Brief Definitive Report

Human B Cell Clones Can Be Induced to Proliferate and to Switch to IgE and IgG4 Synthesis by Interleukin 4 and a Signal Provided by Activated CD4+ T Cell Clones

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Summary

In the present study, it is demonstrated that cloned surface IgM-positive human B cells can be induced to proliferate and to switch with high frequencies to IgG4 and IgE production after a contact-mediated signal provided by T cell clones and interleukin 4 (IL-4). This T cell signal is antigen nonspecific and is provided by activated CD4+ cells, whereas activated CD8+ or resting CD4+ T cell clones are ineffective. 15–35% of the B cell clones cultured with cloned CD4+ T cells and IL-4 produced antibodies; 35–45% of those wells in which antibodies were produced contained IgE and IgG4. In addition to B cell clones that produced IgG4 or IgE only, B cell clones producing multiple isotypes were observed. Simultaneous production of IgG4 and IgE, IgM, IgE, and IgM, or IgG4 and IgE was detected, suggesting that during clonal expansion switching might occur in successive steps from IgM to IgG4 and IgE. In addition, production of only IgM, IgG4, and IgE during clonal expansion indicates that this isotype switching is directed by the way a B cell is stimulated and that it is not a stochastic process.

Antigen-specific T helper–B cell interactions are mediated through binding of the TCR to peptide–class II MHC complexes on the surface of the B cells (1). Productive T–B cell interaction requires T helper cell activation by antigen and lymphokines produced by these cells. However, these activated T helper cells can also interact with B cells in an antigen-nonspecific fashion, which results in B cell proliferation and differentiation (2, 3).

Lymphokines have been shown to play an important role in the regulation of isotype production both in murine and human models (4–8). Human IL-4 induces IgE and IgG4 production in cultures of mononuclear cells (MNC) derived from peripheral blood, tonsils, spleens, or cord blood (6–8). Although IL-4 is the sole inducer of IgE synthesis, it has been demonstrated that a second signal provided by T cells is required, since IL-4 failed to induce IgE synthesis by highly purified B cells (6, 9). Reconstitution experiments carried out with autologous CD4+ and CD8+ T cells indicated that only CD4+ T cells were effective in inducing IgE synthesis in the presence of IL-4 (10). Presently, little is known about the exact nature and contribution of the signal provided by the T cells to IgE synthesis. It has been shown that allogeneic CD4+ T cell clones can replace autologous CD4+ T cells (9, 11, 12).

In the present study, we addressed the question whether IL-4 and the signal provided by CD4+ T cell clones were sufficient to induce cloned surface IgM–positive B cells to proliferate and to switch to IgG4 and IgE production.

Materials and Methods

Reagents. Human rIL-4 (sp act, 2×10^7 U/mg) was provided by Schering-Plough Research (Bloomfield, NJ) (10). The hybridomas producing mAbs against CD2 (OKT11), CD3 (OKT3), CD8 (OKT8), and CD11 (OKM1) were obtained from the American Type Culture Collection (Rockville, MD). The mAbs against CD4 (RIV6) and CD16 (B73.1), and the pair of antibodies recognizing the CD2 molecule (X11.1, D66), were generous gifts of Dr. Kreeftenberg (Rijks Instituut voor de Volksgezondheid, Bilthoven, The Netherlands), and Dr. Moore (DNAX Research Institute, Palo Alto, CA). The antibodies against CD19 (BD3) and CD20 (BB6) were obtained from Dr. Wÿdenes (Centre de Transfusion Sanguine, Besançon, France). The antibodies were purified from ascites by sequential precipitation with caprilic acid and ammonium sulfate. The antibodies against CD19 and CD20 labeled with rhodamine, and antibodies against IgA, IgG, IgM, and IgE labeled with FITC, were purchased from Becton Dickinson & Co. (Mountain View, CA) and Sigma Chemical Co. (St. Louis, MO), respectively.

B Cell Purification. The B lymphocytes were purified by positive selection from PMNC, or by negative selection from spleen cells. PBMC from normal donors were purified by centrifugation

J. Exp. Med. © The Rockefeller University Press • 0022-1007/91/03/0747/04 $2.00
Volume 173 March 1991 747–750

over Ficoll-Hypaque, washed twice, and resuspended in PBS containing 0.1% BSA before being labeled with rhodaminated CD20 antibody. The $CD20^+$ cells were directly sorted with an Epic-IV FACS® (Becton Dickinson & Co.). The sorted population was reanalyzed and contained >98% $CD20^+$ cells. Spleen cells were isolated by centrifugation over Ficoll-Hypaque and labeled for 1 h at 4°C with the following antibodies: OKT11, OKT3, RIV6, OKT8, OKM1, and B73.1 at a final concentration of 1 μg of antibody per 10^6 cells. The cells were washed twice in PBS and incubated with magnetic beads coated with a mAb against mouse Igs (Advanced Magnetics, Cambridge, MA) under gentle agitation for an additional 1 h. The final concentration of bead suspension was 50 μl for 10^6 cells. The non-B cells were removed by applying a magnetic field for 10 min. The purity of the sorted B cell population varied from 93 to 98% as determined by CD20 staining, followed by FACS® analysis.

