Brief Definitive Report

CD8+ T Lymphocytes Provide Helper Activity for IgE Synthesis in Human Immunodeficiency Virus–infected Patients with Hyper-IgE

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Summary

Increased levels of serum IgE and eosinophilia have been described in human immunodeficiency virus (HIV) infection, almost exclusively in patients with CD4+ cell count <200 cells/μl. IgE production is regulated by CD4+ T helper type 2 (Th-2) lymphocytes, producing interleukin 4 (IL-4) and expressing a ligand for the B cell–specific CD40 molecule (CD40 ligand [L]). A shift to a Th-2-like pattern of cytokine secretion has been postulated to be associated with progression toward acquired immunodeficiency syndrome (AIDS). We studied three AIDS patients with very high levels of IgE and almost complete depletion of CD4+ lymphocytes, suggesting that IgE synthesis could not be driven by CD4+ cells. IgE in vitro synthesis by cells from such patients was, however, inhibited by anti-IL-4. We show that both CD8+ T cell lines and the majority of CD8+ T cell clones derived from these patients produce IL-4, IL-5, and IL-6 in half of the cases together with interferon γ (IFN-γ). 44% of CD8+ T cell clones expressed a CD40L, and the supernatants of the clones were capable of inducing IgE synthesis by normal B cells costimulated with anti-CD40. CD8+ T cells in these patients therefore functionally mimic Th-2 type cells and may account for hyper-IgE and eosinophilia in the absence of CD4+ cells. The presence of such CD8+ cells may also provide a source of IL-4 directing the development of predominant Th-2 responses in HIV infection.

Increased levels of serum IgE and eosinophilia have been described in human immunodeficiency virus (HIV-1) infection, almost exclusively in patients with CD4+ cell count below 200 cells/μl (1, 2). Serum IgE levels were raised two- to fivefold above normal values, without accompanying symptoms of atopic diseases, which frequently develop in HIV+ subjects (3). IgE production is regulated by CD4+ T helper (Th) lymphocytes expressing a ligand for the CD40 molecule (4, 5), whose binding induces B cell responsiveness to IL-4 and IL-10. These cytokines, produced by Th-2 cells, are able to promote B cell switch and Ig production (6). CD4+ Th-2 cells are also the main source of other cytokines, such as IL-5, which drives the development and activation of eosinophils, together with GM-CSF (7). A shift from a Th-1-like to a Th-2-like pattern of cytokine secretion has been postulated to be associated with progression towards AIDS (8), with increased production of IL-4, compared with IL-2, in the in vitro response to recall antigens, and raised IL-10 production (9).

Several studies have recently reported IL-4 production by non-CD4+ cell types (10), including CD8+ cells (11, 12). IL-4 produced by non-CD4+ cells is unable to induce IgE responses in IL-4 knockout mice reconstituted with CD4− cells from spleen of congenic type mice (13), although these cells may support already established IgE responses. The effect of depletion of CD4+ cells on IgE production has not been investigated so far. In a selected group of AIDS patients with very high levels of IgE (20- to >100-fold above normal values) we found an almost complete depletion of CD4+ lymphocytes, suggesting that these cells may not be the source of IL-4 driving IgE synthesis. These patients also presented severe atopic symptoms and hypereosinophilia (14), and thus represented a possible model to study the contribution of CD4− cells to Th-2 type of responses. The production of

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/01/0423/06 $2.00
Volume 181 January 1995 423–428

both IL-4 and IL-5 by $CD8^+$ T cells from these subjects, as well as the ability to express a CD40 ligand (CD40L), and to stimulate IgE synthesis by normal B lymphocytes, indicate that these cells can functionally mimic $CD4^+$ Th-2 cells and account for the clinical features presented by AIDS patients with hyper-IgE.

