T Cell Clones from an X-linked Hyper-Immunoglobulin (IgM) Patient Induce IgE Synthesis In Vitro Despite Expression of Nonfunctional CD40 Ligand

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Summary

The induction of immunoglobulin E (IgE) switching in B cells requires at least two signals. The first is given by either of the soluble lymphokines interleukin 4 (IL-4) or IL-13, whereas the second is contact dependent. It has been widely reported that a second signal can be provided by the CD40 ligand (CD40L) expressed on the surface of T cells, mast cells, and basophils. A defect in the CD40L has been shown recently to be responsible for the lack of IgE, IgA, and IgG, characteristic of the childhood X-linked immunodeficiency, hyper IgM syndrome (HIGM1). IgE can however be detected in the serum of some HIGM1 patients. In this study, we isolated T cell clones and lines using phytohemagglutinin (PHA) and allergen, respectively, from the peripheral blood of one such patient who expressed a truncated form of CD40L, and investigated their ability to induce IgE switching in highly purified, normal tonsillar B cells in vitro. Unexpectedly, 4 of 12 PHA clones tested induced contact-dependent IgE synthesis in the presence of exogenous IL-4. These clones were also shown to strongly upregulate IL-4-induced germline e RNA and formed dense aggregates with B cells. Of the four helper clones, three were CD8+, of which two were characteristic of the T helper cell 2 (Th2) subtype. Two allergen-specific HIGM1 T cell lines, both of the Th0 subtype, could also drive IgE synthesis when prestimulated using specific allergen. All clones and lines were negative for surface expression of CD40L, and the mutated form of CD40L was confirmed for a representative clone by RNase protection assay and sequencing. The IgE helper activity could not be attributed to membrane tumor necrosis factor α (TNF-α) although it was strongly expressed on activated clones, and the addition of neutralizing anti-TNF-α antibody did not abrogate IgE synthesis. These results therefore suggest the involvement of T cell surface molecules other than CD40L in the induction of IgE synthesis, and that these molecules may also be implicated in other aspects of T-B cell interactions.

Activated T cells induce IgE switching in B cells via a combination of lymphokines and direct T-B cell contact (1-6). One of the T cell surface structures considered to play a central role in upregulating lymphokine-triggered germ-line e transcript synthesis via CD40 on B cells is the CD40 ligand (CD40L)1, a type II membrane protein with homology to TNF-α and -β (7, 8, and for a review see reference 9). In X-linked hyper IgM syndrome (HIGM1), a childhood immunodeficiency, there are greatly decreased serum levels of IgG, IgA, and IgE, with normal to increased levels of IgM and IgD (for a review see reference 10). This defect in Ig isotype switching has been attributed to a lack of functional CD40L expression on T cells, resulting from either deletions or point mutations within a limited region of the CD40L extracellular domain (11-14). Given that serum IgE is still detectable in some HIGM1 patients, we wished to know whether T cell clones isolated from such a patient could induce IgE synthesis in vitro. Here we report that mitogen-stimulated T cell clones and allergen-stimulated T cell lines isolated from PBMC of a HIGM1 patient are able, in the presence of IL-4, to induce contact-dependent IgE synthesis by pure tonsillar B cells, despite the total absence of functional CD40L. The HIGM1 T cell help for IgE synthesis cannot be wholly accounted for by membrane TNF-α. This

1 Abbreviations used in this paper: CD40L, CD40 ligand; EB-LCL, Epstein-Barr virus-transformed lymphoblastoid cell line; HIGM1, hyper IgM syndrome.
suggests that other T cell surface molecule(s), in addition to CD40L, are able to induce IgE synthesis and may explain why IgE can still be detected in some HIGM1 patients.

**Materials and Methods**

**Patient**

Patient MAB, a 9-yr-old boy, had a family history of X-linked immunodeficiency, and presented with clinical features of HIGM1. At the commencement of study, his total serum IgE titer was 1 ng/ml. Prior testing of PBMC-proliferative responses to a panel of mitogens, recall antigens, and allergens showed vigorous responses to CD40L are able to induce IgE synthesis and may explain why IgE can still be detected in some HIGM1 patients.

