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CELLULAR AND MOLECULAR ASPECTS OF IMMUNOGLOBULIN ISOTYPE SWITCHING

by

Carol Frances Webb

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the Department of Microbiology in
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During differentiation, B cells may switch from the synthesis of IgM to the production of IgG, IgA or IgE antibodies with the same antigen specificity. IgM monomers are composed of two light chain polypeptides and two μ heavy chain polypeptides. The μ heavy chain mRNA is transcribed from four gene segments, three of which encode the variable region portion of the molecule which binds to antigen, while the fourth gene segment encodes the constant region (C_H) of all IgM molecules. Isotype switching involves the expression of the same variable region genes involved in μ production with new constant region genes for γ, α or ε. The exact cellular and molecular mechanisms by which isotype switching occurs are still controversial. The objective of the studies described in this dissertation has been to attain a better understanding of both the cellular and molecular events involved in isotype switching.

More specifically, experiments were designed to determine whether isotype switching occurs directly from IgM to each of the other isotypes rather than in a sequential manner following the order of the C_H genes. Anti-γ_3 treatment of mouse spleen cells was capable of suppressing the mitogen-induced differentiation of IgG_3-producing plasma cells, but had no effect upon the other IgG subclasses, while

anti-μ treatment successfully suppressed the differentiation of cells of all isotypes. These results show that expression of surface IgG_3 is not required as an intermediary step in switching from IgM to the other IgG subclasses, and is consistent with a direct model for isotype switching.

The remainder of the studies address the question of whether C_H genes 5' to the expressed C_H gene are detected in human cells which have already undergone isotype switching. When a panel of Epstein-Barr virus transformed human cell lines producing IgM, IgG_1, IgG_3, IgG_4 and IgA_1 antibodies was examined with human heavy chain gene probes, deletion of C_H genes was observed in every instance. However, the extent of the deletions on the nonfunctional homologous chromosome was variable. These data show that the rearrangements which occur in C_H genes on homologous chromosomes are not coordinately regulated.

Abstract Approved by: Committee Chairman Mary D. Cooper

Program Director Richard D. Cole

Date 5/31/85 Dean of Graduate School R. D. Koozer

For my parents, whose continued belief in me
helped me to believe in myself.

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LIST OF ABBREVIATIONS

C_H - heavy chain constant region gene segment

D - diversity region gene segment

EBV - Epstein-Barr virus

ELISA - enzyme-linked immunosorbent assay

Ig - immunoglobulin

J_H - heavy chain joining region gene segment

kb - kilobases

LPS - lipopolysaccharide

S - switch region

sIg - surface immunoglobulin

USCE - unequal sister chromatid exchange

V_H - heavy chain variable region gene segment

I. INTRODUCTION

The phenomenon of immunoglobulin (Ig) heavy chain isotype switching was discovered many years ago, but many of the cellular aspects and molecular mechanisms involved in the switching process are still incompletely understood. In fact, they have become quite controversial. Isotype switching is preceded by discrete and well-characterized stages in B cell development, and we now know that many of these stages are accompanied by discrete molecular rearrangements in the immunoglobulin genes. For these reasons, isotype switching has become a central issue not only for cellular immunologists, but for molecular biologists who view this system as a good model for studying differentiation and gene expression. A thorough review of the literature pertaining to isotype switching would fill volumes, so this discussion will be limited to aspects of switching which are directly related to the studies described in the following chapters.

Although it is clear that B cells arise first from stem cell precursors located in the fetal liver and later in the adult bone marrow (1-4), the earliest cell which is readily identifiable as a member of the B cell lineage is the pre-B cell. Phenotypically, the pre-B cell is characterized by a large nucleus, sparse cytoplasm and the presence of μ heavy chains in the cytoplasm (5,6). These cells produce no light chains and express no immunoglobulin on the cell

surface. As pre-B cells mature, they begin to synthesize either κ or λ light chains which may then be combined with μ heavy chains to produce a complete IgM antibody. The expression of this molecule on the cell surface as a membrane antigen characterizes the next stage of B cell differentiation. At some time during their differentiation surface (s) IgD is also expressed, and eventually these $sIgM^+sIgD^+$ B cells may begin to express sIgG, sIgA, or sIgE antibodies. This defines the isotype switch at the cellular level. These sIg^+ cells eventually mature into plasma cells which have a large cytoplasm and an eccentric nucleus, and which secrete large amounts of a single Ig isotype.

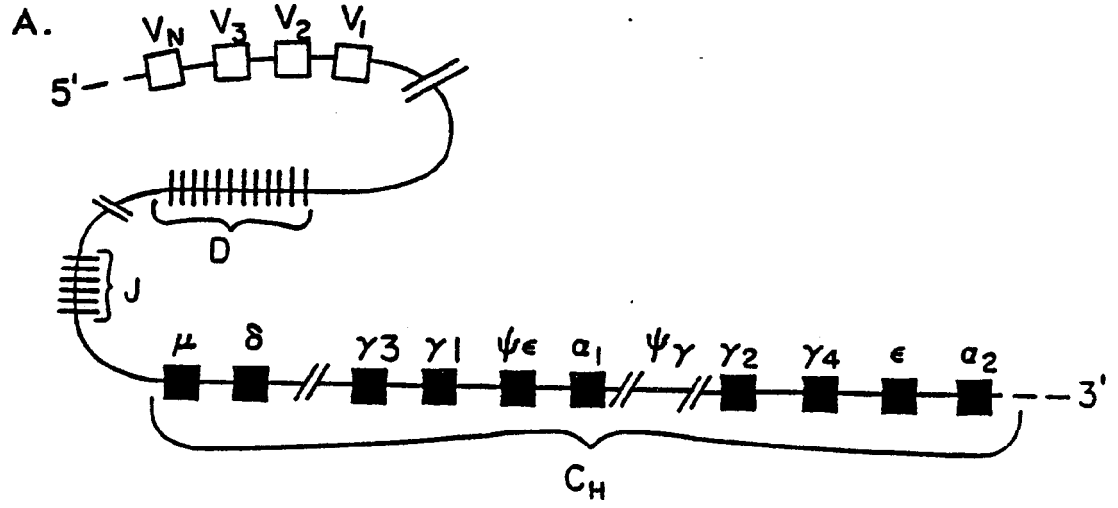
The ability of cells to switch isotypes and to produce antibodies of different isotypes with the same antigen specificity (7) yields a measure of diversity at the functional level. For example, IgM plays an important role in complement fixation, while IgG antibodies are much more readily transported across placental membranes. IgA antibodies, on the other hand, play an important role in mucosal immunity, as demonstrated by individuals who are IgA-deficient and are much more susceptible to certain types of infections than are other individuals (8). And finally, IgE antibodies not only play an important role in allergic reactions, but are also thought to be important in defense mechanisms against intestinal parasites.

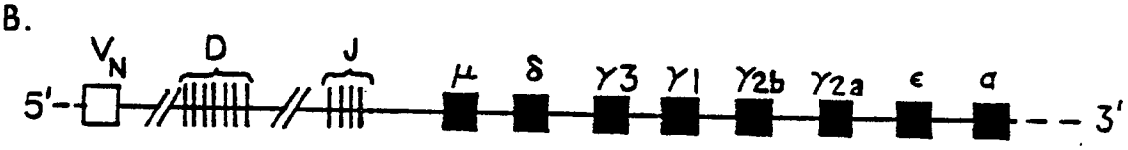
The fact that IgM-bearing B cells are the immediate precursors of B cells producing each of the other heavy chain isotypes has been demonstrated in several model systems. Ontogenetic studies in the avian system have demonstrated that B cells develop in the bursa of Fabricius in a sequential order, with IgM^+ cells arising first,

followed by IgG- and IgA-bearing cells (9,10). These sIg^+ B cells are then seeded into the peripheral tissues in the same order (11,12). The differentiation of cells producing IgG and IgA antibodies has been successfully blocked in studies which have involved removal of the bursa at nineteen days of embryogenesis, before IgG and IgA cells have developed, as well as in studies in which anti-μ antibodies have been used to block B cell differentiation (13,14). Similarly, in both the mouse and the human, the development of IgM-bearing B cells precedes the development of IgG- and IgA-bearing B cells in the fetal liver and bone marrow (1-4,15,16), and in vivo suppression studies have shown that anti-μ antibodies inhibit the differentiation of IgG- and IgA-bearing murine B cells (17-19). Parallel studies in vitro made use of the ability of lipopolysaccharide (LPS) to induce plasma cells producing all isotypes from adult mouse spleen cells, and demonstrated that the addition of anti-μ to such cultures inhibited the induction of plasma cells producing γ and α isotypes as well as μ (20,21).

Due to the recent advances in molecular biology, we now know a lot more about the genetic events involved in producing a μ heavy chain and leading up to the process of isotype switching. The mouse heavy chain constant region genes (Fig. 1) are located on the long arm of chromosome 12 in the order 5' $C\mu$-$C\delta$-$C\gamma_3$-$C\gamma_1$-$C\gamma_{2b}$-$C\gamma_{2a}$-$C\varepsilon$- $C\alpha$ 3' (22-25), while the human heavy chain constant region genes are found on the long arm of chromosome 14 in the order 5' $C\mu$-$C\delta$-$C\gamma_3$-$C\gamma_1$-$\psi\varepsilon$-$C\alpha_1$-$\psi\gamma$-$C\gamma_2$-$C\gamma_4$-$C\varepsilon$-$C\alpha_2$ 3' (26-29). In both mice and humans the μ heavy chain is encoded by four separate gene segments which undergo a series of rearrangements to allow transcription of μmRNA. These gene segments are located in a 5' to 3' direction as

Figure 1. A. Human and murine heavy chain immunoglobulin genes. (A) Human (B) Murine.


A.
V_N V_3 V_2 V_1
5'
D
J
μ δ γ3 γ1 ψε α_1 ψγ γ_2 γ_4 ε α_2
3'
C_H


B.
V_N D J
5'
μ δ γ3 γ1 γ2b γ2a ε α
3'

follows: a variable (V_H) region, a diversity (D) region, a joining (J_H) region and a constant (C_H) region gene segment (Cμ in the case of the μ heavy chain). It is now believed from studies using Abelson virus-transformed fetal liver cells that the first event which occurs in B lineage cells before the pre-B cell stage is the rearrangement of a particular D region exon with a specific J region exon (30). The next event which is thought to occur is the juxtaposition of a particular V region gene segment with the rearranged DJ gene segments, yielding a functional VDJ gene which may be transcribed with the Cμ gene and eventually translated to produce a μ heavy chain. The production of light chains, which defines the next stage in B cell differentiation, is accomplished in an analogous manner; however, there is no known D region segment for the κ or λ light chains in either mouse or humans (31,32). Isotype switching, at the genetic level, occurs when the same VDJ region genes involved in producing a functional μ heavy chain are expressed with a different C_H region gene segment. Thus, antigen specificity is retained in association with a new constant region domain.

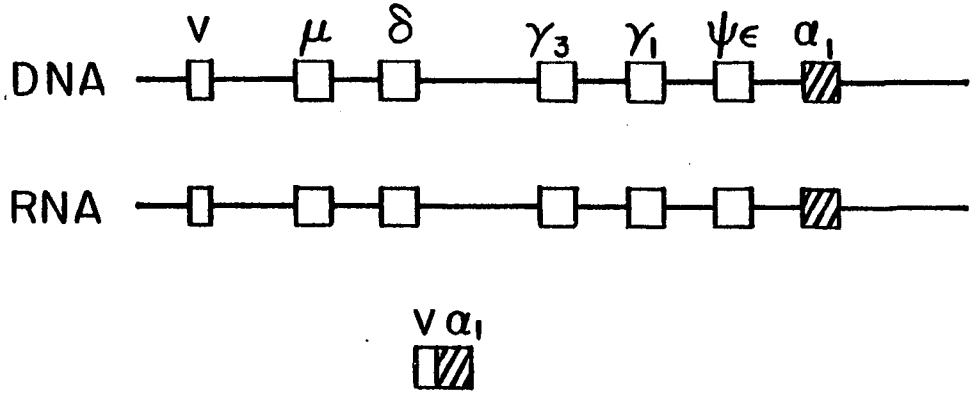
Although mammals are diploid and possess two copies of each of the C_H genes, normal B cells undergo allelic exclusion such that the C_H genes of only one chromosome are expressed in a single cell (33, 34). It has been hypothesized that rearrangements proceed on one chromosome first, and then on the other, until a functional VDJ exon is produced on one chromosome, and that this may be related to allelic exclusion. In fact, it is possible to find examples of abortive rearrangements of the C_H genes on the non-expressed chromosome (35,36).

The exact molecular mechanisms which allow the expression of a rearranged VDJ gene segment with a new C_H region gene are still controversial. Several mechanisms have been proposed (reviewed in 37). These may be divided into two major groups, although many variations and combinations of these mechanisms have also been suggested.

The first basic model is that of RNA processing (Fig. 2). This model proposes that transcription can proceed from the VDJ gene through C_H genes 3' to $C\mu$, forming a transcript which may then be processed to yield a functional mRNA with the new isotype. Although there is good evidence that the coexpression of IgM and IgD on B cells occurs through the cotranscription of $C\mu$ with $C\delta$ (38-40), there are several problems involved in proposing such a model for switching to the more distal C_H genes. The primary problem is one of size. The heavy chain genes in both mouse and humans are thought to span some 180 kilobases (kb) of DNA (22,26), necessitating a transcript of equivalent size in isotype switches to the most 3' C_H genes. The technical problems involved in isolating such a transcript, should it exist, are of course, immense; however, there have been reports of transcripts of increasingly larger sizes from several different systems. For example, the bithorax gene complex in Drosophila may require the transcription of exons which are 70 kb apart (41). It is also possible to envision several variations on an RNA processing model. For example, the physical configuration of a chromosome could be altered in a three-dimensional manner, perhaps by loop formation, such that two distant genes might be brought in close proximity allowing the transcription of both genes, or splicing of two separate messages from different regions of DNA could occur, or one could even imagine a discontinuous transcription mechanism (37).

Figure 2. Hypothetical model for immunoglobulin heavy chain switching by RNA processing.

RNA PROCESSING



The other major model for isotype switching suggests that C_H genes are rearranged in a similar manner to DJ and VD joining such that deletion of intervening DNA sequences is required. Thus, a mouse cell which had switched from IgM to IgA production would have deleted Cμ, Cδ, Cε and all of the Cγ genes. In fact, there is a large quantity of evidence obtained largely from studies using mouse plasmacytomas and myelomas and from a few studies performed using human chronic lymphocytic leukemia cells, which supports a deletion model of switching (42-45). Other studies (46) in which LPS-stimulated mouse splenic B cells expressing $sIgG_3$ were shown to delete both copies of Cμ over a nine-day period have also supported this model.

Sequence analyses of the DNA 5' to the coding regions for the C_H genes have provided a clue as to how such a recombination and deletion process might occur. At approximately 1.5 kb 5' to each C_H gene except Cδ, there exist regions of tandemly repeated DNA sequences which have been called switch (S) regions or switch sites. These S regions span from 2-5 kb and are made up of interspersed regions of GAGGT, YAGGTTG-like and GGGGT sequences (47-49). The μ switch region (Sμ) is homologous, but not identical, to each of the other S regions. Thus, recombination and switching from Cμ to another C_H isotype could be mediated through the pairing of these homologous sequences, and the existence of isotype-specific switch enzymes which could differentially recognize the S-sequences has also been proposed (50,51).

