standard microbiological media. Failure of the organism to induce proliferation of HRT cells at 27°C was probably due to the quiescent nature of HRT cells at this temperature. Interestingly, fungal growth was not markedly inhibited and was independent of HRT proliferation. The ability of *R. seeberi* to induce cellular proliferation appears to require close contact with actively growing organisms and associated mammalian cells, as empty sporangial shells or organisms that attached but failed to grow did not induce this response. When cryopreserved *R. seeberi* were placed in culture, both fungal development and HRT proliferation were delayed by several days in comparison with freshly collected organisms (*12*). This delay appeared to be associated with the lag in development that followed recovery from −70°C storage conditions. Results from Table 1 suggest that a period of development or aging (or both) might be required under some circumstances before spores are capable of further development. This finding may help explain why transmission does not occur directly between hosts (*1*).

Utilization of an in vitro method that supports complete development of *R. seeberi* may facilitate study of its life cycle and assist in development of effective pharmacologic agents for treatment of this infection. The mechanism by which this organism induces cellular proliferation may now be investigated.

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11. After spores were incubated for 101 days at 23°C, with medium changed approximately at weekly intervals, a suspension of several hundred rhinosporidium organisms (average diameter of 15.9 ± 6.5 μm, $n = 25$) per milliliter of medium was made in protein free Dulbecco's modified Eagle medium. No sporangia were identified in this suspension. The nasal mucosa of three male nude mice was scarified and then 0.2 ml of the protein-free rhinosporidium suspension was injected intranasally. Two-tenths of the rhinosporidium suspension was also injected in the right flank of each of these male mice and 0.2 ml was injected intraperitoneally into three female nude mice. Sixty and ninety days after inoculation one male and one female mouse were killed by cervical dislocation. There were no significant macroscopic or microscopic lesions in the mice. The remaining mice were examined periodically for 1 year, and no macroscopic lesions were observed.
12. M. G. Levy and N. Russel-Henry, unpublished data.

13. Funds for this project provided by the state of North Carolina.

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Human Monoclonals from Antigen-Specific Selection of B Lymphocytes and Transformation by EBV

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Hybridoma technology has made it possible to prepare monoclonal antibodies with the use of murine lymphocytes. Attempts to extend this technology to the human level, however, have met with difficulties. A method has been developed for making human monoclonal antibodies of predetermined specificity. Biotinylated antigens (human thyroglobulin or tetanus toxoid) were incubated with human B lymphocytes from peripheral blood. The lymphocytes to which the antigens bound were selected by fluorescence-activated cell sorting. Positively selected (high fluorescence) and negatively selected (low fluorescence) cells were then transformed with Epstein-Barr virus (EBV) and grown in microculture wells. All wells from the positively selected fraction produced antigen-specific antibody (95 to 1800 nanogram-equivalents per milliliter), whereas fewer than 6% of the wells from negatively selected fraction made any detectable antibody (less than 10 nanogram-equivalents per milliliter). When the positively selected EBV-transformed cells were cultured in limiting dilution, clones were obtained that made antigen-specific monoclonal antibodies. By this method, monoclonal antibodies to both foreign antigens and autoantigens can be prepared from the normal human B-cell repertoire.

In 1975 KÖHLER AND MILSTEIN described a method for making antibodies in vitro by fusing spleen cells from immunized mice with mouse myeloma cells (*1*). The resulting hybridomas produced large quantities of antigen-specific monoclonal antibodies. This technique has revolutionized immunology and is widely used. Attempts to extend this approach to the production of human monoclonal antibodies, however, have met with difficulty for several reasons (*2–5*): (i) the lack of good human cells for fusion partners (that is, myeloma cells), (ii) the low frequency of fusion events (approximately 10^{-5}), and (iii) the relative paucity of circulating B cells with given specificity in human subjects who cannot be actively immunized with certain antigens. These factors reduce the probability of obtaining cell lines secreting antibodies of predetermined specificity.

We now describe an alternative approach for making human monoclonal antibodies. It takes advantage of the fact that receptors (that is, immunoglobulin molecules) for antigens are expressed on the surface of B lymphocytes in the normal B-cell repertoire, and, thus, B cells capable of making antibody with a desired specificity can be separated from irrelevant B cells by using the antigen in question as a probe. In our system, purified antigens are biotinylated and incubated with B lymphocytes from peripheral blood. The B lymphocytes to which the antigens bind are selected by fluorescence-activated cell sorting (FACS). The sorted cells are then immortalized by infection with Epstein-Barr virus (EBV) and propagated in culture. Under these conditions B cells from normal individuals can be used to generate clones secreting human monoclonal antibodies of selected antigen specificity.