**T Cell Clones**

PBMC were separated from heparinized venous blood using Ficoll-Hypaque and stimulated for 7 d with either 1 μg/ml PHA (HA-16; Wellcome, Beckenham, UK) or 10 μg/ml dog hair allergen, Alyostal ST-IR (Stallergenes, Paris, France), and 200 U/ml human recombinant IL-4 (Amersham International, Amersham, Bucks, UK). Prestimulation and cloning of PBMC was done in IMDM (Gibco, Paisley, Scotland) supplemented with 10% heat-inactivated AB+ human serum (Centre Transfusion Sanguine, Annemasse, France), 2 mM glutamine (Flow, Irvine, Scotland), 100 μg/ml streptomycin, 100 U/ml penicillin (Gibco), 1% non-essential amino acids, 1% sodium pyruvate, and 1 mM Hepes (all from Sigma Chemical Co., St. Louis, MO), and 1 μg/ml PHA or 10 μg/ml dog hair allergen. Growing colonies were transferred to 200 μl U-bottomed wells (Nunc, Roskilde, Denmark) using autologous γ-irradiated mononuclear cells (106), 100 U/ml human recombinant IL-2 (Glaxo Institute), and 1 μg/ml PHA or 10 μg/ml dog hair allergen. Growing colonies were transferred to 200 μl U-bottomed wells (Nunc) at day 11 and restimulated with 5 × 105 irradiated, allogeneic PBMC, 1 μg/ml PHA, 100 U/ml IL-2, and 200 U/ml IL-4 in CM. Expansion was continued by twice weekly feeding (rabbit IgG, 100 μg/ml streptomycin, 100 U/ml penicillin (Gibco), 1% non-essential amino acids, 1% sodium pyruvate, and 1 mM Hepes (all from Sigma Chemical Co., St. Louis, MO), complete medium. T cells blasts were cloned by limiting dilution in 20 μl Terasaki wells (Nunc, Roskilde, Denmark) using autologous γ-irradiated mononuclear cells (106), 100 U/ml human recombinant IL-2 (Glaxo Institute), and 1 μg/ml PHA or 10 μg/ml dog hair allergen. Growing colonies were transferred to 200 μl U-bottomed wells (Nunc) at day 11 and restimulated with 5 × 105 irradiated, allogeneic PBMC, 1 μg/ml PHA, 100 U/ml IL-2, and 200 U/ml IL-4 in CM. Expansion was continued by twice weekly feeding with IL-2 and IL-4 and by restimulation every 14–21 d with allogeneic PBMC and PHA. Clones were only used in IgE assays at least 14 d after restimulation.

**Immunoglobulin Assays**

**B Lymphocyte Separation.** B lymphocytes were isolated from Ficoll-separated tonsillar mononuclear cells by a two-step negative selection procedure using sheep cell rosetting and magnetic bead depletion (Dynal, Norway) to remove T cells. B cell purity was routinely >99% as determined by FACS®. Cells were resuspended at 106 cells/ml in IMDM as described previously (15). Percoll was used to isolate high density (resting) and low density (activated) B cells. Briefly, tonsillar B cells purified as above were cultured in 2-ml wells (Falcon, Oxnard, CA) with 5 x 105 γ-irradiated (3,500 rad) PBMC and 0.1 μg/ml PHA in CM for 6 d. In some experiments, T cells were stimulated with PHA or dog hair allergen in the presence of an autologous Epstein Barr virus-transformed lymphoblastoid cell line (MAB EB-LCL). EB-LCL were irradiated with 6,000 rad and added to T cells at 3 × 106 cells per well. Stimulated T cells were washed three times in PBS to remove human serum before resuspension in Yssel’s medium at 106 cells/ml. Stimulated T cells were washed three times in PBS to remove human serum before resuspension in Yssel’s medium at 106 cells/ml. Prestimulated T cells (106) were cocultured with tonsillar B cells (106) in Yssel’s medium supplemented with 200 U/ml rIL-4 for 14 d. Supernatants were tested for IgE/IgG by ELISA (16).

To investigate the contact dependency of T cell help for IgE induction, 5 × 106 T cells, prestimulated as described above were incubated with 5 × 106 tonsillar B cells and 200 U/ml Transwells (Costar, Cambridge, MA) with a membrane pore diameter of 0.4 μm. Supernatants were taken from the B cell-containing chamber after 14-d culture and assayed for IgE as described above.

To test if T cell clones could upregulate germline e transcription, 106 T cells, preactivated using auto-EB-LCL and PHA were cocultured with an equal number of tonsillar B cells and 200 U/ml rIL-4 for 5 d in duplicate 2-ml wells (Falcon).

