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B cells from a distinct subset of patients with common variable immunodeficiency (CVID) have increased CD95 (Apo-1/fas), diminished CD38 expression, and undergo enhanced apoptosis

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SUMMARY

We investigated the role of apoptosis in the differentiation failure of B cells from a selected subpopulation of patients with CVID delineated by B cell surface marker analysis, *in vitro* IgE response, and molecular markers of B cell V_H gene repertoire. These patients had altered display of B cell surface molecules that play a role in apoptosis. The patients' B cells had a 4·5–250-fold increase in CD95 (Apo-1, fas) expression and increased CD95 display on their T cells. CD38, a molecule important in preventing germinal centre B cell apoptosis, was reduced on the patients' B cells. The expression of this molecule was inducible on the CVID lymphocytes with retinoic acid. Increased spontaneous apoptosis *in vitro* was observed with the patients' B (23%) and T cells (10%) compared with normal cells (13% and 3%, respectively). Stimulation *in vitro* with IL-4 and CD40 rescued the B cells from apoptosis and allowed for their differentiation. However, IL-4 plus αCD40-driven immunoglobulin production was not quantitatively or qualitatively normal. Failure to overcome apoptosis, a normal step in germinal centre B cell development, may be involved in the lack of differentiation seen in this subset of CVID patients.

Keywords common variable immunodeficiency human humoral immunodeficiency antibody deficiency B cell development immunoglobulin production

INTRODUCTION

CVID delineates a disease spectrum which is dominated by hypogammaglobulinaemia. There is a wide range of phenotypes (and probably genotypes) that are encompassed by this rubric. Most patients have panhypogammaglobulinaemia, but IgM may be preserved or even elevated. In addition to a failure in B cell differentiation, an array of T cell abnormalities have been described [1–3]. Many studies in CVID are difficult to interpret since abnormalities may result either from altered immune cell populations or from the abnormal function of cells that are present [4]. For example, there is often a dramatic shift in the patients' T cells to a predominant CD45RO ('memory type') phenotype rather than CD45RA ('naive type') cells, as well as the less common occurrence of CD8 predominance [5–8].

We have previously used cell surface marker analysis, *in vitro* cell response, and molecular analysis of B cell V_H gene repertoire to delineate a specific subset of patients with CVID. The B cells in these patients are characterized by a bright CD20, L-selectin $(CD62L)^{dim}$ phenotype and a loss of circulating V_H3-bearing B cells [9–11]. Additionally, these patients' cells fail to differentiate under a variety of circumstances, including IL-4 plus CD58 stimulation, but are capable of making some IgE in response to IL-4 plus CD40 [12–14]. Although the percentage of B cells may be somewhat low or normal in these subjects, there is generally an absolute loss of circulating B cells due to lymphopenia of both B and T cells.

Since this subset of CVID patients had reduced numbers of B cells which demonstated a phenotypic and molecular immaturity [9–11], we tested whether the cells from these patients had an altered apoptosis profile. We chose to study CD95 (Apo-1/fas), as this member of the tumour necrosis factor family has been shown to play a critical role in apoptosis in a wide variety of cells, and particularly in the peripheral T and B cell deletion. Additionally, we focused on CD38 expresssion as this molecule is normally highly expressed on germinal centre cells, a cell stage that is central to the defect in our CVID patients and CD38 engagement can prevent apoptosis by such cells. We

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found that these patients' fresh cells underwent increased apoptosis *in vitro* and had altered surface phenotype of apoptosis-related molecules. IL-4 plus CD40 MoAb stimulation rescued their B cells from apoptosis and allowed the cells to undergo differentiation. However, the level and nature of this immunoglobulin differentiation response was quantitatively and qualitatively abnormal, indicating that simply inhibiting apoptosis *in vitro* could not fully reverse the defective B cell responses in these patients. These studies provide further data regarding the immunopathogenesis of the failure of B cell development seen in a particular subset of CVID patients and provide direction as to where the underlying defect lies in these patients' B cells.