The deletion process itself might be explained by either of two mechanisms, both of which could make use of recombination between S and another S sequence. The first mechanism is that of unequal sister chromatid exchange (USCE) (52,53). After DNA replication has occurred

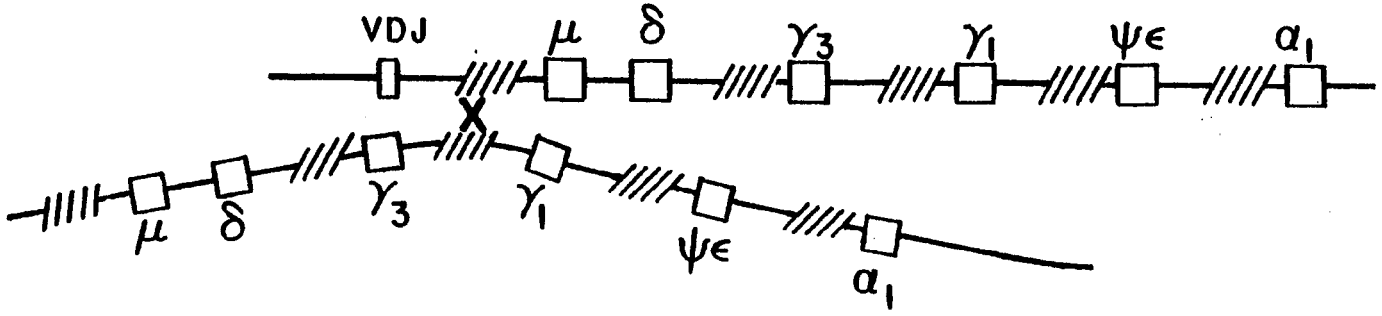
and the two sister chromatids are still joined together at the centromere, one can envision that a Sμ region might recombine with a Sγ or Sα region from the other sister chromatid (Fig. 3). This process would give rise to one sister chromatid containing a functional VDJ-Cγ or -Cα gene, having deleted all of the intervening C_H genes, and another sister chromatid with duplicate copies of Cμ, Cδ and the intervening C_H genes which were lost from the other sister chromatid. Upon cell division, these two sister chromatids would assort into separate progeny cells, giving rise to two separate populations of cells only one of which would be expected to produce the new isotype. This theory has been difficult to test, and absolutely requires that DNA replication accompany each switching event. Studies in which inhibitors of DNA synthesis were added to immature B cells did not prevent isotype switching (20), suggesting that USCE is not the general mechanism for isotype switching, but not ruling out the possibility of its occasional occurrence.

Recombination might also occur in an analogous manner between chromosomal homologs. Although this type of switching has occurred in a few hybridoma cell lines (54), such a model predicts that allotype changes would frequently accompany isotype switches. Serum immunoglobulin analyses in rabbits in which allotypes have been well-defined, have indicated that this is usually not the case (55,56).

The other mechanism by which deletion of C_H genes might occur is by intrachromosomal recombination (Fig. 4) within the switch sites, involving a three-dimensional change in the DNA, i.e., loop formation (reviewed in 37). The DNA in the loop might be either deleted from the chromosome and eventually lost from the genome if it is not

Figure 3. Hypothetical model for immunoglobulin heavy chain switching by unequal sister chromatid exchange. Enclosed boxes signify C_H genes which remain from the original sister chromatid. Slashed lines represent switch sequences.

SISTER CHROMATID EXCHANGE



Progeny Cells

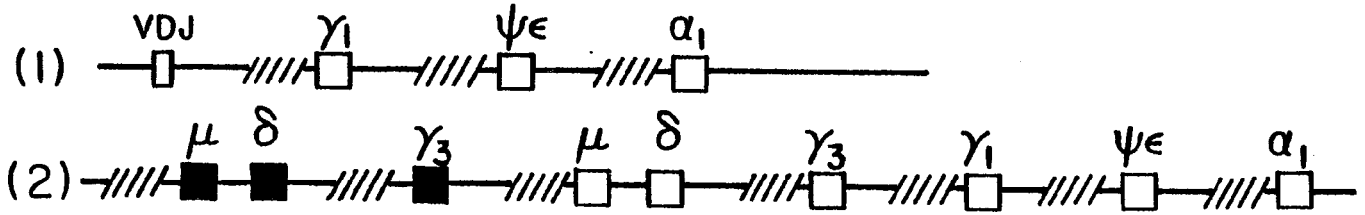
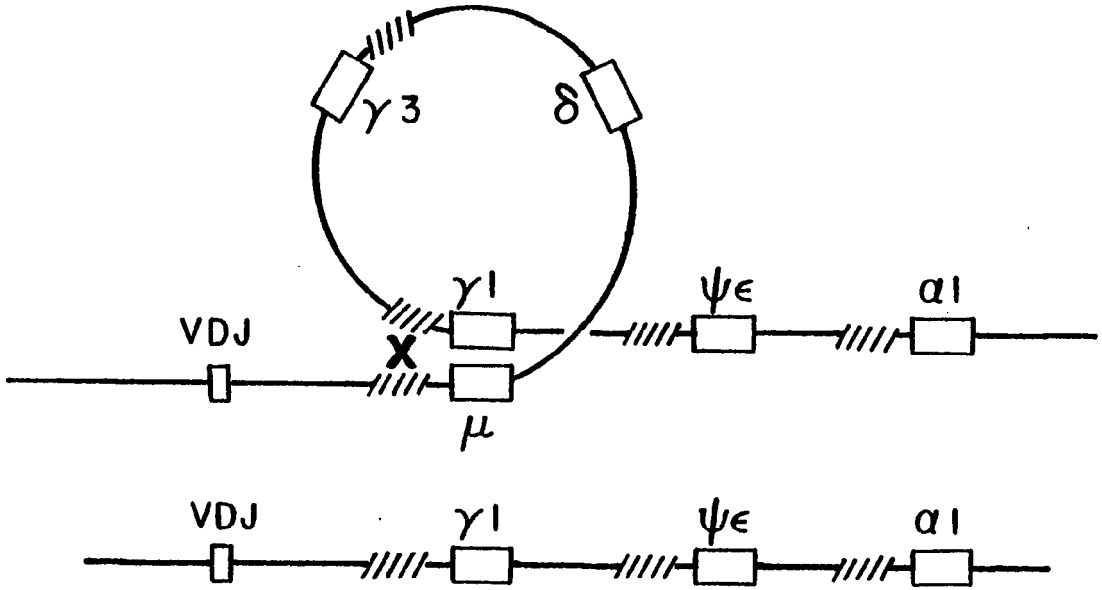


Figure 4. Hypothetical model for immunoglobulin heavy chain switching by intrachromosomal deletion.

DELETION



replicated, it could be retained in an episomal configuration, or it could even be reintegrated elsewhere in the genome. Recent evidence has indicated that reintegration of Cμ may have occurred in an Abelson virus-transformed pre-B cell line which has switched from μ to γ_{2b} synthesis while retaining a copy of Cμ which is not juxtaposed with a J_H sequence (57). Several variations of the deletion mechanism are also conceivable, involving either one or both strands of DNA, as well as one or both chromosomes (37).

In order to explain the presence of multiple isotypes on a single B cell, it has been proposed that the switching process might, in fact, utilize both an RNA processing and a deletion mechanism at separate stages in differentiation (58,59). Thus, RNA processing might be involved at the B cell level, followed by deletion of the intermediate C_H genes when cells matured to a plasma cell stage in which a more efficient method would be advantageous in synthesizing the larger quantities of Ig produced by these cells for secretion. In fact, it is well-established that IgD is co-expressed with IgM by differential RNA processing (40), and the few cases of IgD-producing myelomas which have been examined do show deletion of Cμ genes (38,39, 60). However, IgD differs from the other isotypes in several important ways. It appears to be expressed as a sIg in early stages of all resting B cells, and then is lost when these cells are stimulated to differentiate (15,40). On the other hand, once the other isotypes appear as sIg molecules, they continue to be expressed as membrane proteins and later as secretory proteins as well. Furthermore, the Cδ gene has no switch region sequences, implying that different mechanisms might be important in the regulation of IgD production versus the production of other C_H isotypes.

Early studies which utilized the Abelson virus-transformed murine pre-B cell line 18-81 suggested that RNA processing might play a role in isotype switching in early B cell development (61), but subsequent studies using subclones obtained from this line did not bear out this conclusion (62). Results from this and other Abelson-transformed cells lines showed that switching occurred by deletion of Cμ (57,62, 63), even though Cμ could be retained and integrated elsewhere in the genome (57). Yaoita et al. (58) have proposed that RNA processsing occurs at the B cell level in normal cells which co-express IgM and IgE since deletion of Cμ was not observed in these cells. However, it has since been shown that at least a large number of these cells did not synthesize their sIgE, but had bound exogenously produced IgE to their surfaces via Fc receptors (64). More recently, results from studies in which resting $sIgG_1^+$ mouse B cells were isolated by fluorescence activated cell sorting and examined for the presence of Cμ are also in agreement with an RNA processing model (65). Nevertheless, this model remains controversial due to the difficulties which occur in controlling the binding of cytophilic Ig to cell surfaces and Fc receptors, difficulties in ensuring against crossreactivity of antibodies, and even technical limitations which make accurate sorting for low percentages of sIg^+ cells extremely difficult.

The presence of multiple non-IgM isotypes on a single B cell surface (66), as well as the production of multiple isotypes in clones of B cells statistically derived from one cell, has raised another question concerning the regulation of isotype switching. It has been suggested that switching might occur in a sequential manner following the order of the C_H genes (67-71). Alternatively, it has also been

proposed that switching occurs directly from IgM to each of the other isotypes, and there is evidence to support this mechanism as well (72-75). Studies using the splenic focus assay and limiting dilution analyses of statistical clones have shown a correlation between the number of clones producing multiple isotypes and the order of the C_H genes (69,76,77). These data imply that switching could occur in a sequential fashion through the C_H genes. It is difficult, however, to be certain that the clones described in these studies have arisen from a single B cell precursor. On the other hand, studies in which mouse or human cells were suppressed with anti-isotype reagents, provided evidence for direct switching, since the anti-isotype antibodies were only effective in suppressing their homologous isotypes (72,78,79). Still other evidence from surface staining analyses of IgA- and IgE-bearing cells suggests that switching may occur most often in a direct fashion from IgM directly to IgA or IgE (66).

Interestingly, the same studies have provided evidence for sequential switching within the IgG subclasses. Analyses of mouse B lymphocytes for sIgG have shown that some cells (from 5-35%) express more than one IgG isotype, indicating that multiple consecutive switches are possible within the C_H genes (66). However analyses of the IgG subclasses are complicated by the cytophilic nature of the antibodies, and parallel studies performed after acid elution of cytophilic IgG molecules and Fc receptor saturation of human IgG^+ B cells indicated that only one IgG subclass was present on a single B cell (79). Results obtained from the splenic focus assay in which the frequency of γ subclasses expressed in response to trinitrophenyl-ficoll was examined also showed that the frequency of the γ subclasses

could be correlated with the Cγ gene order, suggesting that γ_3 (the most 5' Cγ gene in the mouse) might play a pivotal role in the expression of the other γ subclasses (76). And in fact, sequence analyses of the S-regions supported this theory. The S sequence was found to be much more homologous to $S\gamma_3$ and Sα than it was to the other Sγ regions. On the other hand, the other Sγ sequences were much more homologous to $S\gamma_3$ than to the Sα and Sμ regions (49).

Scope of Thesis:

The studies described here were begun to address some of the previously discussed controversies about isotype switching. The first experiments have been published in the European Journal of Immunology, and were designed to address the controversy of sequential switching within the IgG subclasses versus direct switching from IgM to each of the IgG isotypes. Results from these studies are described in Chapter II.

The following sections of this dissertation consist of data which have been recently accepted for publication in the Proceedings of the National Academy of Science, a short chapter which contains related material, and additional information pertaining to these studies in the form of appendices. Chapters III and IV are devoted to the development of a panel of Epstein-Barr virus (EBV)-transformed cell lines which produce IgM, IgG and IgA antibodies, as well as one Ig^- line, and the systematic analyses of the C_H gene rearrangements which have occurred in each of these cell lines. Although deletion had been clearly demonstrated in examples of murine isotype switching when these studies were begun, very little data existed concerning the

human system. These studies were undertaken to examine the EBV cell lines for examples of spontaneous isotype switching, to determine whether deletion of C_H genes 5' to the expressed C_H gene occurred in human cells which had switched to the production of non-IgM isotypes, and to determine whether one or both chromosomes were involved in the switching process.

II. EFFECT OF ANTI-γ_3 ANTIBODIES ON IMMUNOGLOBULIN ISOTYPE EXPRESSION IN LIPOPOLYSACCHARIDE-STIMULATED CULTURES OF MOUSE SPLEEN CELLS*

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Abbreviations: C_H: heavy chain constant region Ig: immunoglobulin LPS: lipopolysaccharide FITC: fluorescein isothiocyanate RITC: tetramethylrhodamine isothiocyanate cpm: counts per minute

Abstract

To test the hypothesis that γ_3 is the pivotal isotype for sequential heavy chain switching from μ to each of the γ isotypes, we have compared the effects of anti-γ_3 and anti-μ antibodies on the expression of immunoglobulin isotypes in lipopolysaccharide (LPS)-stimulated cultures of mouse spleen cells. IgM, IgG_1, IgG_{2b}, and IgG_{2a}-containing plasma cells were enumerated by immunofluorescence, and secreted immunoglobulins were measured by radioimmunoassay. Although anti-γ_3 and anti-μ were equally effective in inhibiting the LPS-induced differentiation of IgG_3 plasma cells, anti-γ_3 had no effect on the differentiation of IgM, IgG_1, IgG_{2b}, or IgG_{2a} plasma cells. These results support a direct mechanism of heavy chain immunoglobulin switching.

1 Introduction

The fact that IgM B cells give rise to cells synthesizing other immunoglobulin (Ig) isotypes was established more than a decade ago (1,2), but the mechanism whereby B cells undergo switches in expression of immunoglobulin heavy chain genes remains controversial. Heavy chain constant region (C_H) genes have been located in mice on the twelfth chromosome, 3' to the variable region complex in the order: μ, δ, γ_3, γ_1, γ_{2b}, γ_{2a}, ε, and α (3-7). Plasma cell myelomas synthesizing non-IgM isotypes lack the $C\mu$ and other 5' C_H genes on either one or both chromosomes (8-10). Isotype switches from $C\mu$, which is transcribed with $C\delta$ (5), directly to each of the downstream C_H genes have been suggested in some studies (11-15), while other results are more consistent with the idea of sequential switching in a stepwise fashion from $C\mu$-$C\delta$ to $C\gamma_3$ and then to other C_H genes in the 5' to 3' direction (16-20). Still other studies of isotype switching in clonal assays have suggested a combination of direct switches from $C\mu$ to $C\gamma$, $C\alpha$, or $C\varepsilon$, and for sequential switching from $C\gamma_3$ to other γ isotypes (21-23). A molecular basis has recently been proposed for a switch through $C\gamma_3$ to other $C\gamma$ genes. Switch regions in the intervening DNA 5' to the $C\gamma_3$, $C\varepsilon$ and $C\alpha$ genes exhibit extensive sequence homology to switch sequences 5' to $C\mu$; whereas, the switch sequences preceding $C\gamma_1$, $C\gamma_{2b}$, and $C\gamma_{2a}$ possess extensive homology to repetitive sequences 5' to $C\gamma_3$, but only limited homology to $C\mu$ switch regions (24,25).