Materials and Methods

Patients. We studied three male patients (age 32–59 yr) affected by AIDS, with very high levels of IgE (serum levels were 12,000, 2,600, and 3,400 kU/L, respectively), eosinophilia (900 ± 150 cell/mm^3), chronic diffuse pruritic dermatitis, repeated Staphylococcal abscesses, and Candida infections. These patients had $CD4^+$ cell counts of 0.4, 5, and 0.3% of peripheral blood lymphocytes (PBL), respectively, corresponding to absolute numbers of 1,38, and 4 $CD4^+$ cells/μl. Parasites were absent, both in skin and stools, and no other known cause of eosinophilia was found.

Flow Cytometric Analysis. For double fluorescence analysis cells were stained with the following mAbs, PE- or FITC-conjugated: anti-CD3 (Leu 4), anti-CD4 (Leu 3a), anti-CD8 (Leu 2a, all from Becton Dickinson Immunocytometry Systems, Mountain View, CA). A chimeric molecule generated by fusing the extracellular domain of CD40 to the heavy chain of IgG1 (a kind gift of I. Stamenkovic, Harvard University, Cambridge, MA), was used for the determination of CD40L expression on the surface of T cells, and revealed by indirect immunofluorescence with a FITC-conjugated $F(ab)_2$ goat antiserum to human IgG (Serotec, Oxford, UK). Normal polyclonal human IgG were used as control. Flow cytometric analysis was performed on a Cytoron (Ortho Diagnostic Systems, Raritan, NJ) after electronic gating on lymphocytes.

Mononuclear Cell Cultures. Lymphoprep (Nycomed, Oslo, Norway)-separated PBMC were resuspended in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO BRL), 2 mM L-glutamine (GIBCO BRL) and 10% FCS (Hyclone, Logan, UT) (complete medium) at 10^6 cells/ml. Duplicate cultures of 1 ml were set up for 10 d, with or without the following: cycloheximide (100 μg/ml; Sigma Chemical Co., St. Louis, MO), and goat neutralizing anti-IL-4 (R&D Systems, Inc., Minneapolis, MN), or normal goat serum as control, at different concentrations (1–100 μg/ml). Supernatants were then collected and stored in aliquots at −80°C for up 3 wk until analysis for IgE determinations.

The induction of IgE synthesis by normal B cells from three healthy controls was tested by adding anti-CD40 (mAb 89, 0.5 μg/ml, a kind gift of Dr. J. Banchereau, Schering-Plough, Dardilly, France) with or without IL-4 100 U/ml, or 1:10 dilution of supernatants from six $CD8^+$ T cell clones from patient 1. B lymphocytes were obtained from PBMC by immunomagnetic selection with anti-CD3–coated beads (Dynal, Oslo, Norway). 2×10^5 negatively selected cells were cultured for 7 d and supernatants collected for IgE determination.

Immunomagnetic depletion of $CD8^+$ PBMC was performed by using anti-CD8–coated immunobeads (Dynal). After depletion, $CD8^+$ cells were always <1%. CD8-depleted PBMC were cultured for 7 d at 0.5×10^6 cells/ml and supernatants were tested for IgE synthesis as described.

Generation of T Cell Lines and Clones. Patients' PBMC were stimulated with PHA-P (1 μg/ml; Wellcome, Beckenham, UK) in 24-well flat-bottomed plates (Falcon, Oxnard, CA). rIL-2 (20 U/ml; Boehringer Mannheim, Mannheim, Germany) was then added, and after 6 d, viable T blasts resuspended in complete medium (supplemented with 2-MER 50 μM; Sigma Chemical Co.) were seeded at 0.6 cell/well in U-bottomed 96-wells plates (Falcon). 2×10^5 irradiated (6,000 rad from Cs source) allogeneic mononuclear cells were used as feeders. After 2 wk, growing wells were identified using an inverted microscope, and expanded by weekly splitting and addition of rIL-2 supplemented complete medium. T cell lines were also cultured in bulk with rIL-2 addition.