Experiment 1 was designed to investigate the binding of biotinylated ligands to B

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lymphocytes. The B lymphocytes were purified from peripheral blood of healthy subjects (*6*). Human thyroglobulin (Tg) was purified and biotinylated (*7*). Human B cells were incubated with biotinylated Tg, and after being washed they were reacted with fluorescein isothiocyanate (FITC)–avidin (*8*). After a further washing, cells were analyzed by FACS. Approximately 7% of the cells incubated with biotinylated Tg and FITC-avidin displayed a higher degree of fluorescence than their counterparts incubated with FITC-avidin only (Fig. 1A).

We then isolated the lymphocytes that bound the biotinylated Tg. Cells were sorted, and the positive fraction (high fluorescence intensity) and negative fraction (low fluorescence intensity) were collected and transformed with Epstein-Barr virus (EBV) (*9*) (Fig. 1). These cells were then resuspended and distributed at 4000 cells per well in a 96-well, U-bottom culture plate containing 5×10^4 irradiated (2500 rads) syngeneic human peripheral blood mononuclear cells as a feeder layer. After 18 days of incubation, culture fluids were harvested and assessed for antibody content. All 48 wells from the positively sorted fraction produced high concentrations of antibody to Tg (95 to 200 ngeq/ml) (Fig. 2A). In contrast, only 1 of 48 wells from the negatively sorted fraction produced any detectable antibody (>10 ngeq/ml). Experiments with lymphocytes from three other healthy donors yielded similar results. All of the antibodies that reacted with Tg were of the immunoglobulin M (IgM) class (*10, 11*).

To see if the approach used to obtain B human lymphocytes that reacted with Tg could be applied to other antigens, we obtained peripheral blood from healthy donors who had not recently received a booster dose of tetanus toxoid (TT). The B lymphocytes from these donors were incubated with biotinylated TT and subsequently with FITC-avidin, and finally applied to FACS. Approximately 8% of the cells displayed a higher degree of fluorescence than their counterparts incubated with FITC-avidin only (Fig. 1B). The cells were then sorted, and the positive and negative fractions were transformed with EBV and distributed in culture with feeder layers. After 18 days of incubation, the culture fluids were harvested and analyzed for antibody to TT. All 45 wells from the positively sorted fraction produced high concentrations of antibody to TT (100 to 1800 ngeq/ml), whereas only 3 of 48 wells from the negatively sorted fraction produced any detectable antibody (>10 ngeq/ml) (Fig. 2B). All of the antibodies to TT were of the IgM class (*10, 11*).

To see if antigen-selected, EBV-transformed cell lines would actually secrete monoclonal antibodies, we studied Tg-binding B cells in greater detail. Cells from the positively selected fraction that had been transformed with EBV were cloned in limiting dilutions at ten, five, two, and one cells per well in the presence of an allogeneic-irradiated feeder layer. Over a period of 4 months, 14 clones were derived from three sequential clonings at different numbers of cells per well (Table 1). Cell lines generated after three cloning steps produced amounts of Tg antibody ranging from 62 to 1200 ngeq/ml. All were IgM antibodies with κ light chains. The flow cytometry profile of one of the expanded cell clones (clone P16 2A1.3) suggested that virtually all cells bound Tg (Fig. 1C), compared with the initial uncloned cell population (Fig. 1A).

Positive selection of antigen-specific human B lymphocytes has been attempted previously by using, in particular, erythrocytes coated with selected molecules (*12, 13*). Expansion and cloning of B-lymphocyte cultures by infection with EBV to produce human antibodies has also been used by us and other investigators with variable success (*3–5, 14, 15*). The studies reported here show that antigen-specific lymphocytes from human peripheral blood can be selected by using the appropriate biotinylated ligand, FITC-avidin, and fluorescence-activated cell sorting. The selected B cells can be transformed by EBV, expanded in culture, and cloned to yield cell lines secreting monoclonal antibodies of predetermined specificity. This approach seems to be applicable to the selection and immortalization of lymphocytes producing antibodies to a variety of different antigens. In fact, we have recently prepared EBV-transformed B cells secreting antibodies to insulin and β-galactosidase in addition to Tg and TT. One other advantage of this procedure is that biotinylation of even small peptides does not usually interfere with their binding activity, given the relatively small size of the biotin molecule itself (approximately 341 daltons).

