CLONAL AND MOLECULAR CHARACTERISTICS OF THE HUMAN IgE-COMMITTED B CELL SUBSET

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Allergic and atopic diseases associated with abnormalities in IgE production may represent the most frequent immunological disorders. However, the IgE response and its regulation, the normal role of reaginic Igs, and underlying reasons for the high incidence of abnormal IgE responses are not fully understood. In normal peripheral blood, IgE-committed B lymphocytes are rare. Reported frequencies range from 1 in 3–5 $\times$ 10^3 to as low as one in 10^5 B cells (1–3). While the low frequency of IgE-committed cells is mirrored in low (ng/ml) IgE serum levels, the biological effectiveness of IgE is considerable. After IgE binding to the receptor for the Fc region of the IgE molecule (FcϵRI)[1] on mast cells and basophils (4), the crosslinking of cell-bound IgE by allergen causes release of histamine, kinins, and other products of cell stimulation. This, in turn, leads to allergic reactions that can include anaphylaxis and death (4).

The effectiveness of IgE in this process derives from the high affinity of FcϵR binding (K_d, 10^9 M^{-1}). High affinity FcϵRs are able to bind IgE at 10,000-fold lower concentrations than Fcγ receptors on monocytes can bind IgG (reviewed in reference 5). More recently, a second class of related but distinct FcϵRs has been identified. In contrast to FcϵRI, the binding of IgE to this low affinity FcϵR (FcϵRII) is closer in affinity to FcγR/IgG binding. Cell-bound and soluble FcϵRII-related molecules are presently thought to play a role in the regulation of ϵ gene expression. However, FcϵRII is expressed by several different cell lineages and it remains unclear exactly where and how the expected IgE isotype specificity in regulation is conferred and where and how this process fails in atopy or allergy.

Considerable progress has been made in delineating regulatory T cell functions that govern IgE responses in parasite-infected animals and those in normal or atopic humans (6, 7). Products of certain T cell lines/clones were found to alter IgE gene expression. These cells often express low affinity FcϵRs and produce IgE-binding factors that either suppress or enhance IgE secretory rates, typically in myeloma lines or fresh B cells (8–10). Similar regulatory activities have been detected in T cells from normal and atopic donors (11–13). Some of the regulatory molecules in-

[1] Abbreviations used in this paper: FcϵR, receptor for the Fc portion of IgE; MCS, minimum χ^2; ML, maximum likelihood; PI, propidium iodide; SCE, sister chromatid exchange; sIg, cell surface Ig.

volved have now been molecularly cloned and shown to be analogous to a region of the FcϵR (14).

As the source of reaginic Ig molecules, the B cells committed to IgE secretion must play a central role in the allergic response. Understanding the differentiation pathway that generates IgE-producing cells may be as important to our understanding of allergy as the study of cells at the end of this developmental pathway. With much work focusing on T cell effects and on terminally differentiated IgE-secreting myeloma cell lines, relatively little information is available that characterizes the IgE-committed B cell sublineage.

Investigations of the IgE-producing B cells themselves have been hindered not only by their low frequency but by the difficulty to activate and propagate them in vitro. Standard culture systems have proven unsatisfactory in generating reasonable numbers of progeny cells or cell lines (15, 16). However, we and others (2, 3) successfully activated similar frequencies of IgE-secreting cells in limiting dilution culture systems supplemented with allo-activated T cells or after infection with EBV. These low density cultures have a much higher efficiency than do mass cultures and they reduce suppressive cell interactions that interfere with IgE responses (17, 18).

The use of EBV in this system previously allowed us to generate one stable IgE-secreting clone (K1D5) that unexpectedly produced IgM in addition to IgE and that appeared not to have deleted DNA between VDJ (variable/diversity/joining regions) Cμ, and Cϵ constant region genes (3, 12, 19). This contrasts with the prototype human IgE-secreting cell line, the myeloma U266, which produces only IgE and has deletionally rearranged VDJ in an isotype switch from Cμ to Cϵ (20). In the experiments described here, we asked whether the K1D5 line was a rare or atypical cell in the differentiation pathway of IgE-committed B lymphocytes or whether it represented a normal member of this B cell subset and the pathway itself was in some way distinct from that of other isotypes.

During normal B cell differentiation, cells first express IgM on their surface and then become cell surface Ig$^+$ (sIgM$^+$/sIgD$^+$). Upon antigen activation some cells then switch to the production of other isotypes, most commonly IgG. This H chain switch usually involves transposition of the active V region (VDJ[H]) gene from the expressed Cμ to one of the other constant region genes (21). The exception is IgD, where there is no functional switch region immediately 5' of Cδ (22).

While this mechanism usually guarantees that a given B cell produces only a single isotype or subclass, there have been rare observations of double isotype production in (rodent) B cells. Yaoita et al. (23) detected surface μ^+/ϵ^+ mouse spleen cells by cell sorting. LaFrenz et al. (24) sorted surface IgG$^+$ cells that were shown to later secrete IgM. Sandwich RNA hybridization studies by Perlmutter and Gilbert (25) suggested a low frequency occurrence of nuclear RNA containing both μ and γ or α gene regions in sorted memory cell populations, and a subclone of the mouse lymphoma line BCL$_1$ was shown by Chen et al. (26) to cosecrete IgM and IgG1.

In fluorescent staining experiments, it is difficult to always ascertain that only endogenously synthesized and not cytophilic Ig is detected (27). Nevertheless, these studies suggest that double isotype production can occur in at least some cells, albeit rarely (28–31). Such findings can be accommodated by two alternative models. (a) Perhaps in the process of H chain switch, cells pass normally through a double expression stage but do so very quickly, and therefore remain undetected. (b) All B

cells may be inherently capable of double isotype production but it occurs only on rare occasions, perhaps accidentally (32–34).

In the present study of B cells from normal donors, we have combined cell sorting, EBV-induced transformation, cloning, and molecular genetics techniques to distinguish these two alternatives in the IgE system. Our observations suggest that the coexpression of IgE plus IgM and IgD is the rule in this B cell sublineage. IgE-secreting EBV responders expressed sIgM and sIgD before and after transformation, i.e., during cell sorting, throughout culture and cloning procedures. This was reflected in the genome of IgE-secreting clones as a rearrangement of VDJ to $C\mu$ and the retention of all three ϵ genes in germline configuration. The isotype switch typical of myeloma lines like U266 (20), thus, does not occur in these B lymphoid cells, despite the presence of normal sized ϵ (as well as μ) mRNA. In contrast, in this and other laboratories (35–37), double isotype production was never detected in EBV-transformed IgG and IgA producers, all of which show genomic rearrangement of VDJ to the IgH locus expressed. It therefore appears that IgE-committed B cells regularly reach a stage of multiple isotype expression as a characteristic and stable rather than transient phase in their ontogeny. Although multiple isotype expression may rarely occur in IgG and IgA producers (24, 31), we favor a model where IgE-committed B cells represent a unique B cell subset and perhaps travel a distinct differentiation pathway where deletional switch rearrangement occurs only rarely and late in development.

Materials and Methods

Cell Preparations. PBMC from normal donors were purified on Ficoll-Hypaque gradients, washed and resuspended in RPMI 1640 (Ontario Cancer Institute, Toronto), and supplemented with 10% FCS (HyClone Laboratories, Logan, UT), antibiotics, L-glutamine, and 50 μM 2-ME. Where indicated, B cells were enriched by E-rosette depletion (17). Peripheral blood polymorphonuclear cells were enriched by Plasmagel™ sedimentation and extracted as a source of germline DNA.

Cytofluorometry and Reagents. PBMC were stained with monoclonal (mouse) or affinity-purified polyclonal (goat) antibodies against different human Ig isotypes (Coulter Electronics, Inc., Hialeah, FL or BioCan Laboratories, Mississauga, Ont.). Monoclonal anti-IgE(Fc) antibodies were a gift from Dr. A. Saxon (UCLA) (38). Indirect stains were visualized with fluoresceinated, affinity-purified, goat anti-mouse Ig antibody. Mouse Igs of the same subclass were used as controls. Stained cells were washed with PBS, resuspended in RPMI, and analyzed in an EPICS V cytofluorometer (Coulter Electronics, Inc.). Propidium iodide (PI) was added to exclude nonviable cells from analysis. For culture, individual fluorescence-positive or -negative cells were seeded directly into microwells using an Autoclone™ attachment to the cell sorter equipped with a custom log increment EPROM (Coulter Electronics, Inc.) as described (39, 40). Rhodaminated goat anti-human IgM and goat anti-mouse Ig for cytoplasmic double-staining experiments were obtained from BioCan Laboratories.

