, I. Scher., and J. J. Kenny.** 1983. The asymmetry in idiotype-isotype expression in the response to phosphocholine is due to divergence in the expressed repertoires of Lyb-5$^+$ and Lyb-5$^-$ B cells. *J. Immunol. 131:2468.*
16. **Kenny, J. J., L. J. Yaffe, A. Ahmed, and E. S. Metcalf.** 1983. Contribution of Lyb 5$^+$ and Lyb 5$^-$ B cells to the primary and secondary phosphocholine-specific antibody response. *J. Immunol. 130:2574.*
17. **Storb, U., C. Pinkert, B. Arp, P. Engler, K. Gollahon, J. Manz, W. Brady, and R. L. Brinster.** 1986. Transgenic mice with μ and κ genes encoding antiphosphorylcholine antibodies. *J. Exp. Med. 164:627.*
18. **Kenny, J. J., F. Finkelman, F. Macchiarini, W. C. Kopp, U. Storb, and D. L. Longo.** 1989. Alteration of the B cell surface phenotype, immune response to PC-KLH, and the B cell repertoire in M167 mu plus kappa transgenic mice. *J. Immunol. 142:4466.*
19. **Kenny, J. J., G. Guelde, C. Hansen, and J. J. Mond.** 1987. Synergistic effects of the xid gene in X-chromosome congenic mice. Inability of C3.CBA/N mice to respond to thymus dependent antigens in adoptive transfer assay. *J. Immunol. 138:1363.*
20. **Rusconi, S., and G. Kohler.** 1985. Transmission and expression of a specific pair of rearranged immunoglobulin μ and κ genes in a transgenic mouse line. *Nature 314:330.*
21. **Stall, A. M., F. G. M. Kroese, F. T. Gadus, D. G. Sieckmann, L. A. Herzenberg, and L. A. Herzenberg.** 1988. Rearrangement and expression of endogenous immunoglobulin genes occur in many murine B cells expressing transgenic membrane IgM. *Proc. Natl. Acad. Sci. USA 85:3546.*
22. **Lamers, M. C., M. Vakil, J. F. Kearney, J. Langhorn, C. J. Paige, M. H. Julius, H. Mossmann, R. Carsetti, and G. Kohler.** 1989. Immune status of a μ,κ transgenic mouse line. Deficient response to bacterially related antigens. *Eur. J. Immunol. 19:459.*
23. **Desaymard, C., A. M. Giusti, and M. D. Scharff.** 1984. Rat anti-T15 monoclonal antibodies with specificity for V_H and V_H-V_L epitopes. *Mol. Immunol. 21:961.*
24. **Gearhart, P. J., N. D. Johnson, R. Douglas, and L. Hood.** 1981. IgG antibodies to phosphorylcholine exhibit more diversity than their IgM counterparts. *Nature 291:29.*
25. **Ashman, R. F.** 1973. Lymphocyte receptor movement induced by sheep erythrocyte binding. *J. Immunol. 111:212.*
26. **Herzenberg, L. A., A. M. Stall, J. Braun, D. Weaver, D. Baltimore, L. A. Herzenberg, and R. Grosschedl.** 1987. Depletion of the predominant B cell population in immunoglobulin μ heavy-chain transgenic mice. *Nature 324:71.*
27. **Goodnow, C. C., J. Crosbie, S. Adelstein, T. B. Lavoie, S. J. Smith-Gill, R. A. Brink, H. Pritchard-Briscoe, J. S. Wotherspoon, R. H. Loblay, K. Raphael, R. J. Trent, and A. Basten.** 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature 334:676.*
28. **Goodnow, C. C., J. Crosbie, H. Jorgensen, R. A. Brink, and A. Basten.** 1989. Induction of self-tolerance in mature peripheral B lymphocytes. *Nature 342:385.*
29. **Stein, K. E., D. A. Zopf, C. B. Miller, B. M. Johnson, P. K. A. Mongini, A. Ahmed, and W. E. Paul.** 1983. The immune response to a thymus-dependent form of B512 dextran requires the presence of Lyb-5$^+$ lymphocytes. *J. Exp. Med. 157:657.*
30. **Stall, A. M., P. A. Lalor, L. A. Herzenberg, and L. A. Herzenberg.** 1986. Ly-1 B cells and autoantibodies. *Ann. Inst. Pasteur (Immunol.) 137D:173.*
31. **Hayakawa, K., and R. R. Hardy.** 1988. Normal, autoimmune, and malignant CD5$^+$ B cells: The Ly-1 B lineage? *Annu. Rev. Immunol. 6:197.*
32. **Mond, J. J., and M. Brunswick.** 1987. A role for IFN-γ and NK cells in immune responses to T cell regulated antigens type 1 and 2. *Immunol. Rev. 99:105.*
33. **Chang, S. P., M. Brown, and M. B. Rittenberg.** 1982. Immunologic memory to phosphorylcholine. II. PC-KLH induces two antibody populations that dominate different isotypes. *J. Immunol. 128:702.*
34. **Claflin, J. L., J. Berry, D. Flaherty, and W. Dunnick.** 1987. Somatic evolution of diversity among anti-phosphocholine antibodies induced with *Proteus morganii*. *J. Immunol. 138:3060.*
35. **Claflin, J. L., J. George, C. Dell, and J. Berry.** 1989. Patterns of mutations and selection in antibodies to the phosphocholine-specific determinant in *Proteus morganii*. *J. Immunol. 143:3054.*
36. **Feeney, A. J., S. H. Clarke, and D. E. Mosier.** 1988. Specific H chain junctional diversity may be required for non-T15 antibodies to bind phosphorylcholine. *J. Immunol. 141:1267.*
37. **Feeney, A. J., and D. J. Thuerauf.** 1989. Sequence and fine specificity analysis of primary 511 anti-phosphorylcholine antibodies. *J. Immunol. 143:4061.*
38. **Moore, W., and R. Kautz.** 1986. Data analysis for flow cytometry. In *Handbook of Experimental Immunology, 4th ed.* D. M. Weir, L. A. Herzenberg, C. C. Blackwell, and L. A. Herzenberg, eds. Blackwell Scientific Pub., Ltd., Edinburgh, Scotland.