In our experiments, the clones producing antibodies were stable for a number of months. Some clones, however, stopped producing antibodies or the antibodies they made no longer bound to the relevant antigens. This change may be due to the unstable nature of the transformed cells or to a high rate of spontaneous mutation of the genes coding for the variable portion (antigen-binding site) of the antibody molecule. Repeated cloning of the cells helped to

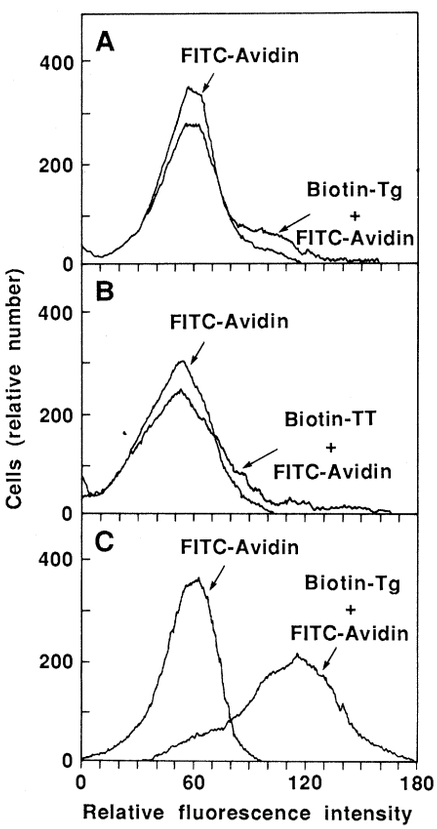

Fig. 1. (A) FACS analysis and sorting of human B lymphocytes reacting with biotinylated human Tg. B lymphocytes were incubated with biotinylated Tg, washed, and then reacted with FITC-avidin or in a parallel procedure reacted with FITC-avidin only (*8*). Cells incubated with FITC-avidin only were analyzed first, and their fluorescence profile was obtained. When Tg-reacted B cells were applied to the FACS and analyzed, a different fluorescent profile was obtained. Channel 90 (relative fluorescence intensity) provided a threshold above which the number of cells incubated with biotinylated Tg and FITC-avidin was much higher than that of cells incubated with FITC-avidin only; this channel was arbitrarily chosen as the cut-off point between "negative" and "positive" cells. Lymphocytes incubated with biotinylated Tg and FITC-avidin were applied to the FACS and sorted. About 10^6 B cells with high fluorescence intensity (positive) and about 10^7 B cells with low fluorescence intensity (negative) were collected in ice-chilled sterile tubes containing fetal bovine serum (FBS). After careful washings with warm RPMI 1640 medium containing 10% FBS (FBS-RPMI), 5×10^5 cells from either fraction were infected with 5×10^5 transforming units of EBV and then distributed in microcultures. (B) FACS analysis and sorting of human B lymphocytes reacting with biotinylated TT. Human B lymphocytes were incubated in ice-cold BSA-HBSS containing or not containing biotinylated TT ($1.8 \times 10^{-8} M$). Both cell samples were then treated with FITC-avidin and applied to the FACS under conditions similar to those described for (A). Channel 90 was again chosen as the cut-off point. After sorting, both positive and negative fractions were infected with EBV and distributed in cultures as described in (A). (C) EBV-transformed cells from clone P16 2A1.3 (5.5.1) (Table 1) were incubated with biotinylated Tg and FITC-avidin or with FITC-avidin only under experimental conditions similar to those described in (A) and then analyzed by flow cytometry.

maintain antigen-specific antibody production.

Most of the clones produced antibodies of the IgM class. This is not surprising since IgM-bearing B cells constitute the majority (>95%) of the circulating B lymphocytes in normal subjects, with only a minor fraction being constituted by lymphocytes bearing and capable of producing IgG, IgA, IgE, or IgD. Clones producing IgG antibodies to Tg, however, were generated from peripheral blood of patients with Hashimoto's thyroiditis (Table 1), and clones producing IgG antibodies to insulin and TT were generated from peripheral blood of patients with type I (insulin-deficient) diabetes and subjects recently immunized with TT, respectively (*16*). Thus, the IgM-producing clones from nonimmunized subjects seem to represent silent, most likely virgin, B cells present in the normal repertoire that can be activated in vitro by EBV. In contrast, the IgG-producing B cells from patients with autoimmune thyroiditis or the IgG-producing B cells from subjects recently vaccinated with TT probably represent actively secreting IgG B cells, memory B cells present in the peripheral circulation, or both.