Determination of IgE in Supernatants. IgE in culture supernatants were measured by a modified paper radioimmunosorbent assay, as described previously (15). Briefly, paper disks coated with anti-IgE (Pharmacia LKB, Uppsala, Sweden) were incubated with 200 μl of the supernatants overnight, then washed and reincubated with 1:4 dilution of ^{125}I-labeled anti-IgE (200 μl; Pharmacia LKB) for 18 h. After four washes, bound radioactivity was read against dilutions of the IgE standards, down to 100 pg/ml of IgE.

In all subsequent calculations, the amount of IgE measured in cultures supplemented with the protein synthesis inhibitor cycloheximide were subtracted from the results of both spontaneous or stimulated samples, to obtain the value of IgE synthesized, disregarding the amount of cell-bound IgE preformed and released in culture.

Cytokine Detection. Supernatants of T cell lines were collected 48 h after restimulation with anti-CD3 (14 ng/ml) or at the same time after addition of 20 U/ml rIL-2, whereas supernatants of T cell clones were studied 24 h after addition of rIL-2 or rIL-2 plus anti-CD3. Cytokine production was determined in supernatants from T cell lines and clones using the following ELISA kits: Hu-IFN-γ ELISA (Hbt, Amsterdam, The Netherlands), IL-4, IL-5, IL-6 ELISA (Quantikine™, R&D Systems, Inc.), IL-10 ELISA (Bender MedSystems, Vienna, Austria). All samples were tested in duplicate and thawed only once. Measurements were performed according to the manufacturers' instructions, and results interpolated from the standard reference curve provided with each kit.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis. Total cellular RNA was extracted as described by Chomczynski and Sacchi (16). RT-PCR was performed as previously described (17). Briefly, cDNA was derived from total RNA (1.5 μg/30 μl reaction), incubated for 10 min at 65°C with 0.1 μg/ml oligo-dT (12-18 mer; Pharmacia LKB). After cooling on ice, 20 U/ml of Moloney murine leukemia virus (M-MLV) RT, 20 mM dithiothreitol (DTT), and 5× M-MLV RT buffer were added, according to manufacturer's instructions (GIBCO BRL). The cDNAs thus obtained were tested for the presence of specific gene sequences in PCR reactions (GeneAmp kit; Perkin-Elmer Cetus, Norwalk, CT) performed in 20 μl volumes, by using defined primers' pairs. 2 μl of the relevant cDNA was amplified in the presence of 0.05 μM final concentration of 5' and 3' primers, 2 mM deoxi-nucleotides and 0.5 U of ampliTaq polymerase and GenAmp 10× PCR buffer. The PCR was performed in a Perkin Elmer thermal cycler for 25 cycles: 40-s denaturation at 94°C, 40-s annealing at 62°C, and 1-min extension at 72°C. The reaction product was visualized by electrophoresis, on 1% agarose gel, using 10 μl of the reaction mixture and 2 μl of loading buffer. 0.5 μg of HaeIII-digested ΦX174 DNA (GIBCO BRL) were run in parallel as molecular weight markers, providing bands at 1,353, 1,078, 872, 603, 310, 281, and 234 bp. Cytokine specific primer pairs were synthesized on synthesizer (Applied Biosystems Inc., Foster City, CA) according to published sequences (17, 18). The primer sequences were RNA specific.

Results and Discussion

Phenotypes of Cells Ex Vivo and in IL-2–dependent T Cell Clones. The dramatic decrease of $CD4^+$ cells in vivo was

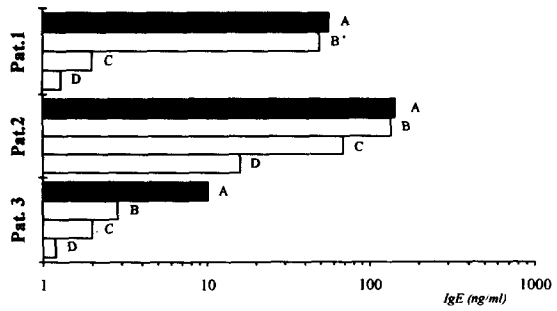

Figure 1. Spontaneous in vitro synthesis of IgE by PBMC from patients with AIDS and hyper-IgE (Pat. 1–3, *A, solid bars*). 10^6 PBMC were cultured for 10 d and IgE content in the supernatants measured by paper radioimmunoassay. The effect of addition of neutralizing goat anti-human IL-4 antibody (1–100 μg/ml, *B–D*) at the beginning of cultures is shown. No inhibition was observed with control unimmunized goat serum. IgE detected in cycloheximide (100 μg/ml)-treated cultures have been subtracted, to obtain net de novo synthesis.