Lipopolysaccharide (LPS), can be used to induce B cell differentiation into IgM, IgG_3, IgG_1, IgG_{2b}, IgG_{2a}, and IgA plasma cells, all of which can be inhibited by anti-μ antibodies (26). We have attempted to determine whether sequential or direct switching occurs in LPS-stimulated murine B cells. If switching from Cμ to the $C\gamma_1$, $C\gamma_{2b}$, and $C\gamma_{2a}$ isotypes involves an intermediate switch to $C\gamma_3$, inhibition of $C\gamma_3$ expression would be expected to suppress all of the Cγ isotypes. In this study, we compared the effects of anti-γ_3 and anti-μ antibodies upon γ isotype expression. The results suggest that while LPS-inducible B cell precursors of all isotypes express IgM, only the IgG_3 plasma cell precursors express surface γ_3 determinants.

2 Materials and methods

2.1 Animals

Inbred CBA/J mice were obtained from Jackson Laboratories, maintained in the animal facility at the University of Alabama at Birmingham, and used at age 6-12 weeks.

2.2 Tissue Culture

Single cell suspensions were prepared from spleens as previously described (27). Lymphocytes were cultured at 2×10^5 cells/ml in Costar 96-well tissue culture plates containing RPMI 1640 with L-glutamine, supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 20% fetal calf serum, 2 μg/ml Fungizone (Flow Laboratories, Rockville, MD), and 5×10^{-5} M 2-mercaptoethanol. Cultures were maintained for five to seven days at 37°C in an atmosphere of 5% CO_2 in air. Bacterial lipopolysaccharide from Escherichia coli O111:1B4

(Sigma Chemical Company, St. Louis, MO) was used at a concentration of 50 μg/ml. Normal goat globulin and goat antibodies to mouse γ_3 and μ were used at a concentration of 100 μg/ml.

2.3 Immunofluorescence studies

Goat anti-mouse class specific antibodies were labeled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (RITC) by techniques described earlier (27). Cytocentrifuge preparations of washed cells were fixed (95% ethanol containing 5% acetic acid for 20 minutes at -20°C), and stained for twenty minutes with fluorochrome-labeled antibodies. After extensive washing, stained slides were mounted with 10% Elvanol in phosphate-saline-buffered glycerol, pH 7.4. Slides were examined with a Leitz microscope with fluorescence epi-illumination and phase contrast optics as previously described (27). At least 1000 cells on each of two slides were counted per data point, except for γ_1 and γ_{2a}, in which case at least 25,000 cells were counted per data point due to the low frequencies of these subclasses in control cultures.

2.4 Antibody preparation

Goat antibody to mouse γ_3 was prepared by hyperimmunization with the myeloma protein Y5606 (γ_3, λ) and adsorption over columns containing a panel of mouse myeloma proteins of IgM, IgG_1, IgG_{2a}, IgG_{2b}, and IgA isotypes. It was then adsorbed onto J606 (γ_3, κ), and eluted with 0.05M glycine-HCl, concentrated, dialyzed against borate-buffered saline, and readsorbed on μ, γ_1, γ_{2a}, γ_{2b} and α columns. Specificity was monitored by Ouchterlony and immunoelectrophoretic analyses and finally confirmed by radioimmunoassay as described below. Goat antibodies against mouse γ_{2b}, γ_{2a}, and

γ_1 were prepared and analyzed in an analogous manner. A monoclonal rat anti-mouse γ_{2b} antibody, received from D. G. Bole, University of Alabama at Birmingham, was used in some experiments.

2.5 Radioimmunoassays

Goat anti-mouse immunoglobulins were labeled with ^{125}I using chloramine T as previously described (28). Murine heavy chain isotype-specific goat antibodies were allowed to bind to flexible polyvinyl chloride 96-well plates (Dynatech) by the addition of 50 µl of a 10 µg/ml concentration to each well, and incubation at 4°C overnight. Plates were washed three times in borate-buffered saline to remove unbound antibodies, covered with 1% bovine serum albumin in borate-buffered saline for two hours at room temperature to prevent non-specific binding, and cell supernatants were added to washed wells. Plates were then incubated at 4°C overnight, washed four times, and overlaid with 50,000 counts/well of ^{125}I-labeled goat anti-mouse immunoglobulin. After incubation at room temperature for four hours, plates were washed four times in borate saline, and individual wells were cut apart and counted in an LKB Ultragamma gamma counter.

All anti-isotype reagents were examined for specificity against a panel of anti-human immunoglobulin mouse monoclonal antibodies of different immunoglobulin isotypes (29), using a method similar to that described above. Briefly, wells were coated with the antigens (in this case human immunoglobulin isotypes), for which each mouse monoclonal was specific, and the mouse monoclonal antibodies were then allowed to bind. After washing, the wells were overlaid with the ^{125}I-labeled anti-isotype reagent to be tested, washed, and counted. All antibodies were titrated against standard concentrations of myeloma

proteins of the corresponding isotype as described by Mongini et al. (30) and experimental values fell in the linear portion of the curves.

2.6 Statistics

Immunofluorescence data was examined for statistical significance using the Yates corrected chi-squared test (31,32). The radioimmunoassay data were analyzed for statistical significance by the two sample Rank test (33). Values of $p<0.05$ were considered significant.

3 Results

Since these experiments were crucially dependent upon the specificity of the anti-mouse γ_3 antibody used to suppress IgG_3 B cells, we first examined this antibody and each of the other anti-isotype reagents for specificity by radioimmunoassay. When ^{125}I-labeled goat anti-mouse heavy chain antibodies were tested for cross-reactivity with a panel of mouse monoclonal immunoglobulins, none of the anti-isotype reagents were cross-reactive with non-homologous isotypes (Table I).

We then examined the inhibitory effects of anti-μ and anti-γ_3 antibodies on LPS-stimulated plasma cell differentiation. Spleen cells from individual mice were split into three fractions and cultured with 50 μg/ml LPS and 100 μg/ml of either anti-γ_3, anti-μ, or normal goat globulin as a control. After five to seven days, cells and supernatants were harvested and examined for the presence of immunoglobulins of each isotype. Mature plasma cells producing each isotype were enumerated by immunofluorescent staining of fixed cells. Anti-μ treated cultures contained only low frequencies of plasma cells of each isotype (Fig. 1). Plasma cells containing IgM were suppressed by

Table 1. Specificity of anti-mouse heavy chain reagents

Mouse Immunoglobulins		^{125}I-Labeled Goat Antibodies to Mouse Heavy Chains: γ_3	γ_{2b}	γ_1	γ_{2a} and γ_{2b}	μ[c]
IgG_{2b},λ	(C3-124)[a]	131[b]	19834	648	34766	1049
IgG_1,κ	(145-8)	119	197	83204	245	480
IgG_3,λ	(1-155-1)	13034	200	254	894	548
IgG_{2a},κ	(IA6-2)	257	229	220	36564	220
IgG_{2b},κ	(14-3-26)	137	N.D.[d]	N.D.	N.D.	N.D.
IgG_3,κ	(TA4-1)	13646	N.D.	N.D.	N.D.	N.D.

[a] Clone numbers of mouse hybridomas (given in parentheses) and monoclonal antibodies are described in ref. 29.

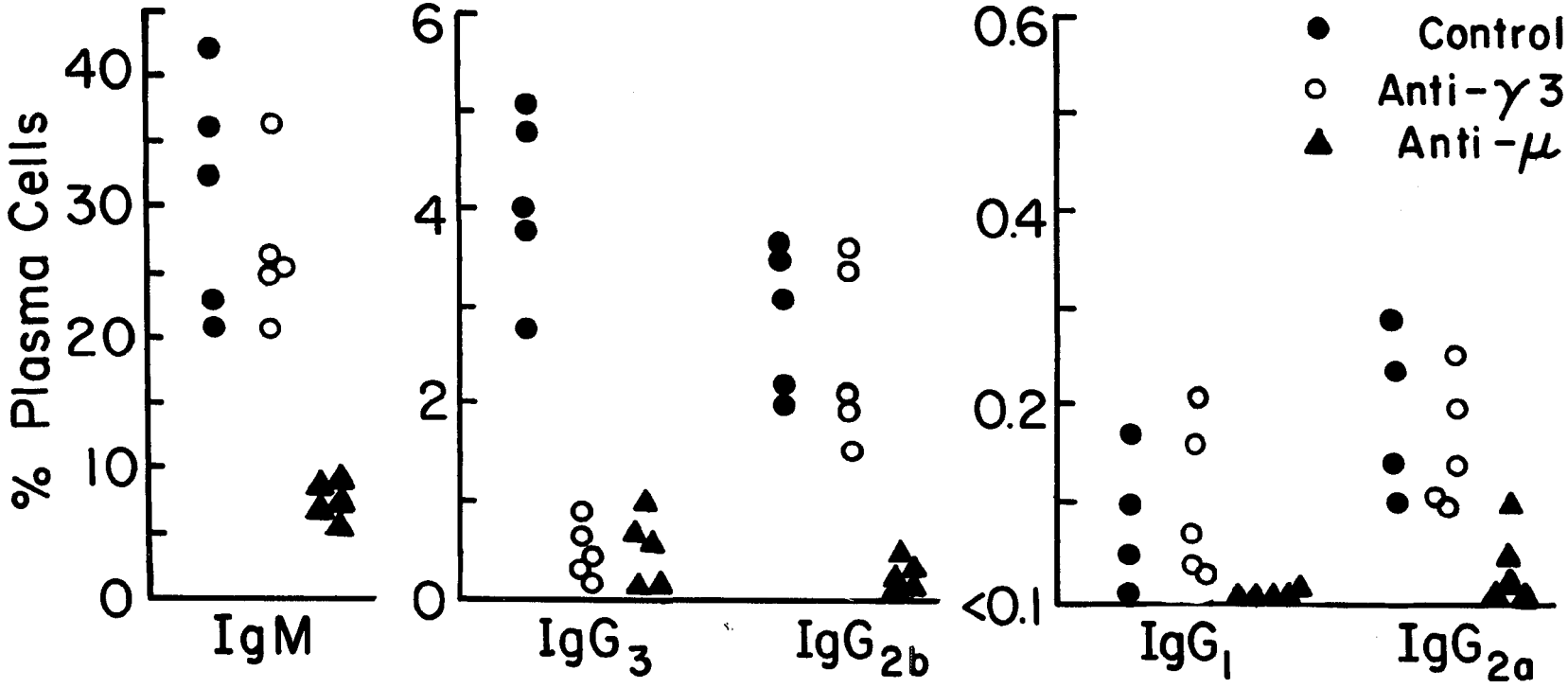
[b] Cpm bound. Background was never greater than 320 cpm.

[c] Anti-μ binding to IgM was 19,250 cpm.

[d] N.D. = not done.

Figure 1. Effects of anti-μ and anti-γ_3 antibodies on LPS-induced plasma cell differentiation. Each data point (conrols represented by closed circles ●, anti-γ_3 treatment by open circles O, and anti-μ treatment by closed triangles ▲) indicates the percentage of plasma cells detected by immunofluorescence in spleen cell cultures from one animal. The culture interval was 5 days in one experiment, and 7 days in four experiments; similar results were obtained in another experiment of 10 days duration (data not shown).

*Monoclonal anti-γ_{2b} was used in this experiment.

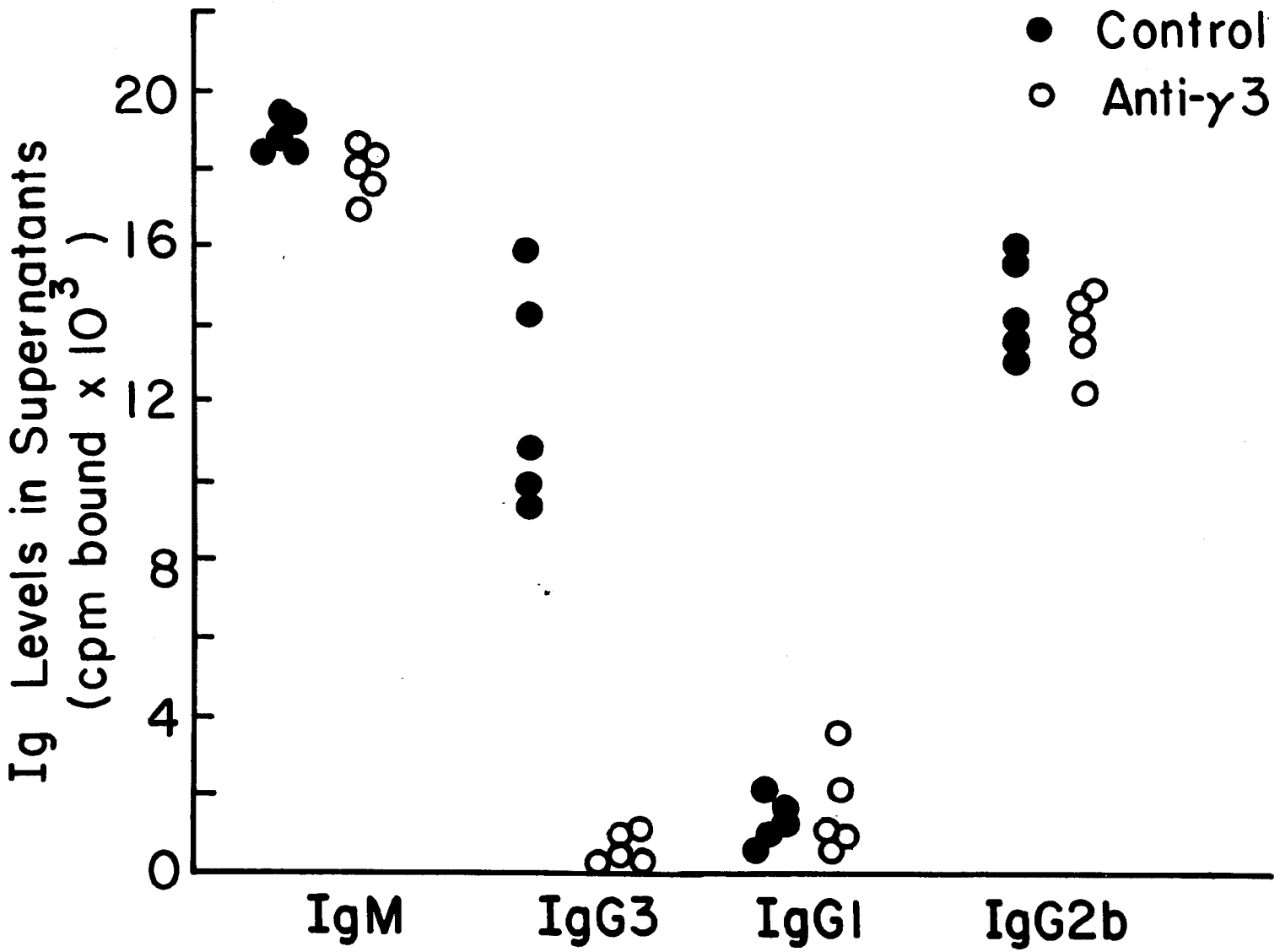

% Plasma Cells
40
30
20
10
0
IgM
6
4
2
0
IgG3
IgG2b
0.6
0.4
0.2
<0.1
IgG1
IgG2a
Control
Anti-γ3
Anti-μ

more than 70%, IgG_3 by 87%, IgG_{2b} by nearly 98%, and IgG_{2a} by about 75% ($p<10^{-14}$ for all isotypes). No IgG_1 plasma cells were found except in one experiment; however, IgG_1 plasma cells were found in very low frequencies in the control cultures.