T Cell Clones. The human T cell clones B21 and B27 are specific for tetanus toxin, and the clones A10 and C5 recognize HLA-B47 and HLA-A3. The $CD4^+$ T cell clones PD49, NP12, and NP44 were derived from atopic patients and recognize specifically *Der p* I, which is the major allergen in extracts of the house dust mite *Dermatophagoides pteronissynus* (Yssel et al., manuscript submitted for publication).

B/T Cell Cocultures. Ig production was measured in cultures where 5,000 cloned $CD4^+$ T cells were added to 5,000 purified B cells or single B cells seeded by automatic cloning using a FACStar Plus® (Becton Dickinson & Co.) in a final volume of 0.2 ml of Yssel's medium supplemented with 10% FCS and 400 U/ml rIL-4.

Limiting Dilution Cultures. Limiting dilution cultures in which B cells were seeded in 96-well round-bottomed plates (Linbro Chemical Co., Hamden, CT). The experiments were performed by seeding decreasing concentrations of purified B cells (from 300 to 0.3 B cells) in the presence of a fixed concentration of 5,000 T cell clones, in a final volume of 0.2 ml of Yssel's medium supplemented with 10% FCS, and in the presence of IL-4 (400 U/ml). The supernatants were analyzed for their Ig content after a culture period of 2 wk. Statistical analysis of the limiting dilution experiments was carried out according to Taswell (13). Ig secretion was determined by ELISA as described (6).

Results and Discussion

We demonstrated previously that purified human B cells cocultured with the allogeneic $CD4^+$ T cell clone A3 (specific for HLA-DR4) and IL-4 produce considerable levels of IgE, whereas B cells (not expressing HLA-DR4) cultured with IL-4 or in the presence of A3 cells in the absence of IL-4 failed to produce IgE, indicating that in addition to IL-4, a second signal provided by allogeneic $CD4^+$ T cells with nonrelevant specificity is required for induction of IgE synthesis (12). A small panel of other $CD4^+$ T cell clones gave similar results. Cocultivation of both positively or negatively selected B cells (5000/well) with $CD4^+$ T cell clones (5000/well) specific for tetanus toxin (B21, B27) or for the house dust mite–derived allergen *Der p* I (PD49, NP12, and NP44) in absence of their specific antigens resulted also in induction of IgE synthesis. However, the $CD8^+$ T cell clones A10 and C5 were ineffective. These results indicate that the capacity to deliver the second signal required for induction of IgE synthesis seems to be a general property of $CD4^+$ T cell clones. The T cell clones needed to be activated (see Materials and Methods) since resting T cells obtained 12–15 d after activation failed to provide B cell help resulting in IgE synthesis (results not shown). The $CD4^+$ T cell clone B21 was selected for further studies.

Clonal Analysis of IgE-producing Cells. To determine whether the IgE levels detected by coculturing B cells with activated $CD4^+$ T cell clones and IL-4 reflected preferential growth and IgE secretion by surface IgE–positive B cells or a real ϵ switch of precursor B cells, the frequency of B cells able to produce IgE were analyzed in limiting dilution experiments. The frequency of IgE-producing cells was found to be 1:15, as shown in Fig. 1 *A*. The frequencies obtained in five other experiments were 1:15, 1:56, 1:17, 1:18, and 1:6. Although some variations were observed from donor to donor, the average frequency of B cells able to produce IgE was 1:22. IL-4 also induces IgG4 synthesis (7). In Fig. 1 *B*, it is demonstrated that IgG4 is produced in our culture system. The frequency of IgG4-producing cells (1:35 in this experiment; average: 1:25) is in the same range as those of IgE-producing cells. These results indicate that the B cells had switched to IgG4- and IgE-producing cells during the culture period, and that the same set of signals that induce B cells to switch to IgE is also inducing switching to IgG4.