accompanied by a relative increase of $CD8^+$ T lymphocytes (58 ± 14%), whose absolute count was only slightly raised, due to the low number of total $CD3^+$ T lymphocytes (average mean, 569 cells/μl). T cell lines were grown from PBMC of the patients, and maintained in rIL-2, after initial stimulation with PHA. Cloning of cells from two lines (patients 1 and 2) was then performed, and growing T cell clones were analyzed 2 mo after seeding. All three cell lines, 24/24 clones from patient 1 and 26/28 from patient 2 were $CD3^+CD8^+CD4^-$, with >99% of the cells expressing this phenotype. The remaining two clones were respectively $CD3^+CD4^+CD8^-$ and $CD3^+CD4^+CD8^+$. The almost complete depletion of circulating $CD4^+$ cells was then reproduced also at clonal level in these patients.

Spontaneous In Vitro Synthesis of IgE by PBMC of AIDS Patients. PBMC from the three patients were cultured for spontaneous IgE production for 10 d, and in all instances, net IgE synthesis in supernatants was present at levels ranging from 10.3 to 144.6 ng/ml (Fig. 1, *solid bars*). No spontaneous net IgE production was detected in cultures from HIV noninfected nonatopic subjects (data not shown). The levels of IgE found in cultures from HIV^+ cases with hyper-IgE were similar to those obtained in cultures of PBMC from patients affected by the primary hyper-IgE syndrome (15), a primary immunodeficiency disease in which clinical features are closely related to those observed in our HIV^+ cases (19), and where an imbalance between IL-4 and IFN-γ production has been reported (15, 19, 20). These two cytokines are known to exert opposing effects on IgE (21), and their balance ought to be critical in hyper-IgE states.

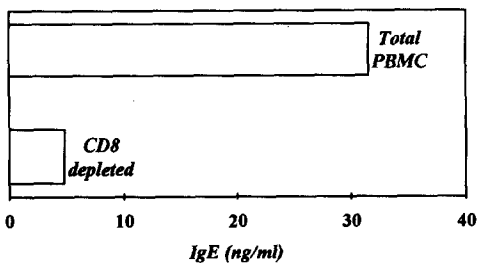

Figure 2. IgE synthesis by CD8-depleted PBMC from a patient with HIV^+ hyper-IgE syndrome compared with unseparated cells. A representative experiment of three is shown.

We therefore asked whether IL-4 was playing a role in the in vitro spontaneous synthesis of IgE occurring in patients severely depleted of $CD4^+$ cells, the principal producers of IL-4 in immune responses. Addition of increasing amounts of neutralizing antibody to IL-4 inhibited IgE production in vitro in a dose-dependent fashion in the range 1–100 μg/ml (Fig. 1, *empty bars*), indicating that presence of IL-4 was essential for de novo synthesis of IgE in vitro. A control nonimmunized goat serum had no effect upon levels of IgE detected in the culture supernatants (data not shown).

$CD4^+$ cells were severely reduced in these patients, and $CD8^+$ cells accounted for 93–99% of all T lymphocytes. We observed that depletion of these $CD8^+$ cells led to a 6–10-fold reduction of IgE synthesis (Fig. 2). We therefore postulated that these cells might be an alternative source of IL-4 in our cases, and might be actively promoting IgE production through IL-4.