Anti-γ_3 treated cultures showed no detectable suppression of IgM producing cells. The number of IgG_3 containing plasma cells was reduced to about 12% of control values, a reduction comparable to that seen in anti-μ treated cultures. Numbers of IgG_{2b}, IgG_1, and IgG_{2a} plasma cells in anti-γ_3 treated cultures were not significantly different from control values ($p\simeq0.25$, $p\simeq0.75$, $p\simeq0.14$, respectively). Viability of the cells in anti-γ_3 treated cultures was comparable to that of control cultures as determined by trypan blue exclusion.

A radioimmunoassay was used to compare the effects of anti-μ and anti-γ_3 on secreted immunoglobulin in cell supernatants (Fig. 2). The results were concordant with data obtained from counting plasma cells in each culture. Supernatants from anti-γ_3 treated cultures contained little detectable IgG_3 ($p<0.01$), whereas IgG_1 and IgG_{2b} were secreted in amounts comparable to those in control cultures ($p \simeq 0.75$ for both IgG_1 and IgG_{2b}). Residual anti-γ_3 antibodies would prevent reliance upon the radioimmunoassay results alone for measurement of IgG_3, but would not affect the concordant reduction in IgG_3 plasma cells or measurements of other immunoglobulin isotypes. IgG_{2a} was not measured separately in this assay, but when antibodies to both IgG_{2a} and IgG_{2b} were used, no differences were seen between control and anti-γ_3 treated cultures (p 0.18). Anti-μ treated cultures, on the other hand, showed greater

Figure 2. Effect of anti-γ_3 upon immunoglobulin secretion in LPS-induced cultures. Each data point (controls represented by closed circles ●, and anti-γ_3 treatment by open circles O) indicates the average number of counts from duplicate samples of supernatant from one experiment. Background cpm were <300 for IgM, IgG_3 and IgG_1, and <1100 for IgG_{2b}.


Control
Anti-γ3
Ig Levels in Supernatants
(cpm bound x 10^3)
20
16
12
8
4
0
IgM
IgG3
IgG1
IgG2b

than fifty percent suppression of secretion of each IgG subclass (Table II).

4 Discussion

The suppression of LPS-induced plasma cells of all immunoglobulin isotypes observed following anti-μ treatment supports the idea that LPS induces differentiation of relatively young B cells, most of which carry IgM on their surface (26,34). Results of other studies indicate that LPS can induce differentiation of B cells as soon as they are formed in mouse fetal liver (35,36).

In the present studies we found that anti-γ_3 was just as effective as anti-μ treatment in inhibiting LPS-induced differentiation of IgG_3 plasma cells, but had no effect on IgG_1, IgG_{2a}, IgG_{2b} or IgM plasma cell differentiation. In contrast, treatment with anti-μ had significant inhibiting effects on differentiation of plasma cells of all isotypes. These results suggest that switching of heavy chain gene expression from $C\mu$ to $C\gamma_1$, $C\gamma_{2a}$ or $C\gamma_{2b}$ can occur without intermediate expression of $C\gamma_3$. This evidence thus directly contradicts the suggestion that multiple heavy chain isotype switches occur sequentially in the order of C_H gene alignment (16-20), and instead favors the conclusion that the major switch pathways are directly from $C\mu$ to each of the other isotypes (11-15). It should perhaps be noted that our evidence is strongest for direct switching from μ to γ_{2b}, since LPS induces fewer γ_1 and γ_{2a} producing plasma cells.

Several studies have suggested that helper T cells enhance the switching process and that this switching occurs sequentially within

Table 2. Immunoglobulin secretion in anti-μ treated cultures

Immunoglobulin Isotype	Control	Anti-μ Treated	Statistical Analysis
IgM	7247 ± 203[a)]	46 ± 48	$p<0.03$
IgG_3	4950 ± 508	437 ± 166	$p<0.03$
IgG_1	1111 ± 249	350 ± 105	$p<0.05$
IgG_{2b}	5907 ± 217	2433 ± 314	$p<0.03$
IgG_{2a} and IgG_{2b}	5320 ± 536	2281 ± 585	$p<0.05$

[a)]Mean (± 1 S.E.) cpm bound minus background in 4 experiments.

the γ subclasses (22,37). Nevertheless, it is difficult to ascertain that new isotypes originated from only one B cell in these studies. Although the use of helper T cells rather than mitogens and a longer study interval may more nearly approximate normal switching conditions, recent in vivo experiments also provide support for a direct rather than sequential switching mechanism. In these studies, clones allowed to develop to senescence by continued passage through murine spleens did not exhibit increased frequencies of cells expressing γ and α isotypes, as would be expected if switching occurred sequentially (38).

The finding that C_H genes in cells expressing both IgM and another isotype are not rearranged has suggested a two-step model for isotype switching (39,40). In this model, cells which express two surface isotypes may do so initially by differential splicing of long mRNA transcripts, while final differentiation into non-IgM producing plasma cells occurs by DNA deletion (41). Our results suggest that cells which express surface IgG_3 are already committed to becoming IgG_3-producing plasma cells, and in this way complement previous studies which show commitment by IgG and IgA B cells to differentiation into plasma cells that produce the homologous immunoglobulin isotype (42-45). This places the additional constraint on a two-step switch model for a mechanism to faithfully integrate the two switch decisions.

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III. IMMUNOGLOBULIN GENE REARRANGEMENTS AND DELETIONS IN HUMAN EBV-TRANSFORMED CELL LINES PRODUCING DIFFERENT IgG AND IgA SUBCLASSES

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ABSTRACT

During differentiation B lymphocytes may switch from the expression of surface IgM to the synthesis of IgG, IgA or IgE isotypes, utilizing a different heavy chain constant region (C_H) gene. The molecular mechanisms by which switching occurs remain controversial. Rearrangements and deletions of C_H genes 5' to the expressed gene have often been observed in the mouse, and more recently in human cells which have switched isotypes. We have used human J_H, $C\mu$, $C\gamma$ and $C\alpha$ probes to examine the extent of the deletions and rearrangements in clones of EBV-transformed human cells which produce IgG_1, IgG_3, IgG_4 or IgA_1. While deletions of C_H genes 5' to the expressed C_H gene were consistently observed, the rearrangement process appeared to be highly variable for the non-productive C_H gene locus: deletion or persistence of 5' C_H genes, combinations of deletion and duplication of 5' genes, and deletions extending to 3' C_H genes. Our results reveal an unexpected imprecision in the isotype switch process.

ABBREVIATIONS: C_H, heavy chain constant region; EBV, Epstein-Barr virus; FITC, fluorescein-isothiocyanate; kb, kilobases.

The immunoglobulin C_H genes in humans are aligned on chromosome 14 in the order 5' Cμ-Cδ-Cγ3-Cγ1-ψε-Cα1-ψγ-Cγ2-Cγ4-Cε-Cα2 3' (1,2). During B lymphocyte differentiation cells may switch from the synthesis of IgM to the synthesis of IgG, IgA or IgE (3,4). This occurs when the functionally rearranged variable exon (V_H) used in conjunction with Cμ to produce the IgM heavy chain is subsequently transcribed with a different C_H gene. The exact molecular mechanisms by which switching occurs are not yet fully understood; several have been proposed. One possibility is that switching is mediated through pairing and recombination between regions of homology in the DNA sequences 5' to each of the C_H genes. These switch sequences exist about 1.5 kb 5' to each C_H gene except Cδ and are composed of short repeated sequences (5,6). Recombination could occur either between switch sites on one chromosome, resulting in formation of a loop and deletion of intervening DNA sequences, or through unequal sister chromatid exchange (4,7-9). Alternatively, it has been proposed that switching may occur via processing of a long RNA transcript containing V_H and all of the C_H genes (10-12).

Most of the evidence to date has been obtained from analyses of mouse plasmacytomas and supports a deletion mechanism on either one or both chromosomes (7,13,14). Studies using lipopolysaccharide-stimulated mouse spleen cells have also shown Cμ deletion in cells which have switched to IgG_3 (15). Evidence obtained from unstimulated mouse B cells favors the possibility that RNA processing is the

initial event in the switching process before B cells begin plasma cell differentiation (11,12).

Fewer studies have been performed using human B cells. Rearrangements of Cμ, Cλ, and Cκ occur in chronic lymphocytic leukemia cells (16,17), and both rearrangements and deletions of C_H genes have been observed in several Epstein-Barr virus (EBV)-transformed lines (18). However, there has been no systematic study to determine the rearrangements and the extent of the deletions in cell lines producing defined IgG and IgA subclasses. We have established from four individuals a panel of EBV-transformed cell lines producing IgM, IgG_3, IgG_1, IgA_1 and IgG_4 antibodies and have analyzed DNA from these lines for rearrangements and deletions of Cμ, the four Cγ, and both Cα genes. Although we have observed one interesting exception in which genes 5' to the functionally rearranged C_H are retained, possibly due to duplication, most of our data are consistent with a deletion model for isotype switching. The data also suggest that the switching process is not a precise, well-defined, orderly deletion confined to the functional chromosome.

MATERIALS AND METHODS

Establishment of EBV-transformed cell lines. Blood mononuclear cells collected from healthy donors were isolated by Ficoll-Hypaque gradient centrifugation, and T cells were depleted by rosetting with aminoethylisothiouronium bromide-treated sheep erythrocytes (19). This B cell-enriched fraction was then resuspended at $2x10^6$ cells/ml in RPMI 1640, and 100 μl/ml of EBV-containing supernatant ($TD_{50}>10^4$) from the B95-8 marmoset cell line was added (20). Cells were incubated

at 37°C in 7% CO_2 overnight, washed, resuspended at 10^6 cells/ml, and grown in RPMI 1640 supplemented with 15% fetal calf serum, L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml Fungizone (Flow Laboratories, Rockville, MD). After 10 days cells were subcloned by limiting dilution onto mouse peritoneal feeder cells in 96-well plates (Costar, Cambridge, MA). Statistical clones (i.e., those obtained from plates showing growth in <30 wells) were screened after 4 weeks for secretion of IgM, IgG, and IgA by ELISA. Sublines producing only one isotype were examined further for cytoplasmic immunoglobulin by immunofluorescence staining with a panel of monoclonal antibodies to human isotypes (21). Fixed cell smears were stained indirectly using fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin as a second layer (22). At least 10^4 cells per slide were examined, and cell lines were used in molecular analyses only after they were recloned so that they produced only one heavy and one light chain isotype. Further analyses for clonal origin of these lines were performed by indirect immunofluorescence staining with a panel of 34 mouse anti-human V_H and anti-idiotype antibodies provided by H. Kubagawa (23, 24). Karyotype analysis was performed by Dr. Andrew Carroll, Medical Cytogenetics, University of Alabama at Birmingham.

DNA isolation and analysis. High molecular weight DNA was isolated (25) and digested to completion with HindIII, BamHI, or EcoRI, as suggested by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, IN). Five μg of DNA was loaded per lane onto 0.7% agarose gels, and fragments were size-separated by electrophoresis at 0.5-1.5 V/cm for 36-48 hours. Southern blots (26) were prepared with

Zetabind membranes as recommended by the manufacturer (AMF, Meriden, CT). Blots were hybridized at 42°C, washed under high stringency conditions, and exposed to XAR5 X-Ray film (Eastman Kodak Company, Rochester, NY). For rehybridization, filters were stripped with 0.4 N NaOH.

Probes. A 1.3 kb EcoRI genomic fragment of human Cμ and a 6.0 kb BamHI/HindIII fragment containing the human J_H exons were provided by J. V. Ravetch (27). The 2.0 kb EcoRI/HindIII fragment containing the germline Cγ4 exons was provided by J. Ellison (28). The Cγ2 probe was a 4.4 kb XhoI/BamHI fragment subcloned into pBR322 from a Charon 28 λ clone also obtained from J. V. Ravetch (29). All probes were restricted with the appropriate endonucleases, separated from vector DNA by electrophoresis, and labeled with [^{32}P] dCTP (30).

Densitometric analyses. Densitometric analyses were performed with a 2202 Ultrascan Laser densitometer and GELSCAN software (LKB Instruments, Bromma, Sweden) in an Apple IIe computer (Cupertine, CA). To avoid discrepancies in intensity due to variations in quantities of DNA per lane or differences in transfer efficiency, analyses were performed on Cγ- and Cα-hybridizing bands within a single lane, and the ratios of these intensities were compared with ratios of the intensities obtained in a control cell line containing two germline copies of each gene. The comparison of peak ratios to ratios obtained from control lines also prevented misinterpretations which might occur due to preferential hybridization of the Cγ4 and Cγ2 probes to those particular subclasses.

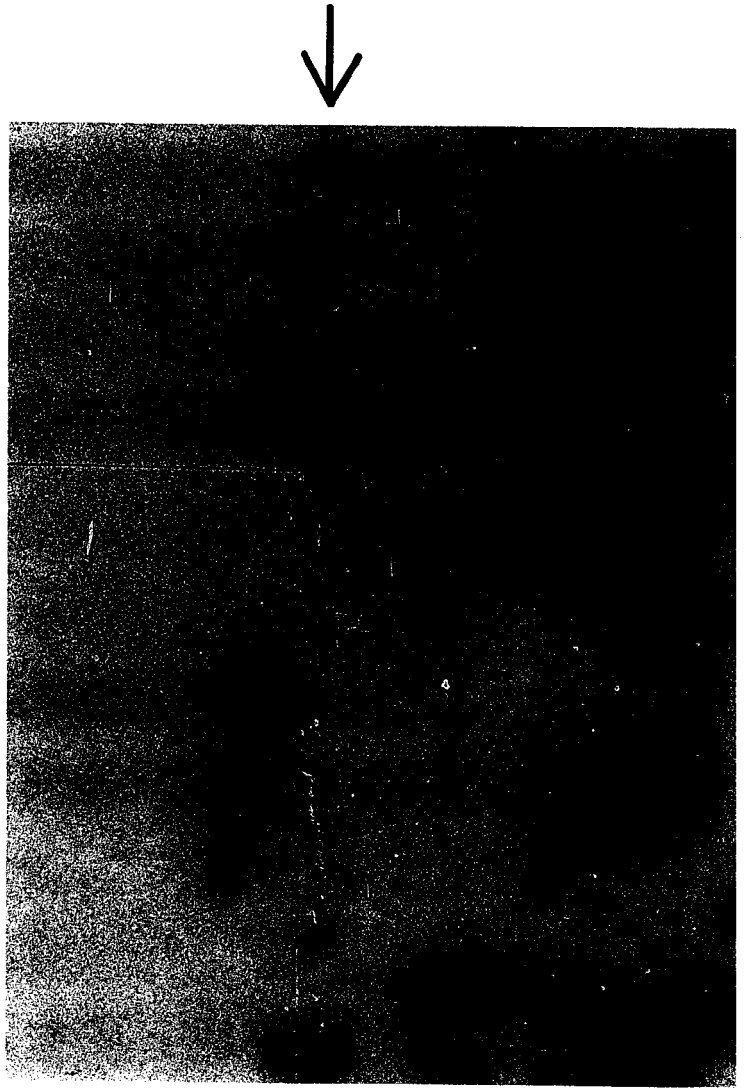
RESULTS

Absence of isotype switching in clones of EBV-transformed B cells. Since the interpretation of any molecular data relies heavily upon the clonality of the cell lines examined, great care was taken to show the clonal origin of each line. At least two rounds of subcloning were performed since immunofluorescence staining after the initial limiting dilution cloning procedure revealed small numbers of cells (from 0.3% to as many as 30% in one instance) producing a second isotype. Cells producing the minority isotype were not observed after subsequent recloning, and in no instance was isotype switching indicated by the appearance of an expanding subpopulation of cells producing a second isotype.