Cloned Human B Cells Proliferate upon Coculturing with T Cells and IL-4. Next, we investigated whether cloned B cells could be induced to proliferate under our culture conditions. To this end, single B cells automatically cloned with the FACS® were cocultured with 5,000 B21 cells and IL-4. One of three B cell cultures was found to contain >1% of $CD19^+$ cells. The average number of B cells varied from 1 to 12% of the total number of cells present in the cultures, which corresponded to ~100–1,000 B cells per well. Fig. 2 represents a typical pattern of a proliferating B cell clone as detected by FACS® staining. The doubling time of the proliferating B cells under these culture conditions was estimated to be 24–36 h.

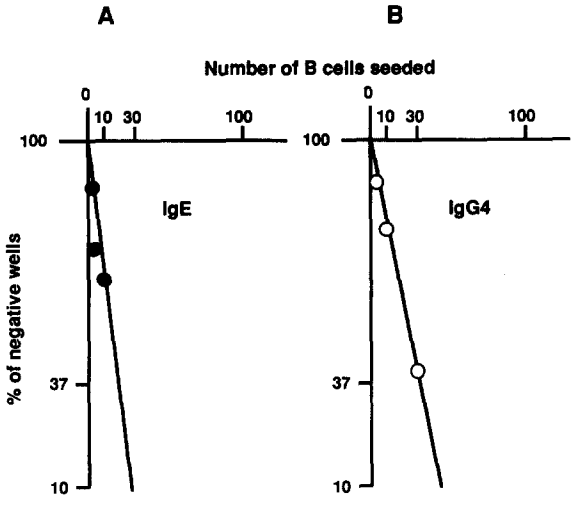

Figure 1. Frequencies of IgE (*A*) and IgG4 (*B*) -producing B cells as determined by limiting dilution experiments. The results are expressed by plotting the percentage of IgE- and IgG4-negative wells against the number of B cells seeded in the culture. The frequency of IgE- and IgG4-producing B cells is determined from the 37% intercept.

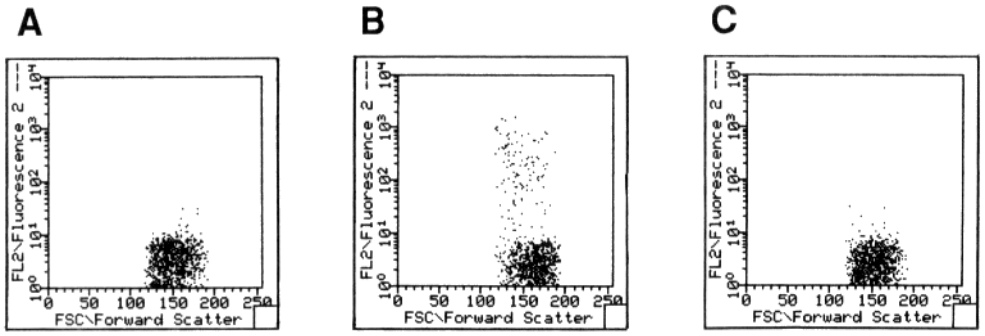

Figure 2. Proliferation of cloned B cells in the presence of cloned CD4+ T cells and IL-4. Single B cells were seeded in the presence of 5,000 B21 cells and IL-4 (400 U/ml) in Yssel's medium supplemented with 10% FCS. After 12 d of culture, proliferating B cells were directly stained with CD19-FITC and analyzed by FACS®. (*A*) Culture of B21 cells in IL-4. (*B* and *C*) Cocultures of cloned B cells and B21 cells in IL-4 reflecting B cell proliferation and absence of B cell proliferation, respectively.

Analysis of Isotype Secretion by B Cell Clones. Cloned B cells could also be induced to switch and to secrete various isotypes in the same well. In a first series of experiments, the frequencies of B cells producing IgM, total IgG/IgG4, IgE, and IgA in the presence of IL-4 and B21 cells were determined. The range of frequencies was found to be similar for IgM, IgG, IgG4, and IgE, varying from 1:14 for the IgG/IgG4–producing cells, to 1:17 for the IgM- and IgE-producing B cells (Fig. 3). Total IgG synthesis reflected predominantly production of IgG4, since virtually all wells containing IgG were positive for IgG4. In addition, IgG4 production constituted >95% of total IgG production (data not shown). In contrast, the frequency of IgA-producing cells in the presence of IL-4 was found to be >1/400, which is compatible with the observation that IL-4 is suppressive for induction of IgA synthesis (Van Vlasselaer et al., manuscript submitted for publication). The probability to have B cell clones derived from one seeded B cell per well was found to be at least 97% for these four isotypes. The various isotypes or isotype combinations produced in cultures initiated from single B cells automatically cloned by FACS® are shown in Table 1. Apparently, 15–35% of the B cell clones seeded are able to produce simultaneously several isotypes, indicating that a switch event occurred in the culture. The surface phenotype of the B cells used for the cloning experiments (Table 1, Exps. 1 and 2) were 50% IgM positive, 98% IgD positive, 3–5% IgG positive, 35–50% IgA positive, and 0% IgE positive (data not shown). After 2 wk of culture, the percentage of isotypes did not change significantly for IgM (36–55%), but a strong increase in the percentage of B cells able to produce IgE (31–38%) and IgG/IgG4 (43–44%) was observed.