PATIENTS AND METHODS

Patients and study design

Five women and four men, CIA numbers 109, 125, 128, 144, 153, 155, 161, 175 and 176 with well documented CVID were enrolled in this study. Patients are identified according to their UCLA-CIA identification number as employed in the earlier publications [9,12,14–16]. Six patients, UCLA-CVID numbers 109, 128, 144, 153, 175, and 176 whose mean age was 42 years (range 28–55 years) were the test CVID patients whose B cells demonstrated a $CD20^{bright}$/L-selectin (CD62L) dim phenotype. Three subjects (ages 44, 50, and 62 years), nos 125, 155 and 161 with CVID whose B cells showed CD20/L-selectin display indistinguishable from normals served as disease controls. These patients were specifically chosen because they represent the normal end of the spectrum in regards to CD20 and CD62L display, yet all had been shown to have 'intrinsic' B cell immunoglobulin production defects *in vitro*. All CVID subjects had circulating B cells, as evidenced by the presence of $CD20^+$ cells bearing surface IgM and IgD. The test group of CVID patients all had low to normal circulating percentage of B cells (range 4–18) and lower than normal absolute number of circulating B cells (range 42–136). All three disease control patients manifested a variety of cell phenotype abnormalities, had a normal percentage of B cells, and one (no. 125) had a decreased absolute number of B cells. An increase in CD45RO expression was seen in all patients (both CVID test and disease control) except disease control no. 161. Only one subject (CVID disease control no. 155) had an increased percentage of CD8 cells (CD4/CD8 ratio 0·5). All the patients were receiving intravenous immunoglobulin (200–400 mg/kg per month) and were free of serious infections. Normal 28–55-year-old control subjects were sex- and age-matched (mean age 41 years). Informed consent was obtained from subjects, and the study was approved by the UCLA Institutional Review Board.

Flow cytometric analysis of surface molecules

Lymphocyte populations were assessed by two-colour flow cytometry as described [16]. Conjugated MoAb directed against CD19 (Leu-16; Becton Dickinson) was used as a pan B cell marker in addition to various CD20 antibodies discussed below. Subsets of B cells were identified using the following unlabelled or FITC- or PE-labelled MoAbs: anti-Leu-8 (L-selectin, CD62L), CD20, CD2, CD45RA (Becton Dickinson); CD45RO (UCHL-1 clone from Dako, Carpenteria, CA); CD38 (clone IOB6) and CD58 (clone IOL58) (Amac, Westbrook, ME). CD40 unlabelled and FITC-labelled was the gift of Dr E. A. Clark (University of Washington, Seattle, WA). CD95 was detected by indirect staining with the first antibody being a MoAb DX2 kindly provided by Dr Louis Lanier (DNAX Research Institute, Palo Alto, CA). CD40L staining were performed on purified T cells stimulated with phorbol myristate acetate (PMA; 5 ng/ml) and employing 5C8 MoAb kindly provided by Dr Seth Lederman (Cornell Medical Centre, New York, NY). Staining was performed in the presence of 50% human AB serum to block potential Fc binding. Median fluorescence for various markers was compared by the Mann–Whitney *U*-test for non-parametric measurements.

Analysis of apoptosis by flow cytometry and DNA laddering

For detection of apoptotic, dead and live B and T cells, staining of surface antigens was first performed. Ten microlitres each of CD3 PE and CD20 FITC in 100 μl of PBS containing 0·2% bovine serum albumin (BSA) and 0·1% NaN_3 were added to 5 $\times$ 10^5 cells, incubated for 20 min at 4°C, washed and the cells resuspended in 500 μl PBS plus 0·1% NaN_3. Then 7 amino-actinomycin D (7AAD; 20 μg/ml) was added 15–20 min before analysis on a FACScan flow cytometer equipped with a 15 mW air-cooled 488-nm argon-ion laser. The green fluorescence was collected after a 530/30 band pass nm filter, the orange fluorescence was collected after a 585/42 nm band pass filter. The red fluorescence from 7AAD was filtered through a 650 nm long pass filter. Electronic compensation was used among the fluorescence channels to remove residual spectral overlap. Data acquisition was done using FACScan research software and 10 000 events were collected on each sample. Multiparameter data analysis was performed with LYSIS II software (Becton Dickinson).