**Northern Blot Assays**

Total RNA was isolated by extraction with RNaZolol (Cinna/Bio-tek Laboratories, Houston, TX) according to the manufacturer’s instructions. Aliquots of RNA corresponding to one third of the isolated RNA were subjected to denaturing agarose electrophoresis, transferred to nylon + membrane, and fixed by UV irradiation as described previously (17). Hybridizations with cRNA probes complementary to Ce or to actin mRNA were performed as described previously (17).

**Measurement of T Cell Cytokines**

106 T cells were cocultured for 24 h with Con A (10 μg/ml, Sigma). Culture supernatants were filtered (0.22 μm) and tested for IL-2, IL-4, and IFN-γ (Glaxo Institute) using ELISA kits (Genzyme, Cambridge, MA).

**Immunostaining**

Surface phenotyping of clones was performed by staining with specific antibodies and flow cytometry as described previously (8). Antibodies used were leu 4-FITC, leu 3a + 3b-FITC, leu 2a, anti-TCR-α/β, anti-TCR-γ/δ, CD3-PF, mouse IgG1 pure, and FITC-labeled and mouse IgG2a pure, all purchased from Becton Dickinson & Co. (Mountain View, CA). Goat anti-mouse FITC was purchased from Bioart (Nanterre, France). Polyclonal rabbit anti-TNFα (MCA255; Serotec, Oxford, UK) was revealed using anti-rabbit IgG1(ab')2-FITC (CalTag Laboratories, San Francisco, CA).

**T Cell Surface Expression of CD40L**

106 T cells were cocultured with 10 ng/ml PMA and 1 μM ionomycin (both from Calbiochem-Novabiochem Corp., La Jolla, CA) for 5 h and stained for expression of CD40L using CD40Fc and fluorescein-labeled goat anti-mouse IgG2a pure, all purchased from Becton Dickinson & Co. (Mountain View, CA). Goat anti-mouse FITC was purchased from Bioart (Nanterre, France). Polyclonal rabbit anti-TNFα (MCA255; Serotec, Oxford, UK) was revealed using anti-rabbit IgG1(ab')2-FITC (CalTag Laboratories, San Francisco, CA).

**Analysis of CD40L RNA by RNase Protection Assay**

An RNA probe complementary to the deleted region of CD40L was generated by PCR amplification of nucleotides 386–603 of CD40L cDNA using the primers CGATAATTCGTTGATACAGATCCCTCAATTCG and CTAGAATTCTGAGCATGCAGTGAGACTCC (8). The cDNA fragment was cloned in the EcoRI site of pBluescript ISK + (Stratagene, La Jolla, CA). A
probe complementary to the CD40L cDNA was generated by transcription in the presence of [32p]UTP (400 Ci/Mmol) of the pBluescript IISK+ template linearized with BamHI. Full-length probe was purified by acrylamide-urea gel electrophoresis. Aliquots of eluted probe (5 \times 10^5 cpm) were coprecipitated with 50 \mu g of total RNA isolated from T cell clones JF7 (8), MAB PHA .3.6, and 20 \mu g tRNA or tRNA alone. The RNase protection assay was performed using a kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

**Analysis of the CD40L cDNA Sequence for T Cell Clone MAB PHA .3.6**

Poly (A)+ RNA was subjected to oligo dT primed reverse transcription. cDNA was amplified by PCR using the primers described above and cloned in the EcoRI site of pBluescript IISK+. The DNA sequence was determined by the dideoxy method (18).

**Results**

**Induction of IgE Synthesis by HIGM1 T Cell Clones and Lines**

T cell outgrowth was seen in 30 out of a total of 300 PHA-stimulated wells. 12 of these T cell colonies, derived from wells seeded with 0.3-100 PHA-stimulated T cell blasts were expanded and tested for their ability to induce IgE synthesis in very highly purified (>99%) tonsillar B cells. In the first assay, T cells were prestimulated with PHA and irradiated allo-PBMC 5 d before coculture with B cells. Unexpectedly, two T cell clones (.14 and .3.6) induced measurable IgE synthesis which was dependent on the presence of exogenous IL-4. No IgE was detected in cultures containing B cells with IL-4 alone (Fig. 1 A). The levels of IgG measured in the same cultures varied among clones. For example, clone MAB PHA .3.6 markedly increased IgG production over the anti-CD40 mAb background, whereas clone MAB 1.14 did not (Fig. 1 A). It is interesting to note that dense cellular aggregates were seen only in cultures found later to contain IgE.