Cytoplasmic Staining. The coexpression of cytoplasmic Igs was examined by immunofluorescent double staining (41). Briefly, cytocentrifuge cell preparations (cytosmears) were fixed in cold alcohol/glacial acetic acid (95:5) and stained (45 min, 22°C) in a humid atmosphere in the dark. For direct staining, rhodaminated antibody against one Ig and fluorescein-conjugated antibody against a second Ig were used. For unconjugated mAbs, a secondary, fluorescence-labeled goat anti-mouse Ig was used (BioCan Laboratories). Smears were preserved in 90% glycerol and examined in an epifluorescence photomicroscope (Carl Zeiss, Inc., Thornwood, NY).

Virus Preparation and Infection. The purification of EBV has been described (40). Briefly, supernatants of the B95-8 marmoset line were filtered, washed and concentrated 100-fold

by ultracentrifugation, and stored at $-70°C$. Virus infectivity was estimated by the ability of diluted virus stock to induce cell growth and IgM production in 4-wk cultures of 10^4 cord blood lymphocytes. B cells were infected either in bulk or by addition of 20 μl of titered virus (30-fold excess) to limiting dilution cultures. In the former procedure, $1-5 \times 10^6$ PBMC were infected with 500 μl of virus (2 h, 37°C) and used for culture after washing.

Culture Conditions.   Limiting dilution cultures were seeded manually or with the Autoclone™. In manual preparations, EBV bulk-infected cells were diluted in complete medium and added to 96-well, microtiter plates (Costar, Cambridge, MA) containing 10^4 irradiated (3,000 rad) DT1.2 cells, a human T-T hybridoma line selected in our laboratory for its ability to support low density B cell cultures (42). In some experiments, 3×10^4 irradiated, PHA-activated cord blood lymphocytes were used as feeder cells with similar results. 46 replicates of typically eight cell doses (10-10,000/well) were fed weekly with 130 μl fresh medium. Week five supernatants were stored frozen until tested by ELISA. Cultures containing >1 ng/ml of a given Ig were considered positive for that isotype (40). Growth transformation was scored microscopically at week five. Control cultures without donor lymphocytes, EBV, or in the presence of cycloheximide were always negative for growth and secretion.

Sublining and Cloning Procedures.   IgE-producing lines from individual fifth-week culture wells were expanded to $\sim 10^5$ cells/ml in 25-cm^2 flasks (Costar). Sublining was performed twice by transferring 1-3% of cells into 24-well culture plates and expansion for 3-4 wk (43). Clones were established by limiting dilution in the presence of DT1.2 feeder cells (44). Subcloning utilized the Autoclone™ to place single, PI^- cells, unstained, or brightly or weakly staining for sIgM into microtiter wells. All cell lines and clones reported had a normal karyotype.

ELISA.   Ig secretion was measured by ELISA as described (40). Briefly, Linbro EIA 96-well microplates (FlowLabs, Mississauga, Ont.) were coated at 4°C with anti-Ig, blocked with 0.1% BSA, (Sigma Chemical Co., St. Louis, MO), washed, and 10-30 μl of sample was added in 0.1 M Tris-HCL containing 2 mM Ca^{2+}, Mg^{2+}, Zn^{2+}, and 0.1% BSA. After overnight incubation and washing, anti-Ig conjugated to alkaline phosphatase (BioCan) was added for 2 h. After washing, bound enzyme activity was measured with 0.8 mg/ml p-nitrophenyl phosphate as a substrate. The optical density (OD_{410}) was read in a Dynatech MR600™ ELISA reader (Fisher Scientific, Toronto) connected via an Apple™ microcomputer to a UNIX-VAX™ system for statistical analysis and quantitation.

Statistical Analysis.   Our criteria for the analysis of limiting dilution experiments have been reported (17, 39, 40). Frequencies were determined by both maximum likelihood (ML) and minimum χ^2 (MCS) methods with UNIX software developed along the arguments of Taswell (3, 45). Single-hit kinetics were assumed at probabilities of $p > 0.1$ and where y-axis intercepts of regression lines were close to zero. In all experiments reported the results determined by ML and MCS differed by <10%. The expected coincidence of two independent events (i.e., the presence of two unrelated clones in a given culture well) was determined by frequency-based contingency analysis and probabilities were estimated using Fisher's exact test (17, 46).

DNA Probes.   Plasmids were routinely purified by equilibrium banding in cesium chloride gradients (47). Excised inserts were isolated by electroelution from agarose gels. The plasmids, pHuμC, pH$\mu\epsilon$C, pHuαC(GMA-5α_1), and pHuJ(H), were obtained from Dr. P. Leder (Harvard University, Boston, MA). The M13δ1 clone was a gift of Dr. T. H. Rabbitts (MRC Laboratory, Cambridge, UK). We subcloned a 240-bp segment into puc-19 (pδ1).

The J(H) probe was excised from pHuJ(H) as a 6-kb Bam HI/Hind III fragment spanning all J regions and the H chain enhancer (48). The Cμ probe includes exons 1, 2, and part of 3 of the constant region gene, and is a 1.5-kb Eco RI/Eco RI insert of pHuμC (48). The ϵ probe encodes all four ϵ constant region exons, and is a 2.6-kb Bam HI/Bam HI fragment from pHuϵC (49). An Xho I/Bam HI Cα_1 fragment of pHuαC(GMA-5α_1), 4.6 kb in length, crosshybridizes with Cα_1 and Cα_2 (50). The δ probe includes both a hinge exon and intron (51). 50-100 ng of probes were labeled with $\alpha[^{32}P]dCTP$ by random priming (52), and all showed sp act of $>5 \times 10^8$ cpm/μg.

Southern Hybridization.   DNA was extracted from polymorphonuclear cells and cell lines according to Davis et al. (53). After digestion with restriction endonucleases (Boehringer Mannheim, Mannheim, FRG), 10 μg DNA was applied to 0.6% agarose gels. Gels were blotted according to Southern (54) onto nylon membranes (ICN Biomedicals, Irvine, CA). Hybridi-

zations were carried out overnight at 42°C in 50% formamide, 5X SSC, Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 100 µg/ml salmon sperm DNA, and specific probe. Hybridization was followed by two, 30-min room temperature washes in 3X SSC and two 20–30-min washes at 65°C in 0.3X SSC. Autoradiographs were developed after typically 3–7 d exposure at −80°C, to x-ray film (XAR-5; Eastman Kodak, Rochester, NY) in the presence of intensifying screens. For repeated hybridizations, blots were stripped for 1–4 h at 65°C in 5 mM Tris containing 10 mM EDTA and pyrophosphate.

Northern Blot Analysis. Total RNA was isolated by the acid guanidinium thiocyanate-phenol chloroform extraction protocol (55). To enhance the yield of specific RNA transcripts, some cultures were exposed (12 h, 37°C) to cycloheximide (100 µg/ml) (56). Poly(A)$^+$ RNA was prepared from the RNA of IgE/M/D-secreting transformed clones by oligo-dT-cellulose chromatography. For Northern blots, 2 or 20 µg of pooled poly(A)$^+$ RNA was separated in 1% agarose gels containing 6% formaldehyde in a 1X MOPS running buffer (0.02 M MOPS [3-[N-Morpholino]propane-sulfonic acid] 5 mM Na Acetate, 0.1 mM EDTA). RNA (10 µg) from the human myeloma cell line U266 was used as a control (20). The samples were subjected to electrophoresis 4 h at 150 V. Ethidium bromide staining identified 23S and 16S bacterial rRNA and 28S and 18S eukaryotic rRNA molecular weight markers. RNA was transferred to nitrocellulose and hybridized overnight in 50% formamide, 5X SSC, Denhardt's solution, 50 µg/ml salmon sperm DNA, and specific probe. Four 5-min room temperature washes in 2X SSC were followed by a 1-h 65°C wash. After hybridization, autoradiographs were developed after after a 2-d exposure at −80°C with the µ probe and 10–14 d after hybridization with the ε probe.

Results

Frequency of EBV-responsive B Cells. Limiting dilution assays permit quantitative analyses of the clonal heterogeneity and isotype diversity among B cells susceptible to the virus (reviewed in reference 40). With minimal uncontrolled, suppressive cell interactions, the efficiency of EBV-induced B cell activation and transformation in these cultures is ~30–100-fold higher than that observed in bulk cultures and with other human B cell activators, reaching as high as >30% of B cells responding (18, 36). Lymphocytes committed to IgE secretion are, in our hands, detected only in such limiting dilution cultures of EBV-infected cells (3). Fig. 1 shows results of two typical limiting dilution experiments with PBMC from normal, healthy donors. Fresh cells were bulk infected with EBV and seeded into limiting dilution cultures. Week five supernatants containing >1 ng Ig/ml were considered positive for that isotype. Most Ig$^+$ wells (>90%) also scored positive for cell growth at this time. The incidence of EBV responders (per B cell seeded, one/frequency) was determined as the cell dose where 37% of cultures were negative (17, 45).