Cytokine Production by PBMC, T Cell Lines, and Clones. Supernatants from PHA-stimulated PBMC of the three patients were analyzed by ELISA, and IL-4 was detected at different concentrations (40–1,600 pg/ml). $CD8^+$ T cell lines also produced IL-4 (150–490 pg/ml) upon stimulation by monoclonal anti-CD3 antibody. mRNAs for IL-4, IL-5, and GM-CSF were detected by RT-PCR, as shown in Fig. 3 for the $CD8^+CD4^-$ T cell line from patient 2. These mRNAs were present both in $CD8^+$ T cells cultured in rIL-2, and after restimulation with anti-CD3; in the latter condition the amount detected seemed to increase. The presence of p55 IL-2 receptor chain RNA showed that the cells were activated in both conditions. IL-5 and GM-CSF are known to promote eosinophil differentiation, growth and activation (7, 22). Thus their production by $CD8^+$ T cells may be related to eosinophilia in these patients. No mRNA for IL-2 and IFN-γ was detected in this cell line, despite maximal stimulation. This cytokine pattern is usually detectable in Th-2 type cells, but it has been reported under certain conditions also in $CD8^+$ lymphocytes (11, 12, 23, 24). Further analysis was made on supernatants of $CD8^+$ T cell clones by ELISA for different cytokines. Data reported in Table 1 demonstrate that a complex mix of cytokine production could be detected in the $CD8^+$ clones from both patient 1 and 2. 67% of the $CD8^+$ clones produced measurable amounts of IL-4, 52% IL-10, and 72% also IFN-γ. IL-5 and IL-6 were consistently detected in all supernatants tested, ranging respectively from 50–>2,500 pg/ml and from 18–>3,000 pg/ml. Not included in the table, the supernatant of the only $CD4^+/CD8^+$ clone from patient 2 contained IFN-γ only, whereas the $CD4^+$ clone from the same patient was a typical Th-2 cell, producing IL-4, IL-5, and IL-10 but no IFN-γ. In 42% of the $CD8^+$ clones we observed both IL-4 and IFN-γ production. This is consistent with previous reports showing that a fraction of alloreactive murine (23) and human