To exclude the possibility that the T cell stimulus for IgE switching might have been provided by the irradiated PBMC used for prestimulation, the IgE helper clones were restested after prestimulation with irradiated autologous EB-LCL and PHA. Tonsillar B cells cultured with these EB-LCL prestimulated T cell clones and exogenous IL-4 were still able to produce IgE. No IgE was detected in cultures of B cells alone, nor in B cells cultured with the irradiated EB-LCL used for T cell prestimulation. During this assay, a further two PHA clones (MAB PHA .3.3 and .3.4) were identified as able to induce IgE synthesis (Fig. 1 B).

To ensure that the IgE measured was not simply the product of IgE precommitted cells, an assay was done as above using high density (resting) Percoll-fractionated tonsillar B cells. Again, IgE was detectable in B cell cultures containing HIGM1 T cell clones, but not in cultures of B cells and IL-4 alone (data not shown).

Fig. 1 B also shows data for two dog allergen-specific HIGM1 T cell lines (MAB DOG 5.2 and 5.5) which induced IgE synthesis when prestimulated using dog allergen and autologous, irradiated EB-LCL as APC. A CD40L-positive, dog allergen-specific T cell clone, OZZ .3.4, isolated from a normal donor (Life, P., V. Schnuriger, S. Estoppey, G. Mazzei, and J.-Y. Bonnefoy, manuscript in preparation), was included in this assay as a second positive IgE control.

With the exception of T cell line MAB DOG 5.2, the levels of IgE produced in the HIGM1 T cell–driven cultures were markedly lower than those in cultures containing either anti-CD40 mAb or the wild-type clone (Fig. 1 B). As described above, dense cellular aggregates were seen in IgE-positive cultures. FACSS® analysis (Becton Dickinson & Co.) showed the aggregates to consist of T and B cells in varying proportions (data not shown). The tendency for dense...
T-B cell aggregates to form in IgE-containing cultures suggested that the help for IgE synthesis was contact mediated. This was confirmed using a Transwell system in which T cells were separated from highly purified tonsillar B cells by a semi-permeable membrane of 0.4-μm pore size. Both chambers contained 200 U/ml of rIL-4. The data in Table 1 clearly demonstrate the contact dependency of the T-B cell interaction in that no IgE was detected when T-B cell contact was interrupted. Also, dense cellular aggregates were again seen in mixed T-B cultures, but were absent when the cell types were separated by a semi-permeable membrane.

To examine whether IgE nonhelper clones either express or secrete factors able to suppress IgE synthesis, two such clones were prestimulated using PHA and coincubated with B cells, IL-4, and anti-CD40 mAb at a concentration optimal for induction of IgE synthesis. Fig. 2 shows that the presence of nonhelper T cells did not suppress IgE production. In the same experiment, the addition of anti-CD40 mAb to an IgE helper T cell clone isolated from a normal donor produced a marked synergistic effect on IgE synthesis (Fig. 2).

### Table 1. Contact-dependent HIGM1 T Cell Help for IgE Synthesis

|                        | IgE (ng/ml) |
|------------------------|-------------|
| B + IL-4               | <0.2        |
| B + IL-4 + PHA 1.14    | 100 ± 15    |
| B + IL-4 + PHA 1.14 (contact +) | 11 ± 4     |
| B + IL-4 + PHA 1.14 (contact -) | <0.2       |

5 x 10⁵ tonsillar B cells (>99% pure) were coincubated with an equal number of PHA-prestimulated HIGM1 T cell clones (PHA 1.14) in 2-ml culture wells either mixed (contact +) or separated by a semi-permeable membrane (contact -). IgE was measured by ELISA at day 14.

### Figure 2

**Effect of HIGM1 and normal T cell clones on anti-CD40-driven IgE synthesis.** 10⁵ tonsillar B cells (>99% pure) were cocultured with 10⁵ T cell clone for 14 d either in the presence or absence of anti-CD40 mAb (1 μg/ml). IgE production was measured by specific ELISA. Data are shown for two HIGM1 clones (PHA 1.1 and 1.4) which reproducibly fail to induce IgE synthesis, and a clone isolated from a non-HIGM1 donor (OZZ.3.4), able to induce IgE. All clones were prestimulated for 6 d using allogeneic, irradiated PBMC and PHA (1 μg/ml) before addition to B cells. All cultures contained 200 U/ml rIL-4.