Under our culture conditions, frequencies of EBV-responsive Ig secretors fall within typical ranges (36, 40). As shown in Fig. 1, IgM was the most common isotype detected with one responder in 20–60 B cells seeded. Approximately 5–10% of IgM secretors coproduced IgD and cultures secreting IgD alone were not observed. IgA and IgG were detected in nearly equal frequencies, ranging from 1 in 60 to 1 in 120 B cells. IgE secretors represent a small but distinct and consistently measurable entity whose frequency averaged 1 in 1,500 B cells in these experiments (range 1:800–1:3000). Overall culture efficiencies (~5–10% of B cells responding) were similar in the experiments described in this report.

Phenotype of IgE-committed B Cells. To characterize the phenotype of IgE-committed cells, we combined cell sorting and autocloning of fresh PBMC stained for various Ig classes (39, 40). Infection with EBV and limiting dilution analysis were then used
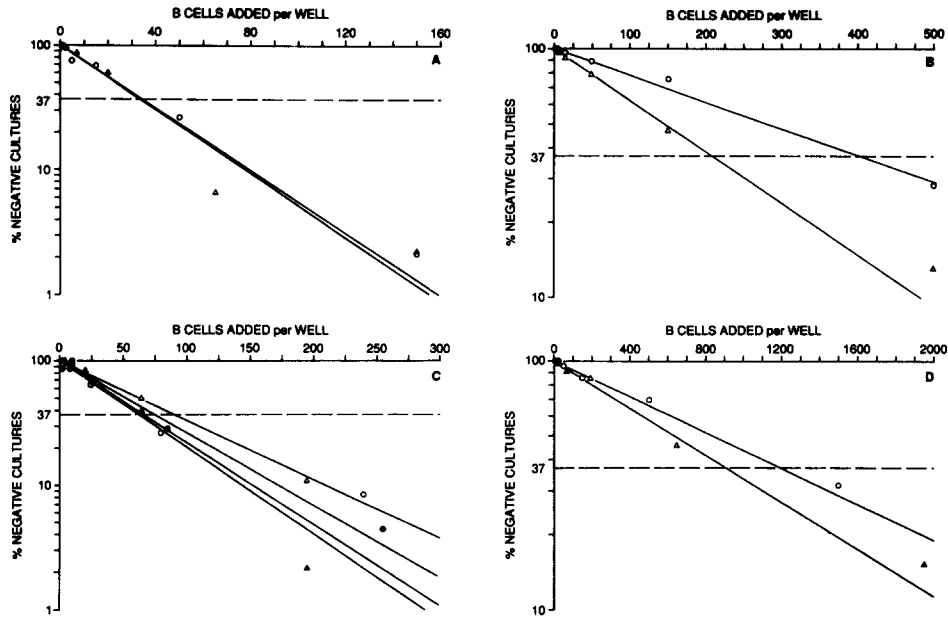
FIGURE 1. Limiting dilution analysis of PBMC. Cells were incubated with EBV and cultured 5 wk when Ig secretion was determined by ELISA. The fraction of cells negative (<1 ng Ig/ml) for each measured isotype is plotted on the ordinate, vs. the number of B cells added per well. Probability values of $p > 0.1$ confirm that cultures follow single-hit kinetics where only one variable (the number of B cells seeded) was limiting the response, i.e., Ig secretion. Shown are two representative experiments of seven performed. A, IgM; B, IgD; C, IgG (*open symbols*) and IgA (*closed symbols*); D, IgE.

to measure the frequency of IgE producers derived from positively or negatively selected populations.

We first determined the fidelity of this approach by staining PBMC with anti-κ or anti-λ L chain antibodies. An average of one in seven B cells that were sorted for the expression of either surface κ or λ were growth transformed in these experiments. Virtually all cultures positive for κ secretion were found in the populations sorted as either κ^+ or λ^-, while nearly all λ secretors had been selected as λ^+ or κ^-. Transformation rates ranged from 1:7 to 1:12 B cells seeded in either population. The frequency of crosscontaminating cells ranged from 1:4,500 to 1:5,200 cells seeded. This indicated that our experimental approach was capable of >98% discrimination (data not shown).

PBMC were stained for sIgM or sIgE and positive or negative fractions were autocloned into limiting dilution cultures. In the example shown (Fig. 2 A), sIgM$^+$ cells represented 9% of the PBMC population. At most, 1% of cells stained weakly sIgE$^+$ (Fig. 2 B). Frequency estimates of IgE secretors after 5 wk of culture showed that >90% of IgE-producing cells had been sorted with the sIgM$^+$ population, while <10% had been sorted as sIgM$^-$ (Fig. 2 C). Surprisingly, cells sorted as sIgE$^+$ contained <30% of the IgE-secreting B cells, while more than two thirds of all such cells detected were found in cultures of sIgE$^-$ lymphocytes. This suggested that IgE-committed B cells expressed sIgM, but not necessarily detectable sIgE, before EBV infection and culture.
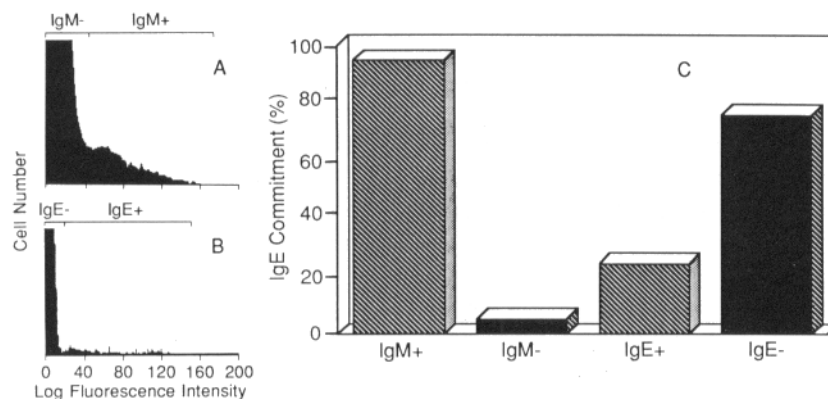
FIGURE 2. Fluorescence histogram of PBMC stained with anti-IgM or anti-IgE antibody. Positive and negative cells were selected in autocloning experiments as indicated. (A, IgM; B, IgE). (C) Phenotype of transformable IgE producers. The proportions of IgE-committed B cells were determined from the frequency of growing IgE producers in each sorted population after 5 wk of culture.

To further define the phenotype of cells giving rise to IgE-secreting clones in culture, enriched B cells were stained and selected for other Ig isotypes as well. Cells were autocloned into microtiter wells as described in Fig. 2. The culture efficiency was estimated using control cultures of 'sham-sorted' cells, stained for sIg, but selected with open gates in the cell sorter (40). In the experiment shown in Table I, the cul-

TABLE I

Precursor Cell Phenotype of EBV-responsive IgE Producers

Phenotype of selected cells	All EBV responders*	IgE producers[‡]
	1/frequency[§]	1/frequency[§]
Sham sorted	1:22	1:1,820
sIgM$^+$	1:6	1:810
sIgM$^-$	1:130	<1:10,000
sIgD$^+$	1:9	1:810
sIgD$^-$	1:180	<1:10,000
sIgE$^+$	1:3,000[‖]	1:3,900[‖]
sIgE$^-$	1:20	1:1,740
sIgG$^+$ or sIgA$^+$	1:110	<1:10,000
sIgG$^-$ or sIgA$^-$	1:17	1:1,680

Enriched B cells were sorted as indicated. Cells positive or negative for a given isotype were seeded into limiting dilution microcultures, infected with EBV, and secretion of all Ig isotypes was analyzed after 5 wk of culture. Frequencies of responding cells were determined using Poisson statistics. Limiting dilution cultures of sham-sorted cells (stained but not gated) provided a control and measure of overall culture efficiency.
* Cells secreting any Ig isotype.
‡ Cells secreting IgE.
§ 1/frequency = Number of B cells seeded that contain an average of one responder cell.
‖ Only two cell doses were available for analysis.

ture efficiency was 4.5% with one responding cell in every 22 cells seeded. Frequencies of B cells secreting any Ig and those secreting IgE are compared in the table.