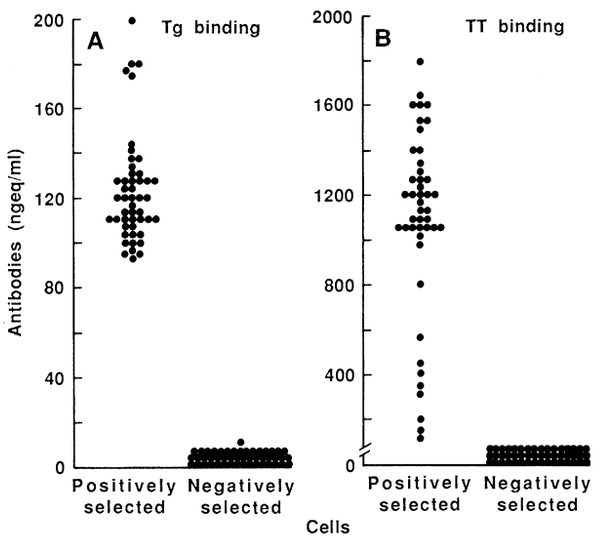

Fig. 2. Detection of Tg- or TT-binding antibodies in fluids from microcultures containing selected B lymphocytes infected with EBV. Enzyme-linked immunosorbent assay (ELISA) was used for the titration of human antibodies to Tg (A) and TT (B). Polystyrene plates (Immunlon II, Dynatech, McLean, VA) were coated with purified Tg (5 μg/ml) or TT (1 μg/ml) in the presence of 0.1*M* carbonate buffer, *p*H 9.5, during a 24-hour incubation period at 4°C. Plates were stored at −20°C until used. Culture fluids were incubated in appropriate antigen-coated plates for 2 hours at room temperature. After the plates were washed with PBS, Tween-20 (0.05%), affinity-purified peroxidase-conjugated goat $F(ab')_2$ fragment to human immunoglobulin was added and allowed to react for 2 hours at room temperature. After the plates were again washed, bound enzyme-linked probes were detected by using *o*-phenylenediamine (OPD) and H_2O_2 as a substrate. The enzymatic reaction was stopped by adding H_2SO_4 at the appropriate time. Reference binding curves for Tg-binding antibodies and TT-binding antibodies were constructed by using human antibodies to Tg and TT derived from Ig fractions from patients with Hashimoto's disease and subjects previously vaccinated with TT, respectively. The specific antibody had been estimated in a precipitation curve with [^{125}I]Tg or [^{125}I]TT labeled by chloramine T. Dots represent the concentration of Tg- or TT- binding antibodies expressed in nanogram-equivalents per milliliter in each of the microculture wells. In both the Tg and TT binding assays, the lower limit of "linear" detectability was 10 ngeq/ml.

Table 1. Human monoclonal antibodies produced by sequentially cloned B lymphocytes that had been positively selected for binding to biotinylated Tg and transformed by EBV.

Clones	Number of cells per well in sequential cloning steps*			Heavy-chain type	Light-chain type	Tg-binding activity (ngeq/ml)†
	I	II	III			
P16 2A1.1	5	10	2	μ	κ	140
P16 2A1.3	5	5	1	μ	κ	62
P16 2A1.5	5	10	1	μ	κ	65
P16 2A1.6	5	2	2	μ	κ	140
P16 2A1.8	5	5	2	μ	κ	1200
P16 2A1.9	5	5	1	μ	κ	920
P16 2A1.13	5	10	2	μ	κ	105
P16 2A1.14	5	2	2	μ	κ	75
P16 2A1.15	5	2	2	μ	κ	65
P16 2A1.16	5	10	2	μ	κ	160
P16 2A1.18	5	10	2	μ	κ	500
P16 2A1.19	5	2	2	μ	κ	80
P16 2A1.20	5	2	2	μ	κ	65
P16 2A1.27	5	10	2	μ	κ	130
P33.7‡			10	γ	κ	>200
P32.10‡			10	γ	κ	>200
P33.15‡			10	γ	κ	>200

*Cells per well at which fewer than 20% of the wells contained growing cells. Cells were cloned sequentially three times by limiting dilutions. †Amount of antibody to Tg produced over a 4-week period in the last cloning step. ‡Characterization of antibodies produced by clones P33.7, P32.10, a P33.15 is included for comparison. These clones were derived from two patients with Hashimoto's disease.