None of the clones examined so far have reacted with any of the 30 anti-idiotype antibodies despite the presence of cells which react with these reagents in bulk transformed cultures from the same individuals (M. Kiyotaki, H. Kubagawa and M. Cooper, unpublished results). However, ME-G_1 is reactive with the monoclonal anti-V_H SA-FD44, while IL-G4 is reactive with the anti-V_H MH-4410. After final subcloning, >70% of the cells in each of the clones stained brightly for cytoplasmic immunoglobulin of the appropriate isotype. While the cell types present in these lines ranged from B lymphocytes to mature plasma cells, most of the cells had a plasmablast morphology.

Deletion of Cμ from cell lines expressing IgG_1, IgA_1, and IgG_4. Hybridization of BamHI- and HindIII-digested DNA from two IgG_1-, one IgA_1- and one IgG_4-producing cell lines with a Cμ probe is shown in Fig. 1. The IgM line (ME-M) shows one rearranged

Figure 1. Hybridization of BamHI- and HindIII-digested DNA from two individuals (ME and IL) with the human Cμ probe. The heavy chain isotype produced by each cell line is indicated after the individual's initials. IL-0 is an immunoglobulin negative line. The arrow designates the germline BamHI-digested Cμ fragment. All size determinations were calculated using a 1.0 kb ladder (BRL, Bethesda, MD) and HindIII-digested λ DNA as size markers.



ME-M
ME-GI
ME-AI
IL-O
IL-GI
IL-G4

BamHI

ME-M
ME-GI
ME-AI
IL-GI
IL-G4

Hind III

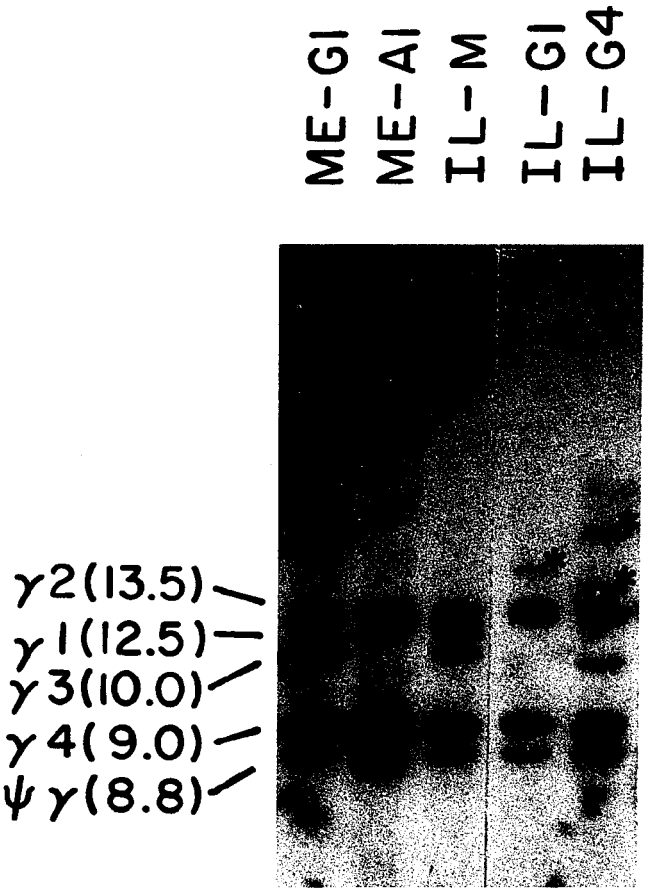
band and one unrearranged germline copy of Cμ. Cμ has been deleted from both chromosomes in the IgA_1 and IgG_4 lines as well as in $IL\text{-}G_1$; however, $ME\text{-}G_1$ shows a single Cμ-hybridizing band. Results from three other cell lines, an IgA_1 line from another individual and an IgG_1 and an IgG_3 line from IL, also show deletion of Cμ from both chromosomes (data not shown).

Rearrangement and deletion of Cγ genes in cell lines expressing IgG and IgA isotypes. Fig. 2 shows the results of hybridization of DNA from the same cell lines with the $C\gamma_4$ probe. All four Cγ genes and the ψγ are sufficiently homologous for the $C\gamma_4$ probe to detect each of these genes, and digestion with BamHI allows the detection of each gene as a discrete band on genomic DNA blots. We assigned Cγ genes to a particular BamHI-hybridizing band according to size, as described by Bech-Hansen (31). Due to polymorphic differences in the introns between the Cγ genes among individuals (31), IgM lines from each individual were used as controls for the germline configuration of the Cγ genes. Only one IgM-producing control is shown, since both individuals ME and IL have identical germline configurations in the BamHI digests.

Deletion from both chromosomes of $C\gamma_3$ and $C\gamma_1$, which are 5' to $C\alpha_1$, is apparent in ME-A1, and deletion of both $C\gamma_3$ genes has occurred in $IL\text{-}G_1$. An IgA_1 line from a third individual has also deleted $C\gamma_3$ and $C\gamma_1$ genes from both chromosomes (data not shown). Each of the IgG-producing cell lines contains at least one rearranged Cγ-hybridizing band as well as several bands in the germline configuration. Rehybridization of the same blot with J_H (Fig. 3) shows at least one of the J_H-hybridizing bands coinciding with one of the

Figure 2. (A) Hybridization of BamHI-digested DNA with the $C\gamma_4$ probe. Asterisks designate rearranged $C\gamma$ bands which hybridize with J_H. (B) Germline organization of the human C_H locus.

A.


ME-GI
ME-AI
IL-M
IL-GI
IL-G4
γ2(13.5)
γ1(12.5)
γ3(10.0)
γ4(9.0)
ψγ(8.8)

B.

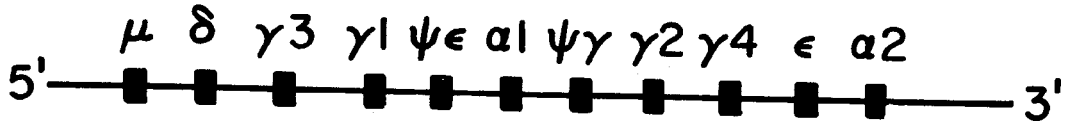
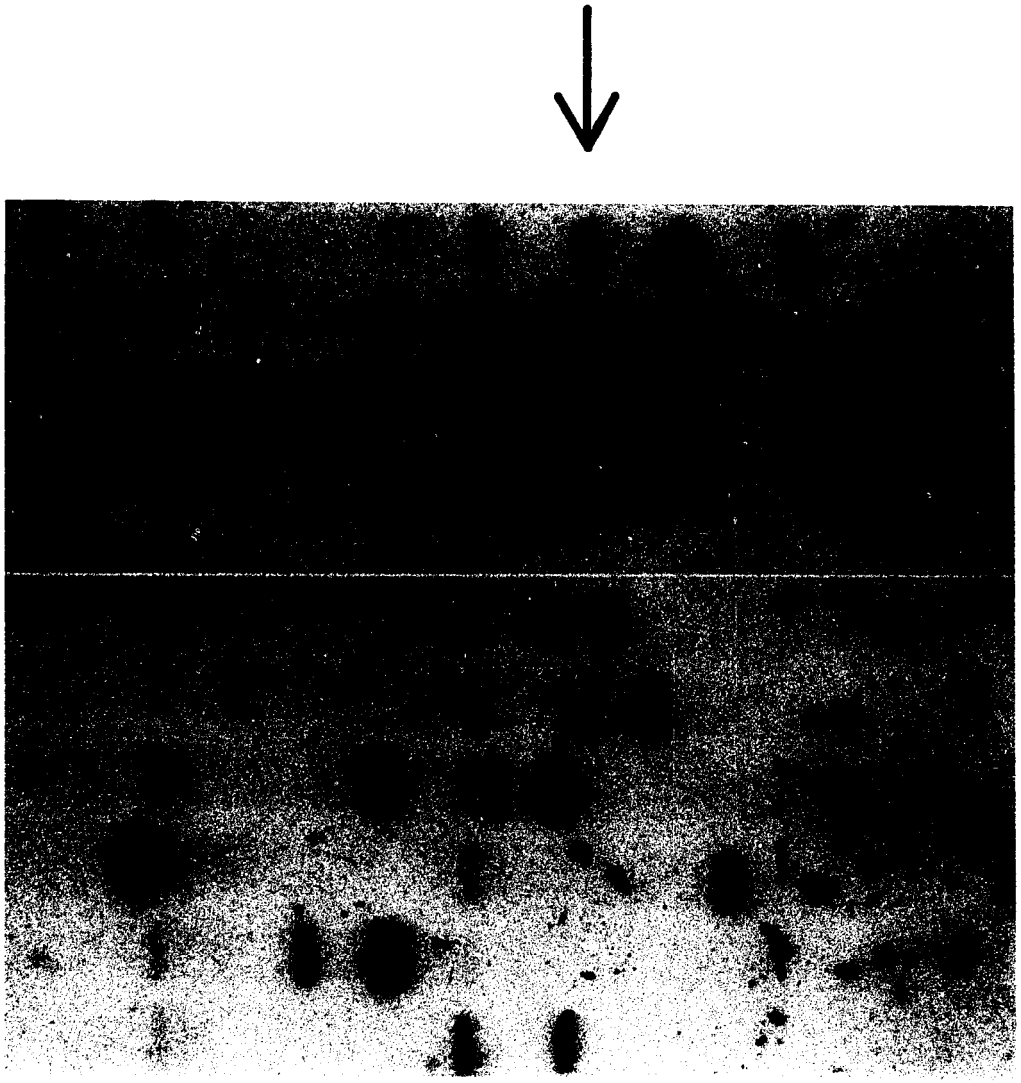

μ δ γ3 γ1 ψε α1 ψγ γ2 γ4 ε α2
5'
3'

Figure 3. Hybridization of BamHI- and HindIII-digested DNA with the human J_H probe. The arrow designates the germline BamHI-digested J_H fragment.



ME-M
ME-GI
ME-AI
IL-O
IL-GI
IL-G4

BamHI

ME-M
ME-GI
ME-AI
IL-GI

HindIII

rearranged Cγ bands in Fig. 2. Therefore these rearranged bands must represent the productively rearranged genes.

Although both IgG_1-producing lines show only one rearranged Cγ band hybridizing with J_H, these two lines differ markedly in the extent of the deletions which have occurred within the C_H locus. $ME\text{-}G_1$ retains at least one copy of Cμ and appears to have undergone rearrangement on only one chromosome. Comparison of densitometric intensities obtained from the control cell line with those obtained from $ME\text{-}G_1$ (Fig. 4) showed that the Cγ-hybridizing bands at the $C\gamma_3$ and $C\gamma_1$ positions in $ME\text{-}G_1$ are probably representative of only one allele and confirmed that deletion of C_H genes occurred on only one chromosome in this instance. $IL\text{-}G_1$, on the other hand, shows deletion on both chromosomes of Cμ and $C\gamma_3$ genes, which are 5' to $C\gamma_1$, and appears to have deleted $C\gamma_1$, as well as C_H genes 3' to $C\gamma_1$ on the nonfunctional chromosome. Densitometric analyses of Cγ-hybridizing bands from $IL\text{-}G_1$ (Fig. 4) indicate that ψγ as well as $C\gamma_1$ may have been deleted on one chromosome, in which case one might also expect that $C\alpha_1$, which is between $C\gamma_1$ and ψγ, would have been deleted on that chromosome. Hybridization of DNA from this line with a $C\alpha_2$ probe and densitometric analyses (Fig. 5) show that there is indeed only one copy of $C\alpha_1$ in this line. Thus, in this instance, deletions have occurred on the nonfunctional chromosome 3' to the C_H gene that is expressed on the functional chromosome.