Most circulating B cells are $sIgM^+/sIgD^+$, as are the majority of EBV-transformable cells (18, 36). This was confirmed in these experiments by the precursor cell frequencies obtained for the different isotypes. Selection of $sIgM^+$ cells before culture resulted in an enrichment of both the numbers of EBV responders overall, and the numbers of IgE producers. Conversely, the $sIgM^-$ population was depleted of both. Similar results were obtained after selection of $sIgD^+$ cells, indicating that the majority of IgE-committed B cells are $sIgM^+/sIgD^+$ before EBV transformation. Again, few IgE producers expressed detectable sIgE before culture. Cells selected as $sIgE^+$ would include $Fc\epsilon R^+$ cells, which bind cytophilic IgE on the surface but are not related to the IgE-committed sublineage (27). The incidence of IgE-committed cells in $sIgG^+/sIgA^+$ populations was below confidence limits established by L chain gating experiments and rare IgE-producing cells in these cultures may reflect cross-contaminating $sIgM^+$ cells. Slight discrepancies in the frequencies of EBV responders in IgG^-/IgA^- and IgM^+/IgD^+ populations may reflect an enrichment of contaminating non-B cells in the negatively selected population.

The phenotype of cells that give rise to IgE-secreting clones in our culture system appear to be over two thirds $sIgM^+/sIgD^+/sIgE^-$, with the remainder $sIgM^+/sIgD^+/sIgE^+$. This phenotype is similar to the IgE/IgM-coproducing KID5 clone (3, 11, 19) and in this respect the clone does not appear to be a rare exception among EBV-responsive IgE producers.

Coincidence of IgE, IgM, and IgD Production. B cell clones producing a given isotype should be independent and thus randomly distributed in our cultures (18, 44). We analyzed limiting dilution culture wells for all Ig isotypes secreted to determine the coincidence of IgE secretion with any other isotypes. At cell doses where 37% of replicate cultures are negative for a given isotype (in the case of IgE secretion, $\sim$1,500 B cells), each positive well receives an average of one precursor cell able to proliferate and secrete that isotype (46). The chance that a given well receives more than one precursor is described by Poisson: if D is the number of B cells seeded, and f the precursor cell frequency, then the probability (P) for the presence of n precursors is $P_n (D,f) = (Df)^n \times e^{-Df/n}$ (reviewed in reference 17).

For coincidence analysis, we arbitrarily chose relatively small B cell inputs of 200 or fewer per well and calculated the probability for the coincidence of two ($n = 2$) IgE-producing clones to be present in these cultures. In one series of experiments, altogether, 1,012 cultures received 200 or less B cells and these yielded 30 IgE-producing clones. Given the average frequency of IgE-committed cells (6.67×10^{-4} or 1:1,500), the probability for the coincidence of two independent clones was $P = 0.01$. This indicated that <1 of 100 IgE^+ cultures was not clonal with respect to IgE-producing cells.

The random chance for two independent clones producing any isotype to be present in the same well is a function of their respective frequency (17, 46). We assumed that B cells are distributed independently and that a given B cell is committed to the production of only one isotype. Since the frequency of, for example, IgM-committed cells is much higher than that of IgE producers (Fig. 1), some IgE^+ wells should contain other clone(s), in particular those secreting IgM. The expected number of IgE-secreting cultures containing an IgM- or IgD-secreting clone was estimated

by contingency analysis (46). Of the 30 wells positive for IgE, ≤15 could be expected to contain an IgM secretor,≤2 of which might produce IgD as well. Observed numbers were 29 IgM/IgE and 30 IgD/IgE-producing wells. Fisher's exact test indicated that the observed coproduction reflected closely linked and dependent events ($p < 0.05$).

Fig. 3 illustrates isotype expression in 79 IgE positive limiting dilution cultures seeded with 20–2,000 cells. At higher cell inputs more wells contained multiple clones of cell secreting an Ig isotype other than IgE. However, at low cell densities IgG- and IgA-producing cells were diluted out, while IgM and IgD production remained unchanged. In seven experiments not a single IgE$^+$ well failed to also secrete IgM and/or IgD and in <2% of cultures, was either isotype (but not both) at or below our detection limits. The distribution of IgG and IgA secretors was entirely independent of IgE secretion in all experiments (data not shown). Furthermore, in each IgE-producing culture, IgM, IgD, and IgE expressed the same L chain, while IgG and IgA showed the expected ($\sim$6:4) κ/λ diversity. Secretory data obtained after 5 wk of growth in culture, as well as the results of sorting data, all suggested that IgM and IgD are continuously coexpressed by virtually all IgE secretors.

Sublining and Cloning of IgE$^+$ Cultures.   It was conceivable that in each individual limiting dilution culture a clone secreting only IgE was present that required the presence of an IgM$^+$/IgD$^+$ clone for transformation or growth. To examine this possibility, we used sublining and cloning experiments of IgE$^+$ limiting dilution cultures. In five different experiments a total of 18 primary cultures were expanded without change in secretory patterns. Of these, nine were sublined at least twice (see Materials and Methods). Contaminating clones secreting IgG or IgA were quickly separated from IgE-secreting clones in the same line and discarded (Fig. 4). Subsequent formal cloning experiments generated many IgM/D/E producers but failed to derive even one single (e.g., IgE$^-$) or double (e.g., IgM/D$^-$) isotype producer, confirming that IgM/IgD/IgE coexpression is a stable, clonal characteristic of IgE producers.

Two models could explain the observation of IgM/IgD/IgE coexpression. All IgE-producing cells could express IgM/IgD/IgE simultaneously. Alternatively, a small subset of cells in the sIgM$^+$/sIgD$^+$ population might continuously switch VDJ to ε in a deletional DNA rearrangement and subsequently express IgE but fail to pro-
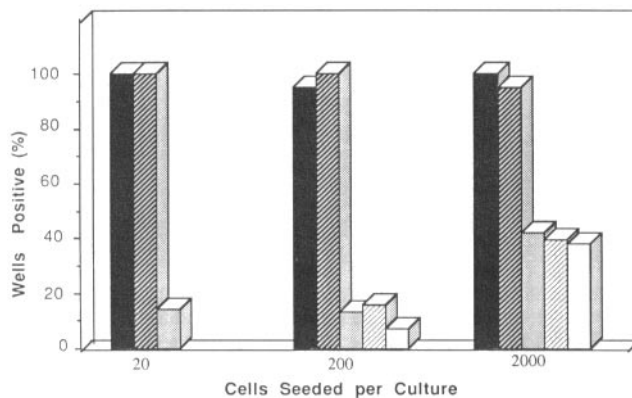

FIGURE 3.   Isotype diversity in IgE$^+$ limiting dilution cultures. Limiting dilution cultures of 20–2,000 EBV-infected cells were tested for Ig secretion after 5 wk. The graph illustrates data from IgE$^+$ cultures and shows the proportion of IgE$^+$ wells containing other isotype(s). Data from seven limiting dilution experiments are pooled. IgE plus: (■) IgM; (▨) IgD; (▦) IgG; (▧) IgA; (□) IgG + IgA.
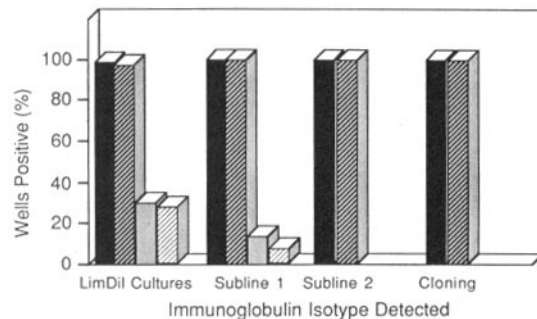
FIGURE 4. Sublining and cloning of IgE⁺ limiting dilution cultures. 18 of 79 IgE⁺ limiting dilution cultures were expanded in culture for 3 wk. Nine were sublined at least twice and cells from two such lines were then formally cloned by limiting dilution. All cultures were analyzed for the presence of other Ig isotypes and the percent of cultures expressing a given isotype in addition to IgE were detected. IgE plus: (■) IgM; (▨) IgD; (▦) IgG; (▨) IgA; (□) IgG + IgA.