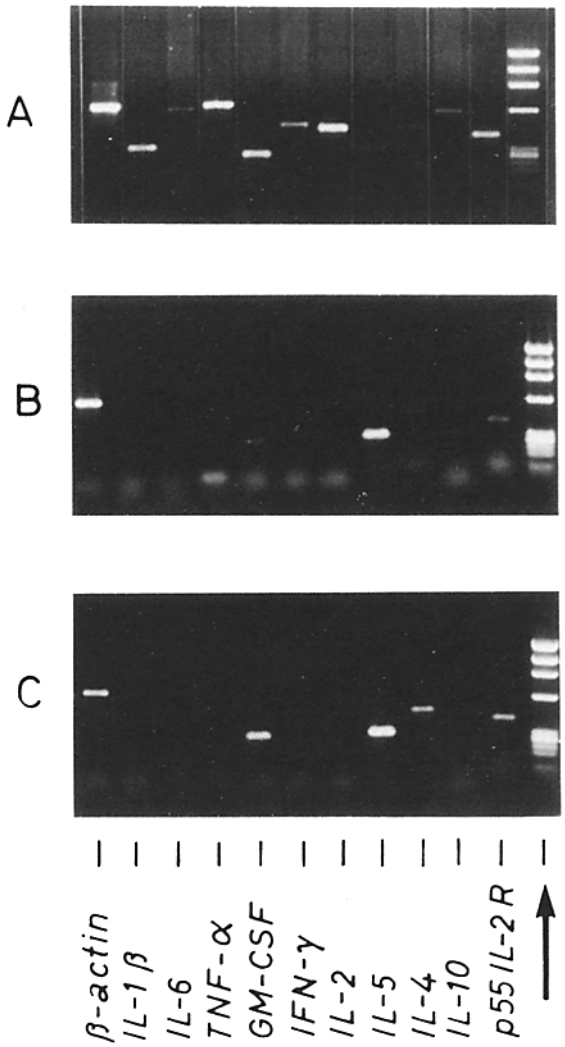

Figure 3. Detection of mRNA for cytokines by RT-PCR in PBMC of a normal control (*A*) and a CD8+ T cell line from patient 2 (*B–C*). (*A*) Normal PBMC tested 48 h after stimulation with anti-CD3 (14 ng/ml) and rIL-2. (*B*) T cell line 48 h after readdition of rIL-2 (20 U/ml). (*C*) T cell line 48 h after restimulation with anti-CD3 and rIL-2. 2 μl of cDNA were subjected to 25 cycles of amplification by using defined primers' pairs synthesized according to published sequences (17, 18). The product of PCR was visualized by electrophoresis on 1% agarose gel. Molecular weight markers are indicated by the *arrow*, providing bands at 1,353, 1,078, 872, 603, 310, 281, and 234 bp.

(24, 25) CD8+ cell clones have the capacity to secrete both cytokines, despite the majority of CD8+ clones produce IL-2 and IFN-γ but no IL-4. Thus, the ability of both T cell lines and about 25% of CD8+ T cell clones to secrete exclusively Th-2 type cytokines after stimulation may be regarded as an abnormal expansion of a CD8+ subset with helper activity. Previous in vivo priming by IL-4 producing Th cells, as demonstrated by in vitro priming of murine CD8+ cells (26) might account for the appearance of this type of cell. In HIV infection a shift to preferential production of IL-4 has been in fact observed (8).

CD40L Expression by Stimulated CD8+ Cells. The possibility of a direct induction of IgE synthesis by CD8+ T cell clones was further suggested by the expression of a CD40L on the surface of the cells. This was tested using a fusion product of CD40 with human IgG1 heavy chain, and detected by cytofluorimetry. 22 out of 50 CD8+ T cell clones examined after 72 h of culture with rIL-2 expressed a CD40L (Fig. 4, illustrating two representative positive clones from each patient). Only a small minority of CD8+ T lymphocytes have been reported to express a CD40L after activation (27), whereas nearly half of the CD8+ clones derived from HIV+ patients with hyper-IgE had a stable expression of this molecule. In a patient with primary hyper-IgE syndrome, only CD4+, but no CD8+, T cell clones expressed the CD40L (data not reported). However, preliminary data from AIDS patients without features of hyper-IgE show that CD40L is expressed by CD8+ clones under the same ex-

Table 1. *Cytokine Production by CD8+ T Cell Clones*

IL-4	IFN-γ	No.	Percent	Percent IL-10+
+	+	17	42.5	76.4
+	−	10	25	60
−	+	12	30	16.6
−	−	1	2.5	0

Supernatants of 40 CD8+ T cell clones from patients 1 and 2 were analyzed by ELISA for cytokine content. IL-5 and IL-6 were present in all, with levels ranging from 50 to >2,500 pg/ml, and 18 to >3,000 pg/ml, respectively. Number and percentage of IL-4 and/or IFN-γ producer clones are given. In the last column, the percentage of clones in each row coproducing also IL-10 is indicated.

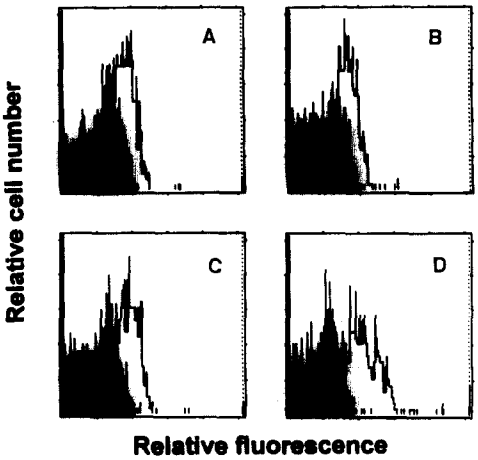

Figure 4. Expression of CD40L on the surface of CD8+ T cell clones from AIDS patients with hyper-IgE. Cells were stained after 72 h of culture in medium with rIL-2 (20 U/ml), with a chimeric CD40-human IgG1 molecule (*clear histogram*) or with polyclonal human IgG (control, *in black*), followed by FITC-F(ab)$_2$ goat anti-human IgG. Mean fluorescence intensity channel was over twice control values on a linear scale in the positive clones. Four representative CD8+ T cell clones expressing a CD40L are shown: nos. 1 (*a*) and 10 (*b*) from patient 1, and nos. 19 (*c*) and 33 (*d*) from patient 2.