### Figure 3

**Wild-type and CD40L deficient T cell clones trigger germline e RNA synthesis.** Cells were incubated for 5 d with IL-4 (200 U/ml) as described in Materials and Methods. Lane 1, tonsillar B cells alone; lane 2, B cells stimulated with anti-CD40 mAb (1 μg/ml); lane 3, wild-type T cell clone OZZ.3.4 alone; lane 4, clone OZZ.3.4 and B cells; lane 5, wild-type T cell clone TT7 alone; lane 6, clone TT7 and B cells; lane 7, HIGM1 T cell clone MAB PHA 1.14 alone; lane 8, clone MAB PHA 1.14 and B cells; lane 9, HIGM1 T cell clone MAB PHA 1.14 alone; lane 10, clone MAB PHA 1.14 and B cells; lane 11, HIGM1 T cell clone MAB PHA 1.14 alone; lane 12, clone MAB PHA 1.14 and B cells; lane 13, irradiated EBV-transformed B cell line MAB-EB alone; and lane 14, MAB-EB and B cells. Aliquots of RNA representing one third of RNA isolated from the cultures were analyzed by Northern blot with cRNA probes specific for C6 or actin mRNA. Cultures included 10⁵ of the following cell types: T cell clones; purified tonsillar B cells; and irradiated EBV-transformed B cells.
Table 2. Surface Phenotype and Lymphokine Profile of IgE Helper HIGM1 and Wild-type T Cell Clones and Lines

| Clone | CD3 | CD4 | CD8 | TCR-α/β | TCR-γ/δ | IL-2 | IL-4 | IFN-γ |
|-------|-----|-----|-----|---------|---------|------|------|-------|
| MAB   |     |     |     |         |         |      |      |       |
| .3.3  | +   | -   | +   | +       | -       | 0    | 85   | 0     |
| .3.4  | +   | -   | +   | +       | -       | 975  | 2,055| 0     |
| .3.6  | +   | +   | -   | +       | -       | 0    | 1,700| 0     |
| 1.14  | +   | -   | +   | +       | -       | 0    | 1,075| 0     |
| 5.2   | +   | 80% | 20% | +       | -       | 0    | 850  | 125   |
| 5.5   | +   | 75% | 25% | +       | -       | 0    | 210  | 0     |
| POD   |     |     |     |         |         |      |      |       |
| .3.4  | +   | +   | -   | +       | -       | 0    | 285  | 810   |
| 5.9   |     |     |     |         |         |      |      |       |

T cell clones and lines were stained for the surface markers shown using standard techniques and FACS® analysis. The mAbs used are described in Materials and Methods. Cells were phenotyped at least 14 d after stimulation with PHA and allogeneic PBMC. To determine lymphokine production, 10⁶ cells were stimulated with Con A (10 µg/ml) for 24 h and supernatants were tested using specific ELISA kits. IgE helper T cell clones OZZ .3.4 and POD 5.9 are from two normal donors.

Each of the four clones and two lines were negative. As a positive control, T cell clones isolated from two normal donors stained strongly positive for CD40L in the same experiment (Fig. 4).

Analysis of CD40L mRNA by RNase Protection. An RNase protection assay was used to differentiate between wild-type and mutated CD40L mRNA. The RNA probe used was complementary to 218 bases of CD40L RNA and was expected to give a mismatch when hybridized with mutated CD40L mRNA from the HIGM1 patient (patient no. 4; reference 11). When hybridization was performed using wild-type RNA from T cell clone JF7, stimulated for 4 h with PMA (10 ng/ml) and ionomycin (1 µM), a major band of 0.2 kb was observed by polyacrylamide-urea gel autoradiography after RNase protection assay. This band was replaced by one of 0.1 kb using RNA isolated from clone MAB PHA .3.6 activated under identical conditions (data not shown). This indicated that the hybrid contained the expected deletion mismatch.

Isolation and Sequencing of Mutated CD40L from T Cell Clones. To show directly that the CD40L was mutated, mRNA was isolated and reverse transcribed from an IgE helper HIGM1 T cell clone (MAB PHA .3.6). A region flanking the expected mutation was amplified using PCR and the product cloned in pBluescript. Five independent cDNA inserts were characterized. As expected, each contained the 10-bp deletion, confirming that clone MAB PHA .3.6 expressed the mutated form of CD40L as described previously (11).

CD40L Expression in HIGM1 T Cell Clones

In view of the unexpected ability of HIGM1 T cell clones to induce IgE synthesis, three approaches were taken to confirm that the clones did indeed express the mutated form of CD40L. This was intended also to exclude the unlikely possibility that wild-type CD40L from irradiated feeders could have contaminated the clones.