FIGURE 4. Sublining and cloning of IgE⁺ limiting dilution cultures. 18 of 79 IgE⁺ limiting dilution cultures were expanded in culture for 3 wk. Nine were sublined at least twice and cells from two such lines were then formally cloned by limiting dilution. All cultures were analyzed for the presence of other Ig isotypes and the percent of cultures expressing a given isotype in addition to IgE were detected. IgE plus: (■) IgM; (▨) IgD; (▦) IgG; (▨) IgA; (□) IgG + IgA.

liferate efficiently and form a distinct subpopulation. To distinguish between these two possibilities, IgE-secreting clones were stained for surface IgM and autocloned into two populations (brightly or weakly/nonstaining). One would predict that if a small, switching subset deleted μ and no longer expressed IgM, then some culture wells would only be positive for IgE. However, subclones in both sorted populations all coproduced IgM and IgE (Table II). No secretion was detected in growth-negative wells. It was interesting to note that the cloning efficiency was much higher in cells sorted for high expression of sIgM, perhaps reflective of an activating effect of anti-μ itself. Since all clones expressed FcεRs (data not shown), autocloning on the basis of sIgE expression was not attempted as we were unable to formally distinguish surface from cytophilic IgE.

In the cloning experiments described above, the amount of IgE produced varied little from well to well, suggesting that all wells contained similar numbers of cells secreting IgE. After expansion of subclones, the accumulation of secreted IgM and IgE in culture was quantitated over a 24-h period with the cells in log phase growth. The amount of 19S IgM secreted was approximately fivefold that of 7S IgE secreted by the same cell. Assuming similar detection sensitivity in our ELISA for both forms, and that five monomers of 7S IgM make up one 19S pentamer, this implies that IgM and IgE are produced at nearly equal rates. This supported results of cloning data and we believe that the IgE production observed is unlikely due to a small cell population continuously switching to ε.

Characteristics of IgE Expression. We next examined the coexpression of IgM and IgE at the level of mRNA. Cycloheximide stabilizes some RNA species, including ε (e.g., 56 and unpublished observation), and we added this drug 12 h before RNA

TABLE II

Isotypes Secreted by Clones Selected for Surface IgM

Isotype secreted	Growing cultures secreting each isotype	
	sIgM⁺ (bright)	sIgM⁺ᐟ⁻ (weak)
IgM or IgE	0:109	0:22
IgM and IgE	109:109	22:22

IgE-secreting lines were stained with anti-IgM and autocloned. Bright cells represented 8% of those sorted and weak cells the remainder. Fifth-week cloning cultures were examined for Ig secretion. Representative data from one of two experiments are presented.
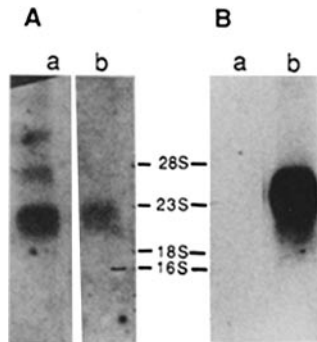
FIGURE 5. Northern blot analysis of mRNA from IgE/M/D-producing subclones of 8G9. (A) Hybridized with the Cε probe: (a) U266 myeloma RNA; (b) 8G9. (B) Hybridized with the Cμ probe; (a) U266; (b) 8G9.

extraction. Poly(A)⁺ mRNA was separated in formaldehyde gels and blotted onto nitrocellulose. Sequential hybridizations with genomic Cε and Cμ probes indicated the presence of both μ- and ε-mRNA species of approximately equal size (Fig. 5). RNA from U266, simultaneously probed, did not contain μ sequences but it contained a major species hybridizing to the ε probe in a similiar-sized band as observed in RNA from IgE-producing B cell lines. Faint additional bands hybridizing to the ε probe were present only in U266 RNA and RNAs coding for IgG or IgA were detected only in IgG- or IgA-producing (control) lines, but not in any of the IgE-producing cells (data not shown).

The cytoplasmic expression of IgM, IgD, and IgE protein was examined at a single cell level by double-fluorescence staining (Fig. 6). Cytosmears were fixed and successively stained with combinations of fluorescein- and rhodamine-conjugated antibodies. About half the cells stained brightly for cIgM (Table III). Using polyclonal and monoclonal anti-IgE reagents with similar results, 60–70% of brightly stained cIgM⁺ cells also stained brightly for cIgE. No cIgE⁺ cells were cIgM⁻, most cIgD⁺ cells were cIgE⁺, and 60–70% of cIgM⁺ cells also expressed cIgD. The latter is comparable with cIgM⁺/cIgD⁺ cells in a control B cell line (clone 34.3), which produces both IgM and IgD but not IgE. Cytoplasmic IgE but not IgM or IgD was detected in the U266 myeloma line, which secretes only IgE. Thus, single cells of IgE-secreting clones express IgM/D and IgE concurrently.

DNA Rearrangements in IgE-producing Lines. Triple isotype–producing cells could be explained by failures of allelic exclusion where IgE and IgM/IgD are transcribed from two active chromosomes. Alternatively, one allele could be transcribed from VDJ-Cμ through to Cε (Fig. 7, *top*) followed by post-transcriptional alternative processing into mRNA, generating μ, δ, and ε species (48–51). To examine the state and stability of rearrangement patterns, we repeatedly analyzed rearrangements of the Ig H chain gene region in a series of lines.

Southern blots of DNA were sequentially hybridized with genomic probes for each Ig class with consistent results. Germline DNA from the original B cell donor and at least one unrelated donor served as controls to distinguish changes in restriction fragment size due to rearrangements or polymorphisms. Several subclones were examined in parallel to examine the stability of transformed IgE producers. Each subclone showed identical patterns in all digests, and on at least two occasions, 6–9 mo apart.

Fig. 8 shows Bam H1–digested DNA of two subclones of the IgE-secreting line
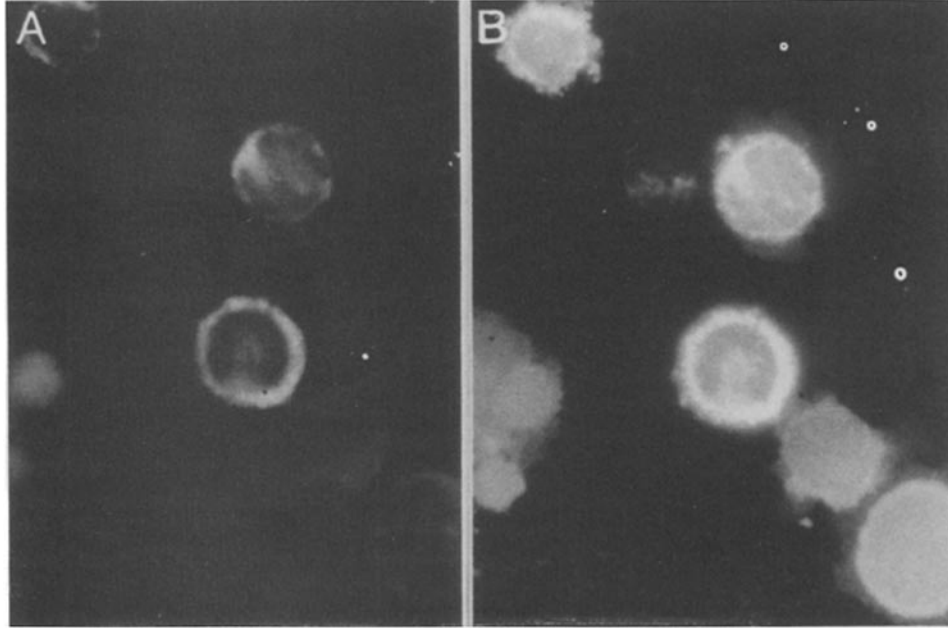
FIGURE 6. Immunofluorescence of double staining for cytoplasmic IgM and IgE. 10^6 cells (>95% viable) were fixed on a slide and stained with affinity-purified FITC, goat anti-human IgE (A) and Rhodamine, goat anti-human IgM (B).