The success in making monoclonal antibodies in mice by hybridoma technology is largely due to the lack of any restriction in repeated immunization of animals and the ease in obtaining spleen cells. Actively immunizing humans with certain antigens is often impossible, as is obtaining human spleen cells. Our studies suggest that antigen selection and transformation of lymphocytes from the normal B-cell repertoire may be an alternative way of preparing antibody-producing clones to desired antigens without in vivo active immunization. In the case of immunized subjects or patients with autoimmune disease, antigen-specific selection and transformation of B lymphocytes with EBV might be useful in generating antibody-secreting clones from cells involved in the ongoing in vivo immune process.

We have shown that a high percentage of lymphocytes in the B repertoire of unimmunized subjects are capable of making antibodies that bind to self or nonself antigens. This result may be explained by two facts: (i) the relatively large size of the antigens used (Tg, 640,000 daltons; TT, 120,000 daltons) means that there are a number of epitopes on each molecule to which lymphocytes from the B-cell repertoire may react; and (ii) it is becoming increasingly clear that a single antibody molecule may react with more than one epitope, but with different affinities (*2, 17*). Thus, in the selection procedure, some lymphocytes might have bound the biotinylated antigens at relatively low affinity and, in fact, have a higher affinity for other antigens (*11*).

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with iron carbonyl particles and subsequent removal of iron-loaded monocytes by a magnet. This mononuclear fraction was incubated in ice with AET (2-aminoethylisothioronium bromide hydrobromide)-treated sheep red blood cells (SRBC) to allow for rosette formation. Non-erythrocyte (E) rosette-forming cells were recovered after application of the whole SRBC mononuclear fraction to a lymphocyte-separating medium gradient. This nonadherent non-E rosetting fraction was constituted of at least 50% B cells, less than 1% monocytes, and variable amounts of lymphocytes with the natural killer (NK) phenotype, as assessed with mouse monoclonal antibodies OKB7, B77.1, and B73.1, respectively. We refer to this non-E rosetting B-enriched lymphocyte fraction as B cells.

7. Human Tg was isolated from normal thyroid tissue obtained at autopsy [S. H. Roman, F. Korn, T. F. Davies, *Clin. Chem.* **30**, 246 (1984)]. Tissue was homogenized in phosphate-buffered saline (PBS) (*p*H 7.2) and centrifuged at 100,000*g*, and the supernatant was applied to a Sephadex G-200 (Pharmacia) column. The Tg was purified to homogeneity by application of the first eluted peak to a Sepharose 6B (Pharmacia) column. Tg was stored in aliquots at −70°C. Semipurified tetanus toxoid (TT) was obtained from the Commonwealth of Massachusetts (Department of Health, Boston, MA) and fractionated to homogeneity by gel filtration on a Sephadex (Pharmacia) G-150 column. Both Tg and TT were labeled with *n*-hydroxysuccinimidobiotin (Sigma) in 0.1*M* carbonate buffer, *p*H 8.5, at a protein-to-biotin ratio of 4:1 followed by exhaustive dialysis against PBS.
8. Approximately 2×10^7 B cells (5×10^6 per milliliter) were incubated for 2 hours in ice-chilled sterile Hanks balanced salt solution without Ca^{2+} and Mg^{2+}, without phenol red, and with 1% bovine serum albumin (BSA-HBSS) containing biotinylated Tg ($4.50 \times 10^{-9}M$). The cells were washed with cold BSA-HBSS and then allowed to react with FITC-avidin ($1.56 \times 10^{-7}M$) in cold BSA-HBSS for 1 hour. A smaller sample of B cells (10^6) was simultaneously incubated with BSA-HBSS devoid of biotinylated Tg and allowed to react with FITC-avidin under similar conditions. After further washing with cold BSA-HBSS, cells from both samples were resuspended at a density of 10^6 cells per milliliter in the same medium and at different times applied to a Becton and Dickinson 440 FACS with an Argon 466 laser.
9. EBV used to infect B lymphocytes was obtained from culture fluids of B95-8 marmoset lymphoma cells incubated at 37°C in the presence of $1.62 \times 10^{-8}M$ 4-phorbol 12β-myristate 13α-acetate (TPA, Sigma). This virus preparation had a titer of 5×10^5 transforming units per milliliter, one unit being the minimum amount of virus-transforming 10^4 purified human B cells.
10. Limiting dilution experiments were performed to assess the degree of enrichment after cell sorting. The ratio of B cells producing IgM reactive with Tg to total B cells producing IgM was: unsorted cells, 1:100; positively sorted cells, 1:3.5; and negatively sorted cells, 1:10,000. The ratio of B cells producing IgM reactive with TT to total B cells producing IgM was: unsorted cells, 1:80; positively sorted cells, 1:2.5, negatively sorted cells, 1:50,000.
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Synchronized Rearrangement of T-Cell γ and β Chain Genes in Fetal Thymocyte Development