Possible duplication of C_H genes. The $IL\text{-}G_4$ line is of particular interest. Although it has deleted both copies of Cμ and shows no germline bands for $C\gamma_3$ and $C\gamma_1$ as might be expected for a line which is actively producing IgG_4, it contains three rearranged

Figure 4. Densitometric scans of autoradiograms of BamHI-digested DNA hybridized with the $C\gamma_4$ probe in Fig. 2

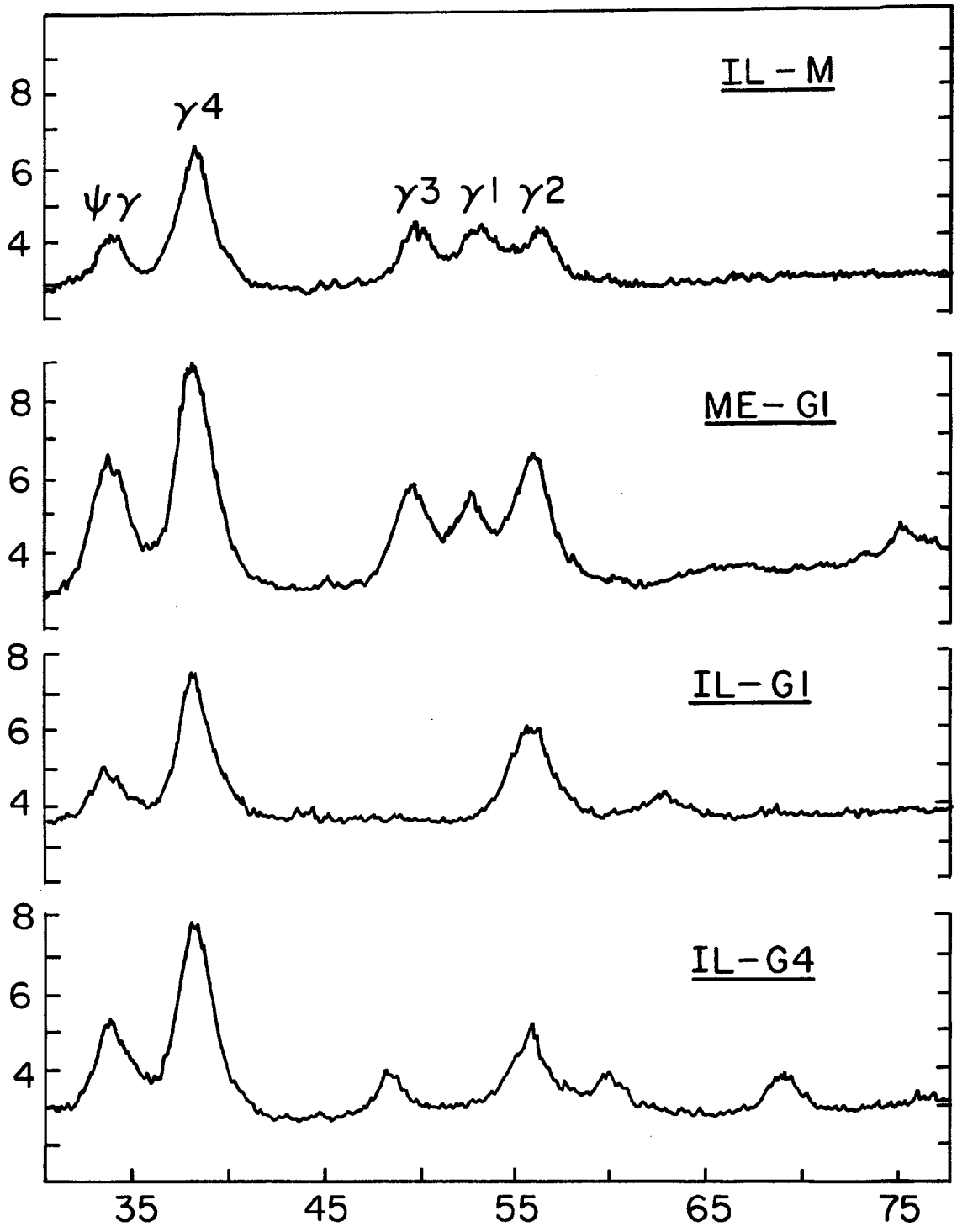
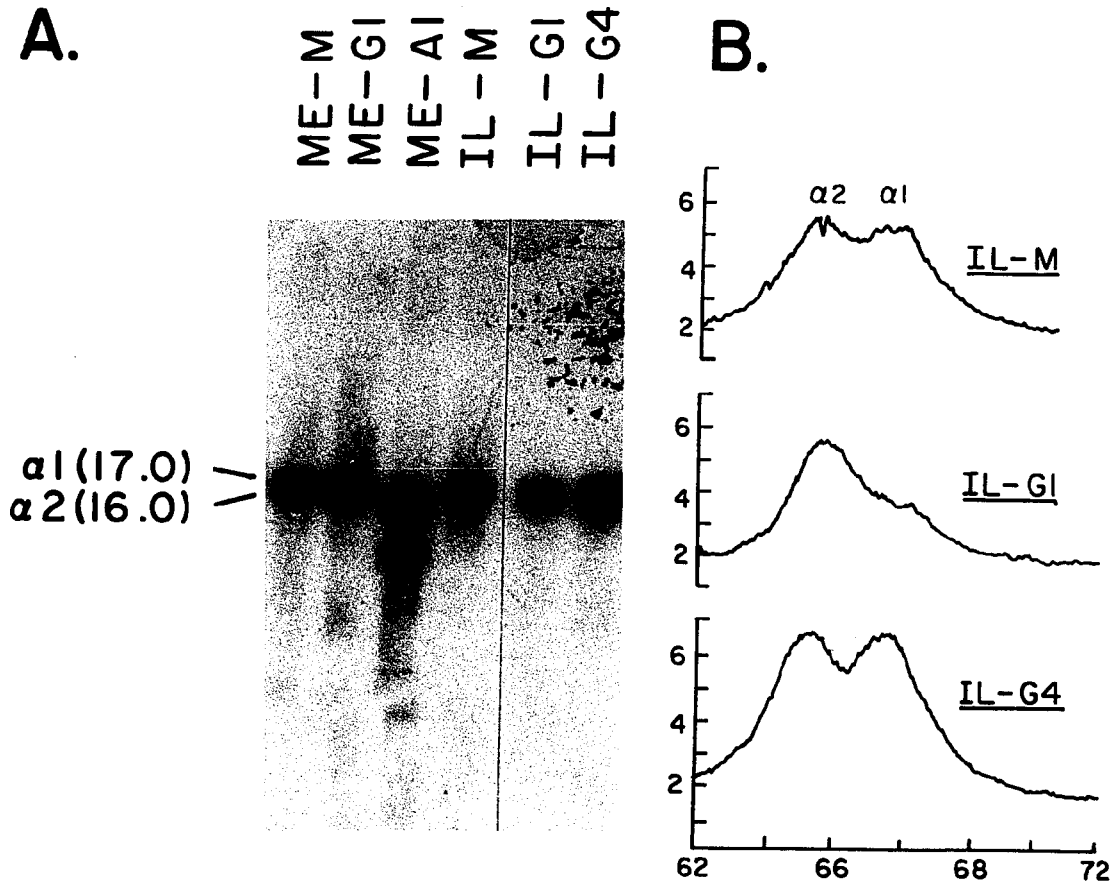

IL-M
ψγ
γ4
γ3
γ1
γ2
ME-GI
IL-GI
IL-G4
8
6
4
35
45
55
65
75

Fig. 5 (A) Hybridization of BamHI-digested DNA with the $C\alpha_2$ probe. (B) Densitometric scans of the Cα-hybridizing bands in (A).


A.
ME-M
ME-GI
ME-AI
IL-M
IL-GI
IL-G4
α1(17.0)
α2(16.0)
B.
α2
α1
IL-M
IL-GI
IL-G4
6
4
2
62
66
68
72

C γ bands. It is also surprising that the $\psi\gamma$ and $C\gamma_2$ genes are retained and that there is a very intensely hybridizing band in the germline $C\gamma_4$ position. While two of the rearranged bands hybridize with J_H, only one of these is likely to be the productive $C\gamma_4$ allele. These data have been obtained consistently from this line and cannot be explained by artifacts or variations in procedure. Eighty percent of these cells stain brightly for IgG_4 and with the anti-V_H antibody MH-4410, suggesting that contamination with another IgG_4-producing line is unlikely. Clonality is also borne out by the molecular data, since hybridization with J_H (Fig. 3) shows only two specifically hybridizing bands rather than the three or more which might be expected from clonal contamination. Nor can karyotypic abnormalities explain these results, since the cells in this line appear to be diploid for all chromosomes, have no apparent translocations such as those seen in Burkitt's lymphoma cell lines, and they have no detectable extrachromosomal fragments (data not shown).

The presence of an intensely hybridizing band in the $C\gamma_4$ position suggests that duplication of at least a portion of the C_H genes is a more likely explanation of the results obtained from this cell line rather than a simple reintegration of a portion of the excised 5' genes, as may have occurred in an Abelson virus-transformed pre-B cell line which switches from Cμ to $C\gamma_{2b}$ and retains a Cμ no longer juxtaposed to J_H (32). Densitometric analyses (Fig. 4) did in fact show that there are probably at least three copies of $C\gamma_4$ in this cell line, one of which is a rearranged band that hybridizes with J_H and represents the productive $C\gamma_4$ gene. The other copies of $C\gamma_4$ are represented by the germline-sized BamHI fragment. A

partial duplication of a fragment containing $C\gamma_1$, $C\alpha_1$, $\psi\gamma$, $C\gamma_2$ and $C\gamma_4$ could explain the detection of $C\gamma_2$ and $\psi\gamma$ as well as the deletion of $C\mu$ from both chromosomes. One possibility is that, although $C\gamma_4$ is rearranged to J_H on one chromosome, the nonfunctional chromosome may have rearranged such that $C\gamma_1$ is joined to J_H, thus retaining all of the 3' C_H genes on that chromosome. The duplication of a large portion of the C_H gene locus on this chromosome and its reinsertion into the genome such that the duplicated $C\gamma_1$ is reduced in size would account for all of our data. Since hybridization of the DNA from IL-G_4 with a $C\alpha_2$ probe (Fig. 5) showed identical numbers of $C\alpha_1$ and $C\alpha_2$ genes, it is likely that the duplication does not extend through $C\alpha_2$.

Our analyses of another two IgG_1^-, two IgG_3^-, and two IgA_1^- producing clones from these and other individuals have shown that deletion of genes 5' to the expressed gene occurs on at least one chromosome in every case.

DISCUSSION

We have established a panel of EBV-transformed cell lines from which we have selected examples synthesizing different C_H isotypes and have determined the rearrangements and deletions which occur in the C_H gene locus. After confirming the clonality of the EBV-transformed cell lines with a panel of monoclonal antibodies specific for isotype, V_H, and idiotype, we tested the clones for the production of any new heavy chain isotype. In the fifteen cases studied in detail we found no indication of isotype switching <u>in vitro</u>, even after serial subclonings.

The identification of the IgG and IgA subclasses produced by our cell lines has allowed us to interpret more precisely the extent of the deletions relative to the rearranged C_H gene on the productive allele. In most of the IgG and IgA clones examined (9/10), rearrangement and deletion appear to have occurred on both chromosomes. However, the data illustrate why there has been so much controversy in this area. Even among clones from the same individual we have found differences in the extent of the deletions occurring on the nonfunctional chromosome, i.e., ME shows deletion on only one chromosome in the IgG_1 line, while deletion has occurred on both chromosomes in the IgA_1 line. There is also no strict correlation between the extent of the deletions on the nonfunctional chromosome and the expressed isotype, since one IgG_1 line shows deletions on the nonfunctional chromosome that extend through C_H genes 3' to the gene expressed on the functional allele. We cannot exclude the possibility that deletions seen on the allelically excluded chromosome are secondary to the actual switching event. However, if the same switch mechanisms govern the DNA deletions and rearrangements on both chromosomes, then our data indicate a lack of isotype specificity in the actual recombination process.

The IgG_4 line is especially interesting in that it has deleted $C\mu$ and has retained $C\gamma_2$, both of which are between $C\mu$ and $C\gamma_4$ in the germline. In addition it appears to have duplicated at least a portion of the C_H locus containing $C\gamma_4$, $C\gamma_2$, $\psi\gamma$ and $C\alpha_1$. A simple sister chromatid exchange model will not explain the apparent existence of three $C\gamma_4$ genes within the line. Unfortunately, the extremely low frequency of IgG_4, IgG_2, and IgA_2 cells in EBV-

transformed cell cultures (33) makes it difficult to determine whether the unusual rearrangements occurring within this cell line are the result of a relatively rare event or whether they may occur more frequently among C_H genes which are more distal to the VDJ exon.

Our previous studies suggested that direct, rather than sequential, isotype switches from IgM are the rule for normal B cells (34-36). Deletions of C_H genes 5' to the expressed gene occur frequently within the lines in the present study, with deletion of $C\mu$, $C\gamma_3$ and $C\gamma_1$ occurring more often than deletions of the more 3' C_H genes. Others have interpreted similar findings to indicate that switching occurs by the orderly, sequential 5' to 3' deletion of these genes (18). However, we do not believe that our data indicate sequential switching within the C_H locus. It is more likely that the infrequent occurrence of deletions of C_H genes 3' to $C\gamma_1$ in these lines reflects the low numbers of IgG_4- and IgG_2-producing cells present in EBV-transformed cultures (33,37 and our unpublished results), even though IgG_2 is a major isotype on peripheral blood B cells.

It is possible that switching could occur by more than one mechanism. We cannot assess the contribution of intrachromosomal recombination versus unequal sister chromatid exchange in the deletions seen in these lines, since sister chromatids assort into separate cells at cell division and antibody-secreting cells were selected during subcloning. The ability to obtain cells producing each C_H isotype by EBV-transformation of circulating B cells and the lack of spontaneous switching within these lines suggest that EBV transformation immortalizes cells which have already switched and which maintain their

normal C_H rearrangements. This suggests that the population of switched cells transformable by EBV do not produce Cγ or Cα isotypes by an RNA processing mechanism, but rather by recombination and deletion of C_H genes. Hence, our data do not contribute to the resolution of the present controversy (10-12,32,38,39) concerning whether or not RNA processing could be involved in cells representative of earlier stages in B cell differentiation.

Our results clearly indicate that the recombination events that occur in conjunction with isotype switching are imprecise, and this is reflected by the diversity of rearrangements observed on the nonfunctional chromosome. Although the ability to switch to the production of antibodies of every isotype is a biological advantage, the imprecision with which rearrangements occur may contribute to aberrant recombinations such as the translocations that are observed in many B lymphocyte malignancies.

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IV. IMMUNOGLOBULIN GENE REARRANGEMENTS IN AN EBV-TRANSFORMED IMMUNOGLOBULIN NEGATIVE CELL LINE

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During the analysis of the EBV-transformed cell lines described in Chapter III, an Ig^- subclone was discovered. Others have also observed Ig^- EBV-transformed cell lines derived from the peripheral blood of X-linked agammaglobulinemia patients, from fetal bone marrow, and from umbilical cord blood (1-4). The results of Southern blot analyses of the C_H gene locus from our Ig^- subclone, IL-0, showed that the μ, J_H, γ and α genes appear to be in the germline configuration. The results and implications of these findings are discussed in the following pages.