8G9, hybridized first with a $C\mu$ probe, then with a probe containing all six of the J(H) region genes (see Fig. 7). Two rearranged J(H) bands were detected. As predicted by the secretory pattern of IgM production, a VDJ has rearranged to $C\mu$ with a change in restriction fragment length from 19 kb (germline) to 28 kb. The second

TABLE III

Immunofluorescent Double Staining of IgE-producing Clones

Clone	IgM/IgE*		IgM/IgE‡		IgM/IgD	
	cIgM	m⁺/E⁺ §	cIgM	M⁺/E⁺ §	cIgM	M⁺/D⁺ ‖
1G7	43	70	30	42	45	68
8G9	35	61	28	40	34	65
34.3	41	0	ND	ND	43	71
U266	0	>90¶	0	>90¶	ND	ND
DT1.2	0	0	0	0	0	0

Cells were stained as in Fig. 6 or with FITC goat anti-human IgD instead of IgE. Monoclonal anti-IgE, to confirm specificity, was visualized with FITC goat anti-mouse IgG. U266 is a human IgE-secreting myeloma line. DT1.2 is a human T-T hybridoma line. The 34.3 line is an EBV-transformed fetal B cell that coproduces IgM/IgD but not IgE. 1G7 and 8G9 are IgM/IgD/IgE coproducing clones.
* Affinity-purified goat anti-human IgE.
‡ Monoclonal mouse anti-human IgE.
§ Percentage of cIgM⁺ cells that are also cIgE⁺.
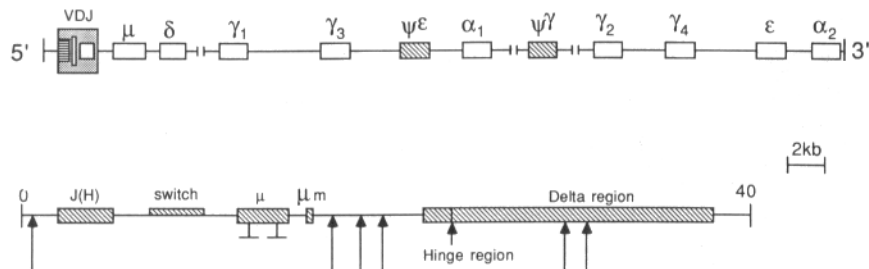‖ Percentage of cIgM⁺ cells that are also cIgD⁺.
¶ Cytoplasmic IgE⁺ only.

FIGURE 7. (*Top*) Physical map of the human Ig H chain gene locus (not to scale). This order was constructed according to the findings of several groups (48, 68, 69, 72) based on sequence data and utilizing information from donors with specific deletions in the IgH locus. (*Bottom*) Restriction map of the region from J(H) to δ determined using overlapping phage clones and DNA sequencing (69). (†) Bam HI; (⊥) Eco RI.

J(H) (20.5-kb fragment) has been assigned to a rearranged Cγ (not shown), and we are presently determining the subclass assignment using specific oligonucleotide probes. Preliminary data indicate a nonproductive VDJ-Cγ rearrangement to Cγ3 or Cγ1.

As shown in Fig. 9, both Cα1 and Cα2 genes, as well as Cδ, are present in germline configuration and are nonrearranged. That Bam HI digests are capable of detecting rearrangements has been previously determined in this and other laboratories (35–37 and unpublished observations) using IgG- or IgA-secreting lines.

The configuration of ε loci was examined by hybridization of several restriction enzyme DNA digests with the ε probe (see restriction maps in Fig. 10) (20, 57–59). The Bam HI digest demonstrates the retention of all three ε germline genes (Fig. 11 *A*). This enzyme does not detect Cε rearrangements, even in U266. However, hybridizations of Xba I digests of DNA from all our transformed lines show germline configuration of both the coding Cε gene region and φε1 on chromosome 14 (Fig. 11 *B*). The U266 IgE-secreting myeloma line has rearranged the functional Cε gene and, as predicted from the genomic map, it has deleted φε1 (Fig. 11 *B*, lane *b*) (20). The restriction map of φε2 (on chromosome 9) is not fully known and this pseudogene is usually not detected in Xba I digests. The presence of φε2 in the lines is confirmed by the Bam HI digest (Fig. 11 *A*) as well as in hybridizations of Hind III (Fig. 12) and Eco RI–digested DNA (not shown).

Fig. 12 illustrates Hind III–digested DNA hybridized with a J(H) probe and then rehybridized with an epsilon probe. Results confirm that all three ε genes are present and maintained in germline configuration (22 kb for Cε and ψε1: 8.9 kb for ψε2).
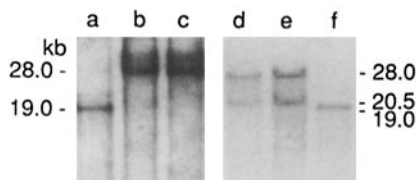

FIGURE 8. Southern blot analysis of DNA from ε-producing clones. 10 μg of DNA was analyzed. Bam HI digests were hybridized with the Cμ probe in *a–c*. Bam HI digest hybridized with the J(H) probe in *d–f*. (*a* and *f*) germline DNA from the original donor. (*b* and *d*) EBV-transformed IgE⁺/IgM⁺/IgD⁺-secreting clone 8G9: subclone 1. (*c* and *e*) 8G9, subclone 2.
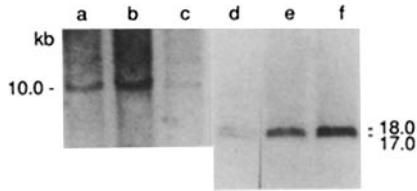
Again, only two rearranged J(H) bands are found (26 and 5.6 kb) and neither lines up with any ε region. The Hind III restriction sites map 2 and 0.5 kb upstream of functional and pseudoepsilon, respectively, and downstream ∼0.5 kb 3' of α₂ genes in germline DNA (68).

The Xba I digest confirms germline configuration of functional ε as far as 10.5 kb 5' of Cε exon 1 in the Cγ₄-ε intron (Fig. 10). This includes a stretch of DNA >7 kb upstream of the ε switch region. A similiar situation is seen with ψε₁, which is present in germline configuration on a stretch of DNA at least 5 kb upstream of its first exon. Downstream both ε genes were mapped on Hind III digests to include Cα₁ and Cα₂, respectively, in expected (germline) position indicating that both the Cε-α₂ and the ψε₁-α₁ intron/exon regions are maintained in germline configuration (Fig. 12 and not shown).

Full-length sequencing of the IgH locus may be required to formally determine if the entire region between VDJ-Cμ and Cε remains intact. However, the fact that only two J(H) bands were detected associated with Cμ on the expressed allele and Cγ on the other, as well as the presence of a 10.5-kb germline DNA region 5' of ε, which includes switch, all suggest that the bulk of DNA may be retained between VDJ-μ and Cε on the expressed allele. The presence of germline ψε₁ and Cα₂ bands at a density equal to Cε and Cα₂ bands suggests that all are present and intact on both alleles and that no major deletions have occurred on the functional allele. Given the presence of ε mRNA, we cannot deny that a V region gene is influencing ε in some manner but a lack of VDJ rearrangement to Cε makes even a model of allelic inclusion unlikely. We orginally suspected that a very large primary nRNA transcript was alternatively processed to yield μ and ε species but alternative mechanisms may have brought a V region gene closer to functional ε.

Similar DNA organizational patterns with rearrangements of VDJ to Cμ and retention of all ε genes in germline configuration were consistently observed in all our IgE-producing lines. We therefore concluded that, regardless of the precise mechanics,
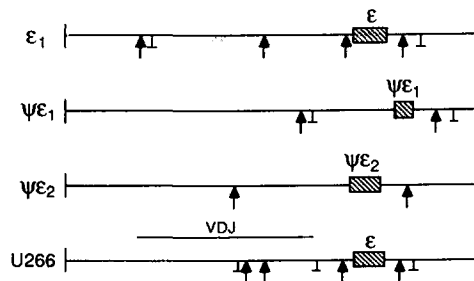

FIGURE 10.　Restriction maps of the human ε genes and of the expressed ε in U266 constructed by combining reported maps (49, 58, 59). Hatched boxes represent exon regions of IgE H chain constant regions. The ε switch region is ∼2 kb 5' of the first ε exon. (↑) Bam HI; (⊥) Xba I.
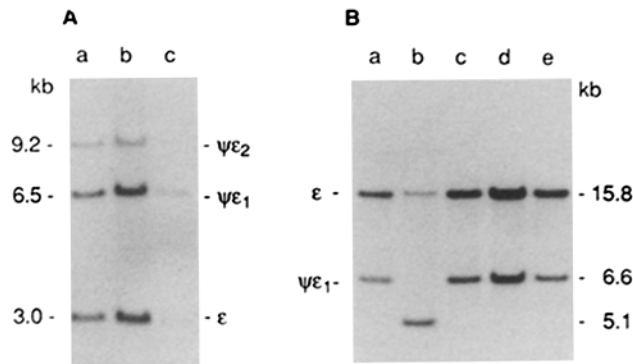
this pattern of gene expression is not a rare exception but represents the typical, clonally stable characteristic of IgE-producing B lymphoid cells. As well, our findings indicate that if DNA rearrangements occur in IgE-producing human B cells, they do not conform with classical deletional patterns, where VDJ(H) regions utilize identified switch regions just upstream of the first coding exon. The absence of nontypical patterns in cells secreting any of the IgG or IgA subclasses (e.g., 36, 38) distinctly sets the IgE-committed B cell sublineage apart from the vast majority of all human B lymphocytes.