Monocytes and debris were initially gated out from the forward scatter *versus* side scatter dot plot display. Apoptotic, live and dead cells were displayed on a forward scatter *versus* fluorescence (channel 3) dot plot after gating on $CD20^+$ B cells or $CD3^+$ T cells. The 7AAD bright smaller cells represent the late apoptotic and dead cells, the 7AAD intermediate smaller cells represent apoptotic cells, and the 7AAD dim larger cells represent the live cells as seen in Fig. 1a. This method has recently been reported in detail elsewhere [17].

To verify that the 7AAD staining pattern of the cells identified distinct populations, cells were sorted on a FACS star plus (Becton Dickinson) on the basis of their 7AAD fluorescence and forward scatter as in Fig. 1a. The sorted cells were then observed by light microscopy and analysed by gel electrophoresis of DNA. Cells in the apoptotic gate exhibited a marked reduction in cellular diameter, condensation of chromatin and nuclear fragmentation, whereas the live cells displayed a normal nucleus and intact cell membrane (Fig. 1b). DNA analysis by gel electrophoresis on sorted cells is shown in Fig. 1c.

Preparation and culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were obtained by density centrifugation and B cells were separated by two cycles of rosetting with AET-treated sheep erythrocytes. Purity of the cells was monitored by flow cytometry.

For analysis of apoptosis, cells were cultured in RPMI 1640 with 10% fetal calf serum (FCS; Irvine Scientific, Santa Ana, CA)