MATERIALS AND METHODS

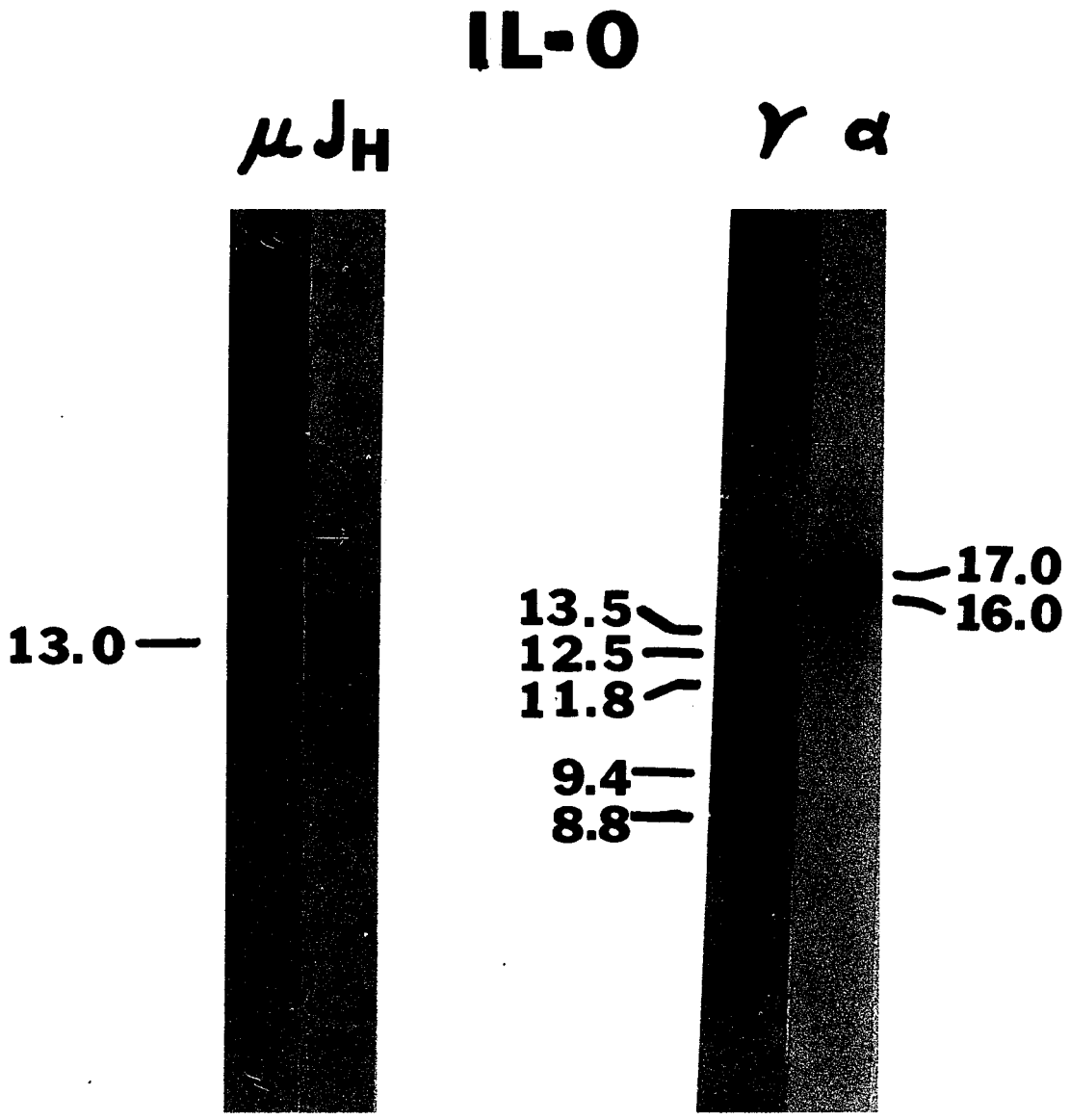
The materials and methods used in these experiments are exactly as described in Chapter III. Briefly, T cell-depleted mononuclear cells were transformed with EBV, subcloned by limiting dilution onto mouse peritoneal feeder cells, and screened for secreted Ig by an enzyme-linked immunosorbent assay. Immunofluorescence staining for cytoplasmic Ig was performed using a panel of mouse monoclonal anti-human antibodies provided by H. Kubagawa, as previously described (5). Southern blot analysis (6) was performed using the μ, γ, J_H and α probes depicted in Appendix 2, and labelled with [^{32}P]dCTP as described previously (7).

RESULTS

The cell line IL-0 was established by the transformation of a B cell-enriched fraction of mononuclear cells isolated from the peripheral blood of a healthy 24-year-old female. After initial cloning and screening procedures, eleven clones were isolated which did not secrete Ig. However, when these clones were expanded and re-examined by immunofluorescence staining for cytoplasmic Ig, cells from all eleven clones produced IgM and either κ or λ light chains. When these clones were re-evaluated, all of the clones secreted Ig, presumably indicating either that Ig^- cells had been overgrown by IgM^+ populations, that maturation into Ig-secreting cells occurred in culture, or that the amount of secreted Ig present at the time of the initial screening procedure was below the levels of detection.

One of these clones was selected for further analysis, and after a second cloning procedure, fourteen subclones were isolated. One subclone, IL-0, contained no cytoplasmic heavy or light chain Ig, while the remainder of the subclones produced either cytoplasmic IgM,λ or IgM,κ. Phenotypically, IL-0 grew in clumps of 16-40 cells and appeared to be identical to the other established Ig^+ cell lines, except for the fact that it grew somewhat more slowly (having a doubling time of 4-5 days rather than 2-3). Analysis of BamHI-digested DNA from this cell line revealed that the μ, J_H, γ and α C_H genes were apparently in the germline configuraton (Fig 1), with a μ and J_H-hybridizing band at 13.0 kb, γ_2-, γ_3-, γ_1-, γ_4- and $\psi\gamma$-hybridizing bands at 13.5, 12.5, 11.8, 9.4 and 8.8 kb, and α_1- and α_2-hybridizing bands at 17 and 16 kb, respectively.

Figure 1. The IL-0 C_H genes are in a germline configuration as shown when BamHI-digested DNA is hybridized with μ, J_H, γ and α probes.


IL-0
μ J_H
γ α
13.0
13.5
12.5
11.8
9.4
8.8
17.0
16.0

DISCUSSION

Cytoplasmic Ig^- cells have been observed in bulk cultures of EBV-transformed umbilical cord and adult peripheral blood B cells (4, our unpublished observations). This Chapter describes a cytoplasmic Ig^- subclone derived from EBV-transformed normal adult peripheral blood B cells. There are several explanations for the lack of Ig production observed in this cell line. First, the cell line may not be of the B cell lineage and could represent an outgrowth of cells from a monocyte or T-cell lineage instead. Secondly, and more importantly, the cell line may be representative of an early progenitor of the B cell lineage in which the Ig C_H genes have not yet undergone rearrangement. Unfortunately, the cell line was lost before analyses of C3, Ia and other B cell surface receptors could be performed, so we cannot entirely eliminate the possibility that these cells are not B cells. On the other hand, several other examples exist of cytoplasmic Ig^- EBV-transformed cells which are thought to represent early B lineage cells. Surface receptor analyses of Ig^- clones isolated from the peripheral blood of X-linked agammaglobulinemia and chronic lymphocytic leukemia patients have implied that these cell lines are derived from the B cell lineage (1,2). Marker studies of these cells indicated that these cell lines were derived from non-malignant cells, and supported data from other studies in which an Ig^- cell line with B cell surface antigens was isolated from fetal bone marrow (3). HindIII-digested DNA from this cell line was analyzed with a J_H probe and shown to contain only one J_H band in the germline configuration.

Thus, the results from other studies are consistent with the idea that IL-0 may represent a "pre-" pre-B cell line. However, absolute proof that these Ig^- lines are B cell progenitors awaits the ability to induce Ig production in one of these lines, or perhaps, to show DJ rearrangments as has been demonstrated in early B lineage Abelson-virus transformed murine cells (8). It will also be necessary to demonstrate that these lines contain C_H genes on both homologous chromosomes, and that they have not simply lost the ability to produce Ig by C_H gene deletion. Further analyses of these and similar cell lines may be useful in delineating the very early stages in B cell differentiation.

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DISCUSSION

The results discussed in Chapter II of these studies demonstrated that surface expression of IgG_3 is not necessary for switching to the other IgG subclasses in the mouse, and supported a model for direct switching from IgM to each of the other γ subclasses. These data do not, however, rule out the possibility that isotype switching, at the genetic level, might occur by a sequential deletion process. As sequence data becomes available for the $S\gamma$ regions, it will be easier to resolve this question. In fact, data from a recent report in which the primary structure of $S\gamma_3$ and a portion of $S\gamma_1$ have been detailed and used in studying the rearrangements which have occurred in the S regions of an IgG-producing myeloma, show a $S\mu$-$S\gamma_1$-$S\alpha$-$S\gamma_1$ configuration in the recombination site (80). Although the occurrence of apparent $S\alpha$ sequences within $S\gamma_1$ is intriguing and has been observed in another instance as well (52), these data also support a direct model of switching within the γ subclasses since $S\mu$ appears to be rearranged directly with the $S\gamma_1$ region.

In order to learn more about isotype switching at the genetic level, a panel of EBV-transformed human cell lines producing IgM, IgG_1, IgG_3, IgG_4, and IgA_1 antibodies was established. The remainder of this dissertation was devoted to the analyses of the C_H gene rearrangements within these cell lines. As discussed in Chapter III, results were consistent with a deletion model for isotype

switching, although the deletion events occurring on the homologous, non-expressed chromosome did not always parallel deletions on the functional homolog. Results from studies which were conducted concurrently with these studies (81) also support a deletion model of switching in human EBV-transformed cell lines; however, the lack of IgG and IgA subclass identification in these studies did not allow the detection of 5' C_H genes which might have been retained, as was demonstrated in our IL-G4 line.

The variability in the extent of the deletions which occur on the non-functional chromosome has raised several interesting questions concerning the recombination involved in isotype switching. If there is a general recombination mechanism, rather than an isotype specific system in which different enzymes are needed for recombination events to each isotype, then it is not difficult to imagine that such a system might also be involved in the aberrant rearrangements which occur between non-homologous chromosomes, i.e., the 8;14 translocation which can occur in Burkitt's lymphoma cells and often involves the activation of the c-myc gene on chromosome 8 (29,82). In fact, recent evidence from a murine plasmacytoma has shown that a segment containing the Ig C_H gene enhancer from the J_H-S region has been inserted 5' to the mouse c-myc locus on chromosome 15 (83). Future studies may show that the recombination process involved in isotype switching may have applications in other recombination processes as well.

The establishment of a panel of EBV-transformed human cell lines provides several advantages for studying isotype switching. The first advantage is that it has provided an opportunity to study switching in

the human system, a system which has not been studied in as much detail as the mouse system. Secondly, transformation with EBV allows the production of cells from a single individual which synthesize each of the isotypes. Despite the fact that IgG_2 and IgA_2 cell lines were not established in these studies, partially because of the low frequency of IgG_2-producing cells in these transformed cultures (see Appendix 3), it may be possible to establish such lines through improvements in cloning techniques and enrichment for these isotypes by either panning or immunofluorescence cell sorting. Although the effect of EBV-transformation upon the C_H gene locus is still unknown, these cell lines were established from normal individuals and may be more likely to reflect normal C_H gene rearrangements than selected myelomas and hybridomas, which are polyploid. Finally, EBV-transformation provides some advantages over the Abelson virus-transformation system, in that it allows the establishment of cell lines representative of the entire spectrum of B lineage cells, from pre-B cells (H. Kubagawa, unpublished results, 84) to plasma cells, and possibly even earlier B cell progenitors as discussed in Chapter IV.

Many aspects of isotype switching were not directly addressed in this dissertation. One such issue of primary importance is the stage of differentiation at which a cell becomes committed to the production of a single isotype and can no longer undergo isotype switching. The results obtained in Chapter II in which anti-γ_3 antibodies were capable of suppressing the differentiation of IgG_3-producing plasma cells suggest that commitment has occurred by the time a B cell expresses $sIgG_3$. The fact that no examples of spontaneous isotype switching were observed within the EBV-transformed cell lines

described in these studies and representative of later stages in differentiation, also supports the idea that commitment to a particular isotype may occur early in B cell differentiation. These data are consistent with results from other studies in which pokeweed mitogen-treated human cells were not observed to switch isotypes (85). On the other hand, other evidence obtained from splenic focus analyses and limiting dilution analyses suggests that activated B cells may be capable of undergoing isotype switching (77,78). Since examples of isotype switching have been observed in Abelson virus-transformed mouse pre-B cell lines (61,62), it may be possible to develop a better understanding of the differentiation stages at which isotype switching can occur in the human by examining EBV-transformed pre-B cell lines for an analogous system.

There are, of course, many other fascinating aspects of isotype switching. One of the questions which remains unanswered is whether T cells and T cell factors are involved in the instruction of B cells to switch or in the selection and expansion of cells already committed to a particular isotype. Still other questions concern the basis of the defects involved in the immunodeficiency diseases, particularly in hyper-IgM immunodeficiency which has been hypothesized to be caused by a switching defect (8,86). It is hoped that these studies have provided a basis for a better understanding of some of the cellular and molecular aspects involved in isotype switching, and that they may serve as a foundation for future studies leading to an understanding of the defects responsible for immunodeficiency diseases. One might even speculate that these results may prove relevant to aberrant recombination defects which occur in malignant transformation.

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Appendix 1

Immunofluorescence and Karyotypic Analyses of EBV-Transformed Cell Lines

Individual	Cell Line	Isotype	% Cytoplasmic Ig^+	V_H Specificity	Karyotype
IL	IL-M	μ,κ	90	U[a]	N.D.
	IL-0	Ig^-	0	-	N.D.
	IL-G1	γ_1,κ	77	U	N.D.
	IL-G4	γ_4,λ	80	MH-4410	diploid
	ILC10	γ_3,λ	75	U	50% tetraploid
	ILγ8	γ_3,κ	86	N.D.[b]	all tetraploid
	ILγ3	γ_1,κ	82	N.D.	N.D.
ME	ME-M	μ,λ	84	U	few polyploid
	ME-A1	α_1,λ	85	U	N.D.
	ME-G1	γ_1,κ	70	SAF-D44	diploid
TC	TC5A6	μ,κ	80	N.D.	N.D.
	TC5G2	γ_1,λ	67	N.D.	N.D.
	TC6BB2	α_1,λ	73	N.D.	N.D.
PD	PDII-M	μ,κ	78	N.D.	N.D.
	PDII-3c	α_1,λ	87	N.D.	N.D.

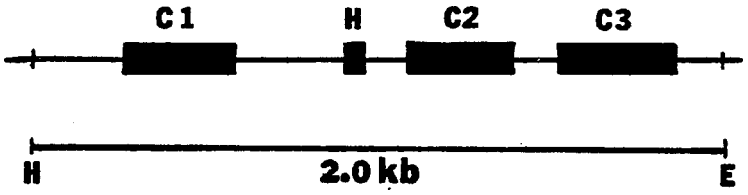
[a]Unknown specificity.

[b]Not done.

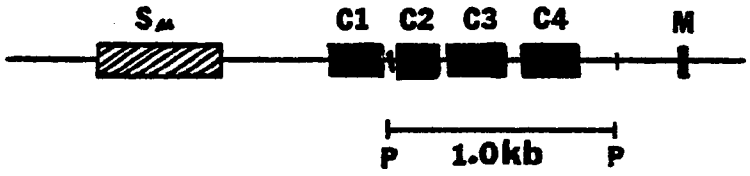
Appendix 2. Human heavy chain probes. Enclosed boxes represent coding regions while dashed boxes represent the switch region sequences. Restriction enzyme sites are indicated as follows: H-HindIII, E-EcoRI, P-PstI, B-BamHI and A-AvaI.