Cell Surface Expression. MAB T cell clones and lines were stimulated under optimal conditions using PMA and ionomycin and examined for surface expression of CD40L using CD40Fc and a FACS® as described in Materials and Methods. a TCR-α/β. The dog allergen–specific T cell lines contained both CD4⁺ and CD8⁺ cells. To determine the lymphokine profile of the IgE helper T cell clones and lines, each was stimulated with an optimal concentration of Con A for 24 h and supernatants were tested for IL-2, IL-4, and IFN-γ by ELISA. Data are given in Table 2. Three clones (MAB PHA .3.3, .3.6, and 1.14) secreted IL-4 only whereas the other (MAB PHA .3.4) secreted both IL-4 and IL-2. Of the two dog allergen–specific HIGM1 lines, one (MAB DOG 5.5) secreted IL-4 only whereas the other (MAB DOG 5.2) secreted both IL-4 and IFN-γ. Also shown in Table 2 are the surface phenotype and lymphokine profile of two wild-type IgE helper T cell clones used in this study. Clone POD 5.9 secretes only IL-4 in similar amounts to HIGM1 clone MAB PHA .3.6 but considerably more than MAB PHA 1.14 and .3.3. In contrast, clone OZZ .3.4 secretes both IL-4 and IFN-γ, yet is a potent and reproducible inducer of IgE synthesis (Fig. 1 B and Life, P. F., unpublished data). Taking these two wild-type clones as representative examples, there was clearly no major difference in IL-4 secretion between these and the HIGM1 helper clones.

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Cell Surface Expression of Membrane TNF-α and IgE Blocking Assays Using Anti-TNF-α

Having confirmed the absence of functional CD40L in the IgE helper HIGM1 T cell clones, we then investigated whether...
the clones express membrane-bound TNF-α, a molecule that has recently been implicated in Ig isotype switching (19, 20). HIGM1 T cell clones and a wild-type clone (OZZ.3.4) were stimulated using irradiated, autologous EB-LCL and PHA and stained daily for TNF-α expression as described in Materials and Methods. FACS® analyses are shown in Fig. 5. For each clone there was a marked induction of TNF-α expression at day 2 which remained until day 8–9 of culture, depending on the clone studied. Neither unstimulated T cells nor irradiated EB-LCL stained positive for TNF-α. The induction of TNF-α expression in these stimulated HIGM1 clones was confirmed by Northern blot analysis (data not shown). To investigate if membrane TNF-α was responsible for the observed HIGM1-induced IgE synthesis, PHA pre-stimulated HIGM1 and wild-type T cell clones were cocultured with tonsillar B cells and IL-4 in the presence or absence of a neutralizing, polyclonal antibody to TNF-α. Data are shown in Table 3. Although anti-TNF-α caused some reduction of IgE in the HIGM1 clone MAB.3.6, this was not dose related and was not seen in the other HIGM1 clone (MAB PHA 1.14) or wild-type clone.

Discussion

At least two cell-derived signals have been shown to be necessary for the induction of Ig isotype switching in B cells. The first signal is given by either of the soluble lymphokines IL-4 (1, 3) or IL-13 (6, for a review see reference 21) which induce germline e transcript expression, but alone is insufficient to trigger secretion of IgE. A second signal is provided by a physical interaction between B cells and activated T cells (2, 4, 5), basophils, and mast cells (22), and it has been shown in both human (23, 24) and murine (7) systems that the CD40/CD40L pairing is able to mediate IgE synthesis in HIGM1, which is characterized by greatly decreased levels of IgG, IgA, and IgE (for a review see reference 10). Here we have been able to show that T cell clones and lines isolated from a HIGM1 patient with very low, yet detectable serum IgE can in fact induce IgE synthesis despite expression of a truncated CD40L.

T cell clones were made from PBMC using PHA and IL-4. The presence of IL-4 in a T cell blast population before cloning has been shown to increase the frequency of IL-4-secreting T cell clones (Th2) (25, and Life, P. L., unpublished observations) and in the current study, four of the six clones tested were of the Th2 type.

4 clones out of 12 tested were able to induce IgE synthesis by highly purified tonsillar B cells (>99% pure), but only in the presence of exogenous IL-4. There did not seem to be a relationship between the ability of a clone to induce IgE and its ability to increase IgG synthesis.

It is important that neither IgE synthesis nor B cell proliferation was induced in IL-4-containing cultures where preactivated T cells were separated from B cells using a semi-permeable membrane. This demonstrated the contact dependency of the T cell-derived signals and excluded the possibility that soluble factors were responsible for the observed IgE induction.