Similar data were obtained with single CD20+, surface IgM-positive B cells sorted from MNC that were double stained with CD20 rhodamine and anti-IgG, anti-IgA, and IgE-FITC antibodies, and cloned at one B cell per well by automatic cloning. Analysis of the isotypes produced by B

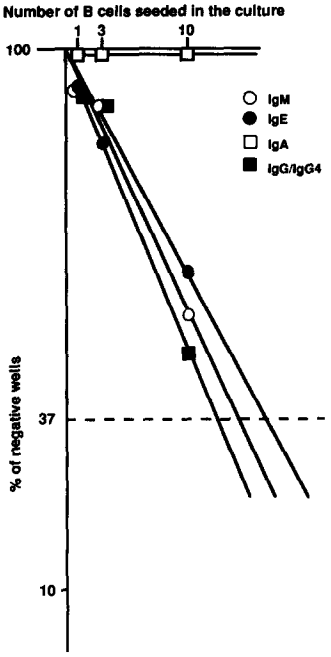

Figure 3. Frequencies of B cells producing IgM, IgG/IgG4, IgE, and IgA in the presence of cloned CD4+ T cells and IL-4. Each well was assayed for the presence of IgA (□), IgM (○), IgG/IgG4 (■), and IgE (●) by ELISA. The results were expressed by plotting the percentage of negative wells for each isotype as a function of the number of B cells seeded in the well. The frequencies were determined as the number of B cells that gave 37% of negative wells for the appropriate isotype.

Table 1. *Determination of Isotype Production in Cultures Initiated from Single B Cells Cloned with the FACS*

	Percent of producing wells			
	Exp. 1	Exp. 2	Exp. 3*	Exp. 4*
IgE	12	29	15.4	23
IgE + IgG/G4	2.4	3.5	7	4.5
IgE + IgM	9.7	4.7	4.4	6.4
IgE + IgG/G4 + IgM	7.3	1.1	7.4	2.2
IgM	34	22	22.4	25
IgM + IgG/G4	17	7.7	12.7	10.5
IgG/G4	17.6	32	30.7	28.4
IgA‡	ND	0	0	0
Total:	100	100	100	100
No. of B cell clones analyzed:	47	85	267	228

B cell clones were obtained as described in Materials and Methods. The probability that Ig production in the wells was derived from a single B cell was >97%. Data shown are isotype production in wells derived from one producing B cell.

* IgM surface-positive cells seeded by automatic cloning (1 cell/well).

‡ Alone or in combination with the other isotypes.

cell clones generated from surface IgM-positive cells indicated 34–36% of the clones produced IgE, 46–58% IgG/IgG4, and 44–47% IgM (Table 1, Exps. 3 and 4). These results confirmed that surface IgM-positive B cells underwent switching in the culture. Collectively, 35–45% of the wells in which Ig was produced contained IgE and IgG4, whereas IgM was synthesized in 35–65% of the wells. Although 12–15% and 18–32% of the wells contained only IgE and IgG4, respectively, multiple isotypes were found to be produced by the cloned B cells. Simultaneous production of IgG4 and IgE (2–7%), IgM and IgE (4–10%), IgM/IgG4 (8–17%), and IgM, IgG4 and IgE (1–7%) could be measured. One possible explanation for these findings is that during clonal expansion, IgE-producing cells are not directly generated from IgM-positive cells, but that switching first to IgG4 and then to IgE occurs. This notion is supported by recent reports in which analysis of switch circular DNA excised by Ig class switching of murine B cell induced by TGF-β or IL-4 indicated that isotype switching might occur in successive steps (14, 15).

We thank Janet Dillon for her careful preparation of the manuscript. Jim Cupp and Josephin "Dixie" Polakoff are gratefully acknowledged for their expert FACS® assistance.

The DNAX Research Institute of Molecular and Cellular Biology is supported by Schering-Plough Corporation.

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Received for publication 17 October 1990 and in revised form 27 December 1990.

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