Human Heavy Chain Probes

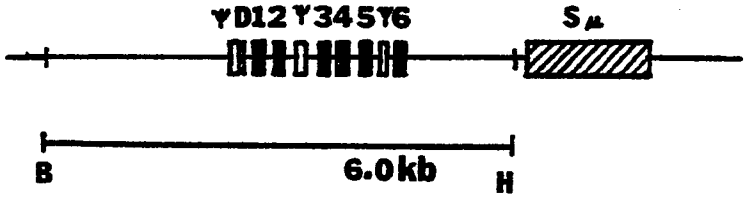
Cγ4



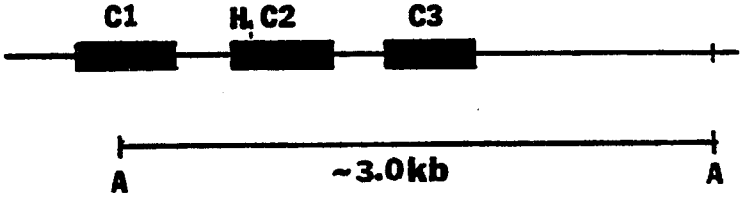
Cμ



J_H



Cα2



Appendix 3

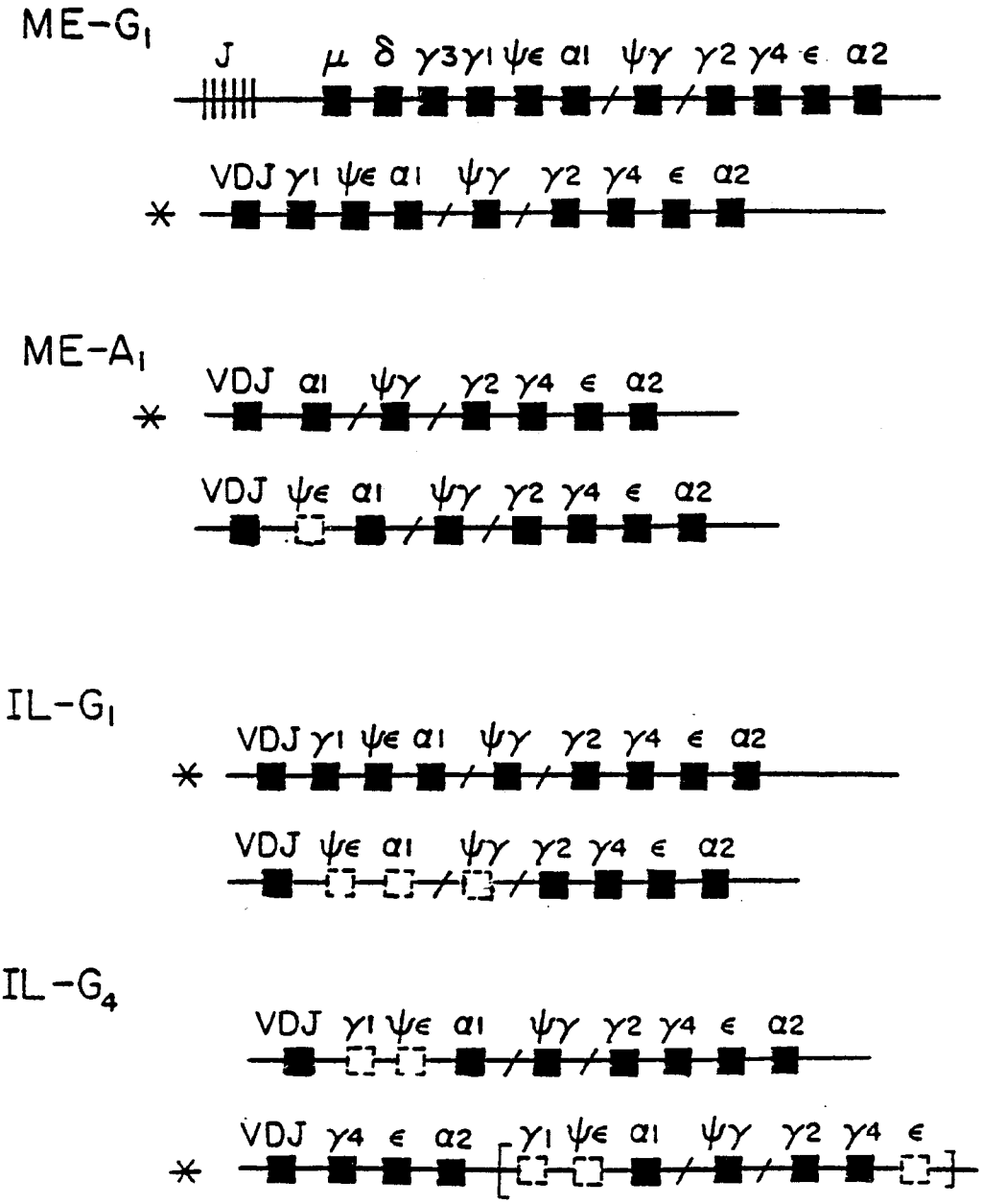
Isotype Distribution in EBV-Transformed Bulk Cultures

Ig Isotype	% Cytoplasmic Ig^+ Cells	
	Individual TC	Individual IL
μ (SA-AB6-4)[a]	34.8	48.5
δ (δ-1A6-2)	2.3	6.4
γ_1 (NL-16)	24.0	23.0
γ_2 (GOM-2)	0.01	<0.01
γ_3 (C3-8-80)	0.28	7.7
γ_4 (#6020)	N.D.[b]	2.0
γ (B1t-G01-4)	23.8	34.0
ε (ε-RB6-2)	<0.01	<0.01
α_2 (14-3-26)	18.4	5.4
α_1 (1-155-1)	8.2	8.1
κ (SA-BA1-M)	49.6	59.6
λ (1-155-2)	46.4	35.4

[a]Murine monoclonal anti-human isotype reagent used for immunofluorescence staining with FITC-goat anti-mouse Ig. (Kubagawa, H., et al. J. Clin. Immunol. 2:264).

[b]Not done.

Appendix 4. Hypothetical models of the heavy chain immunoglobulin gene loci in four EBV-transformed cell lines. Asterisks denote the functional chromosome. Open boxes represent genes which may or may not be present.


ME-G1
J
μ δ γ3γ1 ψε α1 ψγ γ2 γ4 ε α2
VDJ γ1 ψε α1 ψγ γ2 γ4 ε α2
ME-A1
VDJ α1 ψγ γ2 γ4 ε α2
VDJ ψε α1 ψγ γ2 γ4 ε α2
IL-G1
VDJ γ1 ψε α1 ψγ γ2 γ4 ε α2
VDJ ψε α1 ψγ γ2 γ4 ε α2
IL-G4
VDJ γ1 ψε α1 ψγ γ2 γ4 ε α2
VDJ γ4 ε α2 γ1 ψε α1 ψγ γ2 γ4 ε

Appendix 5

Composite List of EBV-Transformed Cell Lines

Source	Cell Line	Date Established	Ig Isotype[a)]	Comments
Individual IL (peripheral blood)	IL0	11-15-82	all	Bulk culture before cloning
	ILC1	2-20-84	$\gamma_1, \gamma_2, \gamma_3$	Not stained for L chains
	ILC5 (IL-G4)	"	γ_4, κ	Reacts with anti-V_H MH4410
	ILC5B1	12-15-84	γ_4, κ	
	ILC5B11	"	γ_4, κ	
	ILC5D8	"	γ_4, κ	
	ILC10	2-20-84	γ_3, λ	
	ILγ4F9(IL-G1)	12-12-82	γ_1, κ	
	ILγ1	9-11-84	γ_1	Not stained for L chains
	ILγ2	"	γ_1, γ_3	" " " " "
	ILγ3	"	γ_1, κ	
	ILγ31F12	12-15-84	γ_1, κ	

a)Isotypes assigned by immunofluorescence staining for cytoplasmic Ig.

Appendix 5 (continued)

Source	Cell Line	Date Established	Ig Isotype	Comments
Individual IL (peripheral blood)	IL4γ	9-11-84	γ_1	Not stained for L chains
	IL5γ	"	γ_1	" " " " "
	IL6γ	"	γ_1	" " " " "
	IL7γ	"	γ_1	" " " " "
	IL8γ	"	γ_3,κ	
	IL4°1	1-3-83	μ,κ	Originally did not secrete; later μ^+
	IL4°2	"	μ,λ,κ	Originally did not secrete; later μ^+
	IL4°3	"	μ,κ	Originally did not secrete; later μ^+
	IL4°7	"	μ,κ	Originally did not secrete; later μ^+
	IL4°7-2 (IL-0)	7-17-84	none	Subcloned from IL4°7; lost
	IL4°7-3	"	μ,κ	" " "
	IL4°7-4	"	μ,κ	" " "
	IL4°7-5	"	μ,κ	" " "

Appendix 5 (continued)

Source	Cell Line	Date Established	Ig Isotype	Comments
Individual IL (peripheral blood)	IL4°7-7	7-1-84	μ,κ	Subcloned for IL4°7
	IL4°7-8	"	μ,κ	" " "
	IL4°7-9	"	Ig^-, few μ	" " "
	IL4°-10	"	μ,κ	" " "
	IL4°7-11	"	μ,κ	" " "
	IL4°7-12	"	μ,δ,κ	" " "
	IL4°7-13	"	Ig^-, few μ	" " "
	IL4°9	1-3-83	μ,λ, few μ	Originally did not secrete; later μ^+
Individual ME (peripheral blood)	U01	10-19-82	$\mu,\alpha,\kappa,\lambda$	
	U01'(ME-A1)	11-19-82	α_1,κ	Subcloned from U01
	U01"(ME-M)	"	μ,λ	" " "
	U02	10-19-82	-[b]	Secreted μ

b)-, not done.

Appendix 5 (continued)

Source	Cell Line	Date Established	Ig Isotype	Comments
Individual ME (peripheral blood)	U03	10-19-82	$\mu,\alpha,\kappa,\lambda$, few γ	Lost
	U03'	12-2-82	μ,λ	
	U03's	1-9-84	μ,λ	
	U03-4	7-17-84	$\mu,\alpha,\lambda,\kappa$	
	U03-5	"	μ,κ,	
	U04	10-19-82	γ_1,κ	
	U04' (ME-G1)	12-2-82	γ_1,κ	Reacts with anti-V_H SAFD44
	U05	10-19-82	γ_1,κ,λ	
	U013	"	μ,λ	
	U019	11-12-82	$\mu,\alpha,\kappa,\lambda$	(Subclone of U01)
Individual JO (peripheral blood)	J014	10-18-82	α,λ	
	J024	"	α_1,κ	
Individual CT (peripheral blood)	CT2B1	3-7-83	μ,λ, few α	
	CT3C7	"	$\mu,\alpha,\lambda,\kappa,\lambda$	

Appendix 5 (continued)

Source	Cell Line	Date Established	Ig Isotype	Comments
Individual CT (peripheral blood)	CT1D3	3-7-83	μ, κ, λ	
	CT1E4	"	μ	Not stained for L chains
	CT1E6	"	μ, α	
	CT1E8	"	$\mu, \alpha, \lambda, \kappa$	
Individual CM (peripheral blood)	CME	11-4-82	-	Bulk culture, not cloned
	CM2	12-12-82	μ, κ	
	CM4	"	μ, κ	
	CM5	"	μ, κ	
Individual BR (peripheral blood)	BR3A8	3-29-83	μ	Not stained for L chains
	BR3E1	"	μ	" " " " "
Individual PD (peripheral blood)	PDII3Cα	3-27-83	α_1, κ	Original culture transformed by M. Crain
	PDIIM	12-15-84	μ, κ	

Appendix 5 (continued)

Source	Cell Line	Date Established	Ig Isotype	Comments
Individual JB (peripheral blood)	JB3D9	1-9-84	γ_1,γ_4	Original culture transformed by M. Crain Not stained for L chains
	JB3D9-1	12-15-84	-	Original culture transformed by M. Crain Not stained for L chains
	JB4F11	1-9-84	γ_1	Original culture transformed by M. Crain Not stained for L chains
	JB4G12	"	γ_1	Original culture transformed by M. Crain Not stained for L chains
	JB6B8	"	μ,γ	Original culture transformed by M. Crain Not stained for L chains
	JB6C1	"	μ,λ	
Individual JG	J. Glosser	5-23-84	all	Peripheral blood, bulk culture from family studies. For DNA analyses, see G. Borzillo.

Appendix 5 (continued)

Source	Cell Line	Date Established	Ig Isotype	Comments
Individual BG	B. Glosser	9-16-84	all	Peripheral blood, bulk cultures from family studies. For DNA analyses, see G. Borzillo.
" RG	R. Glosser	5-23-84	all	Peripheral blood, bulk cultures from family studies. For DNA analyses, see G. Borzillo.
" MG	M. Glosser	9-16-84	all	Peripheral blood, bulk cultures from family studies. For DNA analyses, see G. Borzillo. IgG_2-Deficient?
Spleen 1	TC spl.	9-1-83	all	Bulk culture, thrombocytopenia patient
	TC6AF3	12-15-84	α_1	Not stained for light chains
	TC5D4	"	γ	" " " " "
	TC6BB2	"	α_1,λ	
	TC6AC11	"	γ	Not stained for light chains

Appendix 5 (continued)

Source	Cell Line	Date Established	Ig Isotype	Comments
Spleen 1	TCAG11	12-15-84	γ_1	Not stained for light chains
	TC5B5	"	γ_1	" " " "
	TC5A6	"	μ,κ	
	TC5G2	"	γ_1,κ	
Individual IC	IC0	4-7-83	-	Bulk culture, pre-B leukemia patient, no EBV
	ICE	"	$\mu,\gamma,\alpha,\kappa,\lambda$	Bulk culture, pre-B leukemia patient, with EBV
Individual BB	11-57	11-9-83	μ,κ,λ	Bulk culture, X-linked agammaglobulinemia patient, 6.2% pre-B cells
Fetus 3 (bone marrow)	FB3	1-31-83	μ,λ,κ	12-14 week-old fetus, very few pre-B's
	FB3-3C1	4-29-83	-	
	FB3-1B10	"	-	

Appendix 5 (continued)

Source	Cell Line	Date Established	Ig Isotype	Comments
Fetus 3 (bone marrow)	FB3-1A10	4-29-83	-	
	FB3-1A5	"	-	
	FB3-2B7	"	-	
	FB3-2F10	"	-	
	FB3-1B1	"	-	
	FB3-1B3	"	-	
	FB3-1A1	"	-	
	FB3-2A6	"	-	
Fetus 8 (bone marrow)	FB8-5E11	3-2-83	-	14 1/2 week-old fetus
	FB8-5A10	"	none	
Fetus 13 (bone marrow)	FB13-4D8	3-2-83	μ,κ, few λ	16 1/2 week-old fetus
Fetus 26 (bone marrow)	FB26	5-3-83	μ,λ,κ, few γ	17 week-old fetus, some pre-B's

Appendix 5 (continued)

Source	Cell Line	Date Established	Ig Isotype	Comments
Fetus 27 (bone marrow)	FB27	5-3-83	μ,λ,κ	15 1/2 week-old fetus, few pre-Bs
	FB273C5	7-27-83	-	
	FB273H1	"	-	
	FB2-3H5	"	-	
Fetus 28 (bone marrow)	FB28	5-3-83	μ,λ,κ,few α	15 week-old fetus
	FB281F2	7-27-83	-	
	FB281D5	"	-	
Cord blood	M.K. Cord blood	2-29-84	all	Established by M. Kiyotaki
Individual H. Ky	Hiromi (M.K.)	3-7-84	all	Duplicates of normal adult peripheral blood bulk cultures established by M. Kiyotaki
" St.	Steve (M.K.)	"	"	Duplicates of normal adult peripheral blood bulk cultures established by M. Kiyotaki

Appendix 5 (continued)

Source	Cell Line	Date Established	Ig Isotype	Comments
Individual Bea	Beatrice (M.K.)	3-7-84	all	Duplicates of normal adult peripheral blood bulk cultures established by M. Kiyotaki

GRADUATE SCHOOL
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