It is interesting to note that three of the IgE helper clones were CD8+ two of which were characteristic of the Th2 subtype. Generally, CD8+ cells have been associated with the suppression of IgE and production of Th1-type lymphokines (26, and for a review see reference 27). However, activated CD8+ cells can produce high levels of IL-4 (28–30) and CD8+ cells have been shown to differentiate into a CD4−CD8− phenotype that not only produces high levels of Th2 lymphokines but also drives IgE synthesis (31). However, the CD8+ clones described in the present study did not lose CD8 expression. Moreover, CD8+ clones from allergic donors can trigger IgE synthesis (32) and express CD40L (8). In the present study however, CD40L was presumed, and later proven, to be absent.
Figure 5. Expression of membrane TNF-α on HIGM1 T cell clones. IgE helper HIGM1 T cell clones (MAB PHA .3.4, .3.6, and 1.14) and a wild-type clone (OZZ .3.4) were stimulated for 48 h using autologous, irradiated EB-LCL and PHA (1 μg/ml) and analyzed for TNF-α expression using a polyclonal rabbit anti-TNF-α antibody and goat anti-rabbit IgG(ab')2-FITC as a second step. As a negative control, cells were stained using nonspecific rabbit IgG. Also shown are irradiated MAB and OZZ EB-LCL stained at the same time as the clones. FACS histograms represent data from 5,000 cellular events.

Dog allergen-specific T cell lines isolated from the same patient were also shown to induce IgE synthesis. The two lines studied in detail (MAB DOG 5.5 and 5.2) were predominantly CD4+, had undetectable surface CD40L, and secreted IL-4 and IL-4/IFN-γ, respectively. These findings confirm and extend those using PHA-stimulated clones and suggest possible physiological relevance.

HIGM1 clones were prestimulated for 6 d using a suboptimal dose of PHA in the presence of irradiated, allogeneic PBMC before use in IgE assays. This raised the concern as to whether activated T cell membranes from PBMC might be responsible for the observed IgE synthesis. We addressed this point by demonstrating that HIGM1 clones could still trigger IgE synthesis if prestimulated using autologous EB-LCL, negative for CD40L. Although the autologous EB-LCL were positive for CD23 (data not shown), known to positively regulate, though not induce, IgE production through CD21 ligation (33, 34), irradiated EB-LCL alone plus IL-4 were, as expected, reproducibly unable to induce IgE synthesis in tonsillar B cells. Therefore, the co-signal for IgE synthesis must have been supplied by the HIGM1 clones and lines themselves. HIGM1 T cell clones also induced IgE synthesis in high density tonsillar B cells isolated using a Percoll gradient. These cells are considered resting (35) which argues strongly against IgE production from IgE precommitted or activated cells.

IgE synthesis is preceded by transcription of the germline e RNA, and a relationship has been proposed between this expression and subsequent switching to Ce (36, 37). We were able to show that HIGM1 T cell clones and IgE helper T cell clones that express wild-type CD40L did not differ overall in their ability to upregulated IL-4-induced germline e RNA

Table 3. **Anti-TNF-α Antibody Does Not Abrogate HIGM1 T Cell Help for IgE Synthesis**

| Clone | OZZ .3.4 | MAB PHA .3.6 | MAB PHA 1.14 |
|-------|----------|--------------|--------------|
|       | IgE ng/ml| Assay 1      | Assay 2      |
| B alone | <0.2     | <0.2         | <0.2         | <0.2         |
| B + anti-CD40 | 110 ± 6  | 68 ± 10      | 295 ± 36     | 42 ± 30      |
| T + B | 64 ± 14  | 18.5 ± 5     | 11 ± 4       | 18 ± 4       |
| T + B + anti-TNF-α | 60 ± 20 | 10 ± 3       | 15 ± 6       | 15 ± 4       |

*1 μg/ml*  | 93 ± 18  | 8 ± 2        | 10 ± 2       | 14 ± 4       |

T and B cells were cocultured for 14 d in the presence of 200 U/ml rIL-4 as described in the legend to Fig. 1. Rabbit anti-TNF-α (dialyzed overnight against PBS at 4°C before use) was present throughout the assay and used at the concentrations indicated. Clone OZZ .3.4 is from a normal donor, clones MAB PHA .3.6 and MAB 1.14 are from the HIGM1 patient. As a positive control in each assay, anti-CD40 mab was used at 1 μg/ml. Negative control wells contained B cells and IL-4. Cell supernatants were tested for IgE by specific ELISA.
expression. Generally, though, the levels of IgE obtained in HIGM1 clone-driven cultures were less than those seen using wild-type clones or anti-CD40 mAb.