Discussion

In the present report we describe the subset of normal, human B cells committed to IgE production. It is characterized by (a) the expression of IgM and IgD on the surface; (b) stable secretion of IgM, IgD, and IgE after activation by EBV in lim-

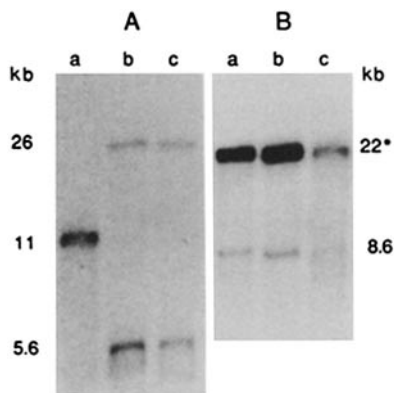Figure 12. Southern blot analysis of DNA from ε-producing clones and lines. 10 μg of DNA were analyzed. (A) Hind III-digested DNA hybridized with the J(H) probe; (a) germline DNA from the donor of 8G9 subclones (IgE⁺/IgM⁺/IgD⁺); (b) 8G9: subclone 1; (c) 8G9, subclone 2. (B) Hind III-digested DNA hybridized with the ε probe; (a) 8G9: subclone 1; (b) 8G9, subclone 2; (c) germline DNA from the donor of 8G9 subclones.

iting dilution cultures; (c) EBV transformability; (d) continued triple isotype secretion in progeny cells and subclones; (e) coexpression of cIgM, cIgE, and cIgD in the cytoplasm of single cells; (f) rearrangement of VDJ to $C\mu$ on the active chromosome; (g) absence of classical deletionel rearrangements with retention, in germline configuration, of all three IgE gene loci; and (h) the presence of normal-length IgE and IgM mRNA species.

The recently described IgE-secreting line, K1D5, was created from similar limiting dilution cultures that permit the outgrowth of low frequency precursors (3, 18, 19). This line secreted not only IgE, but also IgM. Since clonal IgG- and IgA-secreting lines from the same and many other experiments in our and other laboratories (35, 37) never showed coexpression of IgM, we wondered if IgE-committed B cells were somehow different from B cells committed to other isotypes of the secondary response. The experiments presented here extend our initial observation by defining this subset among fresh B cells, following its development at functional, phenotypic, and molecular levels through activation, transformation, and eventual cloning. All IgE-committed cells examined continued to coexpress μ, δ, and ϵ throughout this process. No evidence was obtained for the presence of classical deletional switch rearrangements to $C\epsilon$, setting this subset apart from cells expressing other isotypes 3' of $C\delta$.

All isotypes may have the capability to coproduce IgM/IgD and another isotype. Herzenberg et al. (29) proposed a model whereby cells activated to an 'early' memory B cell stage transiently produce sIgM, sIgD, and sIgG and rapidly lose sIgM and sIgD expression in a 'late' memory B cell stage. Yaoita et al. (23) suggested as well that such coexpression is transient and is an essential precursor to deletional switch. This model may explain the presence of rare multiple isotype-positive cells (31). Although we do not detect such cells in purified human IgG^+ or IgA^+ populations (36), these cells could have previously moved through this stage. These cells may be inherently infrequent or difficult to detect because the transition period is very short. Alternatively, such cells may be EBV resistant or EBV infection may promote a deletional switch. However, even if all isotypes go through this stage, then IgE producers do so much less transiently. They may perhaps require more or different signals for a transition from an earlier to a later, switched B cell stage.

In an alternative model, the deletional switch from VDJ-μ to VDJ-ϵ is an uncommon event, while deletional switch is the rule only for γ and α subclasses. Rather than rare cells frozen in a transitional stage of differentiation, the IgE-producing cells described here may be the typical IgE-committed B cells. In atopic patients B cells are found in circulation that secrete IgE spontaneously and at a high rate, without activation by EBV (12, 13). Current limiting dilution, as well as sorting experiments in our laboratory, indicate that these cells continue to express IgM and IgD, supporting our view that cells committed to IgE production do not frequently switch but may produce IgE by RNA-processing mechanisms similar to coproduction of IgM and IgD (Cheema, A., T. McKenzie, and H.-M. Dosch, manuscript in preparation). The size of such primary transcripts is not known. In this model, myeloma lines such as U266 would be examples of rare terminally differentiated (switched) IgE-producing B cells.

It is conceivable that activation by EBV in our system is generally biased toward cells that coproduce IgM, IgD, and IgE rather than those that express only IgE.

We believe this to be unlikely since frequencies of IgE-committed cells stimulated in the presence of alloreactive T cell clones were similar to those observed by us with EBV, indicating that the majority of such cells were being detected in either system (2). If there was a specific bias, one might expect to detect double or triple isotype-secreting cells of other Ig classes. We have not observed IgG or IgA secretors in cultures selected for presence of sIgM or sIgD, nor have we or others (35–37) observed IgM secretion in IgG- or IgA-producing clones. Cytophilic IgE on IgM producers may have biased sorting experiments (60). This is not likely relevant in our studies, since the majority of IgE-secreting cells were in the sIgE⁻ population and very few crosscontaminating cells were seen in any sorted population. Our data, therefore, imply that IgE producers are inherently different from IgG- or IgA-committed cells and, even if multiple isotype expression (such as IgM/IgD/IgG) occurs in other isotypes, the frequency and stability of triple isotype–secreting IgE-committed cells must be much higher.

Supportive evidence that deletional switch is not a priori required for IgE secretion comes from studies of murine lines that spontaneously switch in vitro. Subclones of two IgG2a-secreting mouse hybridomas independently began secreting IgE and ceased IgG2a production without changing DNA restriction endonuclease patterns as determined by Southern blotting (29). Subclones that had switched to other isotypes had almost invariably deleted the C(H) gene coding for the original isotype and none showed retention of the parent configuration.

If IgE producers do not formally switch VDJ to ϵ, why then does ϵ have a 5' switch region that seems to be functional, as evident in myeloma lines like U266 (20). Suggestions that rearrangements would be uncommon because of switch region homology with Cμ switch or because of their position on the chromosome have largely been discounted (61). Signals required for the initiation of IgE transcription in IgM⁺/IgD⁺-unstimulated cells may be similar to those that are responsible for transitory coproduction of IgG and IgA with IgM. However, signals causing the latter cells to switch and delete intervening DNA, seem to be missing in or ignored by IgE-committed B cells.

We propose that switch to ϵ is under stringent control at the molecular level of the B cell. This control mechanism could, however, be susceptible to external (perhaps T cell–mediated) triggers. It is possible that, in vivo, switched IgE-secreting cells are more common and lead to high IgE levels in atopic patients. This presently seems less likely since at least the bulk of (circulating) IgE producers in such patients appear to express IgM and IgD as well (see above). However, the cells responsible for antigen-specific IgE secretion in atopics have not been examined as yet.

Delayed or infrequent switch has implications for affinity selection during switch and for the preferential expansion of high affinity clones (61). Since IgE is potentially a harmful molecule, the absence of switch rearrangements could act as a safeguard by reducing the number of high affinity clones and diluting the effectiveness of the overall IgE response. The concurrent presence of IgM/IgD antibody of the same specificity would further reduce the functional role of IgE through competition for allergen. In conditions where IgE is potentially helpful, as during parasitic infections, it will be interesting to determine if localized B cells secreting IgE have switched, perhaps in response to specific factors elaborated during the infection.

Coexpression patterns of IgM/IgD/IgE were clonally stable at both the secretory

and molecular level in repeated analysis throughout a period of >1 yr. As far as analysis of both chromosomes is possible without allelic markers, it appeared that the single remaining $C\delta$ and both copies of $C\alpha_1$, $C\alpha_2$, $C\epsilon$, and both $\psi\epsilon$s remained in germline configuration and no new rearrangements or deletions were evident (see Fig. 7). Only rearrangements of VDJ to $C\mu$ were detected and, using five different restriction enzymes, never to $C\epsilon$. The unproductive rearrangement of VDJ to $C\gamma$ was recently confirmed to be to $C\gamma_3$, using polymerase chain reaction and subclass-specific oligonucleotides (unpublished).