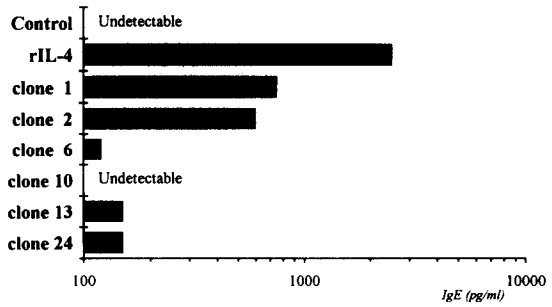

Figure 5. In vitro synthesis of IgE by normal B cells induced by anti-CD40 and rIL-4 or $CD8^+$ T cell clones supernatants. B cells were negatively selected from normal PBMC by immunomagnetic depletion with anti-CD3–coated beads. 2×10^5 B cells were cultured for 7 d in the presence of anti-CD40 (mAb 89, 0.5 μg/ml), and rIL-4 (100 U/ml), or 1:10 dilutions of supernatants of $CD8^+$ T cell clones nos. 1, 2, 6, 10, 13, and 24 from patient 1. IgE levels below 100 pg/ml are indicated as undetectable.

perimental conditions (Paganelli, R., manuscript in preparation).

The presence of $CD8^+$ lymphocytes expressing a CD40L, and able to produce IL-4, IL-5, and GM-CSF suggests that these cells are directly capable of causing the main features of this HIV-associated syndrome, i.e., hyper-IgE production and eosinophilia.

Induction of IgE Synthesis by $CD8^+$ T Cell Clones Supernatants. Since both IL-4 and IFN-γ were produced, alone or in combination, by the majority of T cell clones examined, we tested the effects of the addition of clone supernatants on the in vitro production of IgE by normal B lymphocytes. B cells purified from a normal individual were cultured for 7 d in the presence of anti-CD40, without or with either 100 U/ml rIL-4 (positive control), or 1:10 dilutions of six different $CD8^+$ T cell clones supernatants from patient 1. All six selected clones produced IL-4 (15–60 pg/ml), but five of the six also secreted IFN-γ. In five cases the addition of the supernatants resulted in the stimulation of IgE synthesis above control cultures with anti-CD40 alone (Fig. 5). This shows that $CD8^+$ T cells from HIV^+ patients with hyper-IgE have the characteristics of Th-2 type cells, directly inducing IgE synthesis by normal B cells. These cells may be able to drive hyperproduction of IgE, as well as eosinophilia, in patients with almost complete depletion of $CD4^+$ lymphocytes. It is possible that $CD8^+$ cells endowed with Th-2 type competence arise earlier in the course of HIV disease, and may be the source of IL-4 directing $CD4^+$ cells to shift toward a Th-2 phenotype (28). The expansion of this subset of $CD8^+$ cells may account for the clinical aspects manifested by a small proportion of patients with advanced stage of HIV disease. Alternatively these $CD8^+$ cells may appear as a consequence of the prevailing Th-2 type of response in HIV infection (8), and this would be consistent with the presence of increased IgE levels in the late phase of HIV disease, parallel to the decrease of $CD4^+$ cells (2, 3).

This work was supported by a grant from Istituto Superiore di Sanità (VI° Programma AIDS, 1993, Contract no. (8204-01).

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Received for publication 3 June 1994 and in revised form 15 September 1994.

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