Taken together, these findings suggested that a molecule(s) other than CD40L on the HIGM1 clones can upregulate IL-4-induced germline ε mRNA expression and synthesis of mature IgE.

Three approaches were taken to ensure that the observed IgE helper activity could not be attributed to contaminating wild-type CD40L from irradiated allogeneic PBMC used during routine cell expansion. First, CD40L was undetectable on IgE helper HIGM1 clones, stimulated under conditions optimal for CD40L surface expression. Furthermore, more stringent analysis of an IgE helper HIGM1 clone (MAB PHA.3.6) by RNase protection assay and molecular cloning and sequencing confirmed the presence of the mutated form of CD40L.

If CD40L is nonfunctional in the HIGM1 clones, then what is the nature of the T cell surface molecule(s) able to trigger IgE synthesis? Factors other than CD40L such as EBV and hydrocortisone are able to induce Ig isotype switching in the presence of IL-4 (38, 39). Also, we (Fig. 2) and others (40, and Henchoz, S., unpublished observations) have shown that anti-CD40 mAbs synergize with T cell clones to increase Ig switching, suggesting that CD40L is not the only T cell structure able to drive this response. It has been shown recently that membrane TNF-α, expressed on activated CD4 + T cells, can provide a costimulatory signal for IL-4–induced Ig production (19) even when CD40L is undetectable (20). In this study we showed that membrane TNF-α could be induced on IgE helper HIGM1 and wild-type T cell clones. However, we were unable to abrogate IgE synthesis using up to 10 μg/ml of neutralizing anti-TNF-α polyclonal antibody, present throughout the assay. In support of these negative findings, we also observed that COS cells, transiently transfected with full-length TNF-α using the pCDLSRα296 vector and expressing surface TNF-α, induced tonsillar B cells to proliferate, but not to secrete IgE in the presence of IL-4 (Gauchat, J.-F., unpublished data).

Additional support for a secondary mechanism of Ig isotype switching is emerging from many sources. Very recently, Wen et al. (41) demonstrated high titers of T cell–dependent Ig isotypes, including IgE, in mice congenitally deficient in α/β T cells. IgE help was provided by IL-4–secreting γ/δ + T cells which did not express CD40L. Also, anergized human T cell clones specific for Der p1 allergen no longer induced IgE synthesis in the presence of IL-4 despite expression of CD40L (42). Indirect support for a secondary mechanism of Ig isotype switching comes from the unexpected finding that an anti-CD40L mAb did not alter the concentration of total IgG in mice immunized with collagen (43). An interesting observation from our study was the tendency for IgE helper HIGM1 T cell clones to form dense aggregates with B cells in IgE-producing cultures. Contact between T and B cells is obligate for IgE induction (2) and the above finding suggests that IgE-helper HIGM1 clones may express an important surface structure(s) that mediates and/or induces B cell contact. Certainly, the induction of IgE using HIGM1 T cell clones was a contact-mediated event as separation of T and B cells by a semi-permeable membrane abrogated synthesis.

We have shown that T cell clones, isolated from HIGM1 PBMC can both induce IgE synthesis and secrete high levels of IL-4 in vitro, yet IgG, IgE, and IgA levels are barely detectable in vivo. This discrepancy suggests that the absence of CD40L has wider implications for aberrant immune function in vivo in addition to an inability to synthesize switched Ig isotypes. Indeed, CD40L is required for germinal center formation in humans (10) and mice (44) and rescues B cells from apoptosis (45). Also, CD40 provides a strong coactivating stimulus to CD4 + T cells, resulting in generation of CTL and selective expansion of CD4 + cells (46). Reciprocally, activated T cells induce expression of B7/BB1 on B cells through a CD40-dependent signal (47). The overall T cell repertoire in HIGM1 may also be abnormal as stimulation of CD40, which is constitutively expressed on human thymic epithelial cells, induces lymphokine secretion (48) which may in turn affect thymic function. Last, a direct effect of CD40L that could contribute to immunodeficiency may be an impaired bactericidal function of monocytes, since it has recently been shown that ligation of CD40 on monocytes with CD40L potentiates both cytokine production and tumoricidal activity (49). Taken together, the above findings show that T cell clones and lines, isolated from a HIGM1 patient are able to induce IgE synthesis in vitro despite the absence of full-length, functional CD40L, and suggest the existence of other T cell surface structures capable of inducing IgE switching.
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