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Kinetics of mouse T-cell γ gene rearrangements in ontogeny were determined as an approach to understanding the possible role of these genes in the development of fetal thymocytes. Two of these genes (Cγ1 and Cγ2) rearranged rapidly during days 14 to 17 of the gestational period in BALB/c mice. Moreover, these rearrangements seemed to be tightly synchronized with rearrangements of T-cell receptor β chain genes in the same cells. It is suggested that the early transcriptional activity of γ genes, which precedes that of β chain genes, may not reflect the functional activation of these genes. Nevertheless, productive and therefore potentially functional γ gene rearrangements precede surface expression of T-cell receptors in the thymus by 2 to 3 days, which is compatible with a role for γ gene products in thymocyte development prior to antigen-specific stages.

WHEN ATTEMPTS WERE BEING made to clone the genes of the α and β chains of T-cell receptors for antigen, a third, structurally similar group of genes was found and designated γ (*1, 2*). The organization of these γ genes in variable (V), joining (J), and constant (C) segments placed them within the immunoglobulin-like superfamily of cell-surface receptor genes. Beyond this, T-cell specific rearrangement and expression and high concentrations of γ messenger RNA (mRNA) in fetal thymus populations at days 14 and 15 suggested a role for these genes early in T-cell development (*1, 3–5*). At least in humans, the protein chains encoded by γ genes have now been found (*6, 7*), but their possible functions have remained uncertain.

To help clarify this issue we have analyzed the developmental period in which γ genes rearrange to form the complete VJC-structures that are necessary for function. This study was carried out with a large panel of mouse fetal thymus hybridomas made by fusing thymocytes from fetal mice of different ages to the AKR thymoma, BW5147. These hybridomas appear to be representative of the thymocyte populations from which they were prepared because, for example, the occurrence of β gene rearrangements in the hybridomas correlates well with the kinetics of these events in unfractionated thymocytes (*8, 9*). The studies we report here allowed us to establish the point in fetal life at which γ protein might begin to function and to correlate this time with other events in T-cell differentiation, in particular β chain gene rearrangements (*10*) and intrathymic expression of the α/β heterodimeric receptor for antigen (*11*).

There are at least three highly homologous γ constant regions in the mouse genome (*12*), located on chromosome 13 (*13*). A fourth Cγ gene with little homology to the others has recently been found (*14*). We have analyzed rearrangements associated with the two functional genes of the three Cγ genes that were first described by Hayday *et al.* (*12*). These Cγ segments have been found on three restriction fragments after digestion of germline DNA with the restriction enzyme Eco RI, and have been named correspondingly Cγ 10.5, Cγ 13.4 and Cγ 7.5. In the meantime, a more simple nomenclature has been used by several groups, in which the four known γ constant regions have been assigned arbitrary numbers. In this report we have followed the terminology used recently by Iwamoto *et al.* (*14*): Cγ 10.5 = Cγ1, Cγ 3.4 = Cγ2, Cγ 7.5 = Cγ3, a nonfunctional gene, and the new γ constant region = Cγ4.

Consistent with previous studies we found that Cγ1 and Cγ2 were frequently rearranged in our hybridomas. However, somewhat surprisingly in the view of previous examinations of γ mRNA levels in early fetal thymocytes, we found rearrangements involving Cγ1 and Cγ2 to be tightly synchronized with T-cell receptor β chain gene

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