The hypothesis that a V region gene may have been brought into a region that is too far upstream of ϵ to be detected by the enzymes used is attractive. However, using both Xba I (Fig. 11 B) and Eco RI (not shown) digest, the region $\sim$10.5 kb 5' of ϵ was found to be in germline configuration, including a 7.5-kb DNA stretch upstream of the ϵ switch region. More importantly, we can assign each of the only two J(H) bands detected to a fragment containing either a rearranged $C\mu$ or $C\gamma$. In Eco RI digests these J(H) bands (28 and 9.6 kb) do not contain an ϵ gene, and functional ϵ is detected as a (germline) 35-kb band in Eco RI digests (not shown). Nevertheless, the production of both μ and ϵ mRNA species indicates that some mechanism is controlling coexpression of V and ϵ. Although not a classical deletional rearrangement, some other mechanism may determine that ϵ is preferentially transcribed with μ and δ.

Differential RNA processing of a large primary transcript has been suggested in a number of systems to account for coexpression of two products that share genetic precursors, for example, for the coproduction of IgM and IgD (62). In the mouse BCL_1 B_2 line, coexpression of IgM and IgG1 was found to be controlled exclusively at the RNA level (63). Formal proof that such mechanisms are responsible for coexpression of μ, δ, and ϵ requires the isolation of a primary transcript that includes all three gene regions. If no deletion or manipulation of intervening DNA has occurred then this transcript would be very large (>200 kb) and its detection awaits improvements in technology.

Alternative mechanisms are conceivable, however. For example, the ϵ exons could have been brought closer to the $C\mu$-δ region by an inversion mechanism, allowing for transcription of a small primary transcript. An inversion requires two break points but, rather than deleting the intervening DNA, it remains in the chromosome next to the rearranged segment in inverted form (64). Inversions have been described in several systems, including globin and TCR genes, and they are especially common in Ig gene loci (65, 66).

For example, if DNA from the 5'-ϵ-α_2-3' region inverted into a region farther upstream, then ϵ would be brought considerably closer to VDJ-μ, yet all other C genes would appear to be in germline configuration. DNA digested with Hind III reveals a germline 22-kb band (see * in Fig. 12) that hybridizes with both ϵ and α_2. Since the region between ϵ and α appears to be intact, α_2 would have to be included in such an inversion and there would still be at least 65 kb of DNA to transcribe. In this model the expressed allele would be transcribed in two directions: 5' VDJ-$C\mu$-$C\delta$ 3' and 3' $C\alpha_2$-$C\epsilon$ 5'. The latter would be read in the direction opposite to its germline orientation. To accept this model one would have to account for the lack of functional expression of the intervening $C\alpha_2$ and explain how a functional IgE molecule were to be constructed from the inverted and complementary DNA strand.

One hypothesis that may account for coexpression of three isotypes is that C(H) genes might be translocated downstream closer to ϵ in a nonclassical recombination, i.e., without using recognized switch sequences. If one assumes that the productive allele contains only Cμ, Cδ, Cϵ, and Cα_2, then every other C(H) gene should be present in only one copy, on the other allele. However, the densities on Southern blots between ϵ and $\psi\epsilon_1$ and between α_1 and α_2 do not appear different, thus not providing evidence for fewer copies of $\psi\epsilon_1$ and α_1 genes. Thus, a translocation on the functional chromosome appears unlikely. Karyotypic abnormalities were absent in our clones.

Duplication of the expressed chromosome as a mechanism accounting for coexpression of μ and ϵ also seems unlikely. We have found in each of our clones only two distinct J(H) bands, both of which were nonambiguously assigned to non-ϵ-rearranged C(H) regions. A rearrangement arising after the duplication would not account for ϵ expression on either allele beyond those mechanisms already discussed. It is conceivable that a duplication of a functional ϵ gene may have occurred, however, perhaps by a gene conversion event.

Gene conversion normally involves two closely related genes interacting such that a portion of the sequence of one gene is converted into the sequence of the other (67). This seems unlikely as the conversion would have to include an intron region 10.5 kb upstream and 16 kb downstream of ϵ in addition to the 2-kb exon region. Extensive mapping of germline cosmid clones, switched myeloma lines, and fortuitous deletions in individuals within the H chain cluster has led to the current construction of the human C(H) gene region (Fig. 7) (68, 69). This does not formally rule out that a second functional ϵ gene may be present, perhaps in the unmapped region between the J-Cμ-Cδ cluster and Cγ_3. A mechanism of mRNA processing of a single transcript would then involve a smaller, appended region of perhaps <40 kb. However, such a gene would have to either be of equal size to a known ϵ gene and much of its surrounding intron stretches or be so different that it failed to hybridize to our ϵ probe; both of which seem highly unlikely.

The possibility that unequal crossing over (sister chromatid exchange [SCE]) leads to ϵ expression may be considered. This mechanism has generally been viewed as a germline event. However, somatic cells generating switches during SCE can result in a duplication of the expressed C(H) gene. This may alter the 5' to 3' order of C(H) genes (and may be one mechanism responsible for inversion) or commit the cell to production of a particular C(H) gene on the 3' side of deletion (70). It could therefore account for expression of IgE.

The consistent frequency of IgM/IgD/IgE-secreting cells detected in our system remains a puzzle since the mechanisms considered here are all uncommon. Preliminary observations in atopic patients suggest that triple isotype $\mu/\delta/\epsilon$ expression may be common in vivo. A recent limiting dilution study of mouse IgE-secreting cells demonstrated that IgE-producing clones secrete IgM and IgE in the same proportions as our clones ($\sim$5:1) and are blocked by anti-IgM antibodies, consistent with the view that IgE expression may follow unique but similar pathways in either species (71). Until approaches such as pulsed field gel electrophoresis provide us with more information on the order of C(H) genes in our clones, we favor a model of alternative processing of a single primary transcript, possibly transcribed from a Cϵ gene that has been brought closer to the VDJμ-δ region and requiring a smaller

primary transcript. Whatever the mechanisms responsible for multiple isotype production in IgE-committed B cells, our observations seem to rule out classical recombination/deletion events, such as those seen in the majority of B lineage cells and in the rare IgE-secreting myeloma lines U266.

In Northern blots of poly(A)$^+$ RNA hybridized with μ and ϵ probes, the detection of ϵ mRNA required a sevenfold longer development period. Levels of μ and ϵ mRNA are therefore markedly different. This dichotomy was also observed in the BCL1.B2 clone (63) and in cells that coexpress sIgM and sIgD where the level of μ membrane mRNA is 10 times that of δ despite higher levels of membrane IgD (72). It is possible that transcription of μ is preferentially terminated immediately 3' of Cμ exons and that IgE is expressed as the result of infrequent RNA polymerase unloading downstream of Cϵ. However, observations in the μ/δ system suggest that mRNA expression is both translationally and post-translationally regulated (62).

The mRNA species may also have different half lives or be preferentially processed to μ. Indeed, our experiments suggest that ϵ mRNA is less stable and may be specifically degraded or regulated by a protein, since it is stabilized in the presence of cycloheximide (unpublished). If this observation can be confirmed it would suggest that major regulatory events are occurring at post-transcriptional levels. Experiments show as well that IgE and IgM protein are produced at rates that do not reflect the disparity in mRNA levels. Results of cytoplasmic staining suggested that the translation or export rate of IgE protein may be more variable than that of IgM as most cells stain brightly for cIgM, while only 60–70% of these are also brightly cIgE$^+$. Characterization of the differential mechanisms controlling IgM and IgE production could lead to therapeutic strategies for the selective manipulation of IgE expression. This could represent a powerful tool in the management of atopic disease.

Summary

We have followed the pathway of the IgE-committed B lymphocyte from fresh, unstimulated peripheral blood, through EBV activation, transformation, and eventual cloning. Using cell sorting in conjunction with limiting dilution culture systems, we found that: (a) cells that are selected in the cell sorter and secrete IgE in culture are sIgM$^+$/sIgD$^+$. They secrete all three isotypes after EBV activation and continue to do so stably in culture; (b) individual IgE$^+$ cells in culture coproduce IgM, IgD, and IgE and cytoplasmic Ig of each isotype can be detected in single cells; (c) no rearrangement was observed of VDJ to ϵ in any of six lines tested. DNA between the rearranged VDJ-μ and -ϵ appears to be overall intact, including a region 10.5 kb upstream and 18 kb downstream of the 2-kb ϵ coding region and; (d) mRNA of μ and ϵ species is of normal and comparable size. In contrast to IgG- and IgA-producing clones, multiple isotype expression appears to be both frequent and stable in cells committed to IgE production. We propose that IgE-committed cells represent a unique B cell sublineage whose differentiation pathway may be more strictly regulated than that of other isotypes with regard to the signals required for classical, deletional switch recombination that has been observed in rare IgE-producing myeloma cell lines.

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