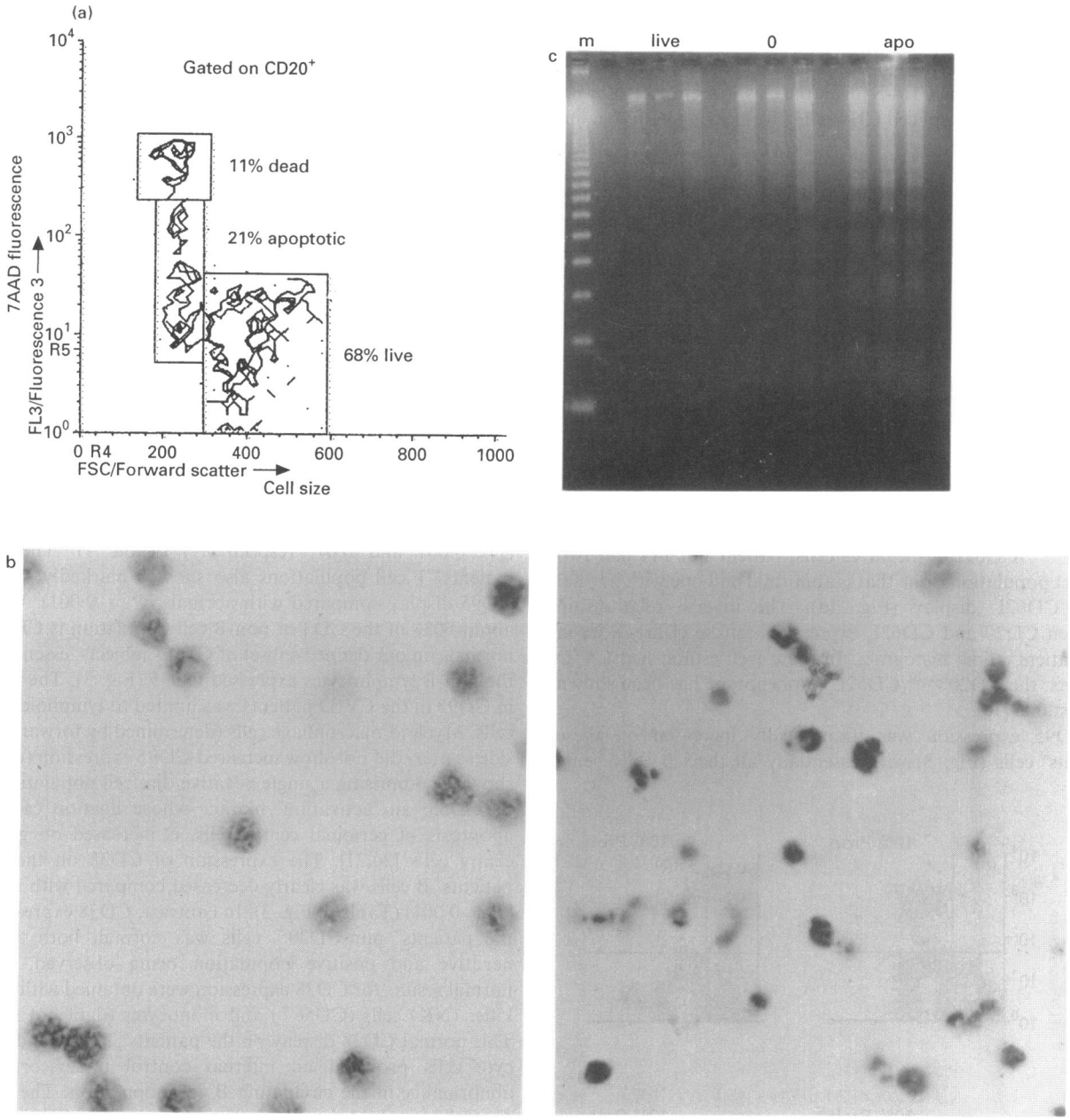

Fig. 1. Flow cytometric analysis of apoptosis. (a) Cells that have been gated for CD20 staining and then analysed for dead, apoptotic, and live cells employing cell size *versus* 7 amino-actinomycin D (7AAD) log fluorescence intensity. (b) Photomicrographs of apoptotic (right) and live cells (left) recovered following sorting based on gates as shown in (a). (c) DNA analysis of sorted live cells (live), unsorted cells (0) and sorted apoptotic cells (apo). The left-hand lane shows size markers (m).

and 1% penicillin/streptomycin and glutamine for 1–3 days. IL-4 (100 U/ml) plus αCD40 (0·1 μg/ml) were added at the start of cultures to assess their ability to inhibit apoptosis.

For immunoglobulin production, cells (1×10^6) were cultured in replicate in 1 ml of RPMI 1640 with 10% FCS and 1% penicillin/streptomycin and glutamine. Cells were stimulated with either media alone, IL-4 (100 U, a gift from Dr Eugene Medlock, Amgen, Thousand Oaks, CA) plus αCD40 (0·1 μg/ml, MoAb G28–5 graciously provided by Dr E. A. Clark), or IL-4, αCD40 plus IL-10 (500 U/ml, a kind gift from Dr Kevin Moore, DNAX). Supernatants were collected on day 12 for IgG, IgM and IgE production as assayed by solid-phase ELISAs as reported [16].

Assessment and quantification of ϵ heavy chain mRNA isoforms

Alternate splicing of the single human functional ϵ heavy chain gene results in multiple different ϵ mRNA isoforms and resulting proteins [18]. We developed modified reverse transcriptase-polymerase chain reaction (RT-PCR) techniques to quantify the amounts of the different ϵ mRNA isoforms produced, as detailed elsewhere [19]. Two different RT-PCR assays were performed The first PCR, termed PCR1′, was used

to amplify the three ϵ mRNA isoforms CH4-M1′-M2, CH4-M2′ and CH4-M2′ and the house keeping gene β-actin. The second PCR, termed PCR2′, was used to amplify two other ϵ mRNA splices; the CH4-S form (the classic secreted isoform), and the alternative spliced CH4′-CH5 form along with β-actin.

Statistical analysis

Statistical analysis were determined using the Mann–Whitney *U*-test for non-parametic values, as data for flow cytometry, apoptosis and ϵ mRNAs have not been shown to follow a normal distribution and the numbers of samples was small as well.

RESULTS

Patients display a 'characteristic' phenotype profile

A defined subset of CVID subjects whose B cells failed to respond normally with differentiation/immunoglobulin production *in vitro* and displayed diminished CD62L and increased CD20 intensity were investigated [9,15,16] (Fig. 2, Table 1). While most of these patients had a single population of $CD20^{bright}/CD62L^{dim}$-negative cells, patient no. 144 had two distinct populations, one that is abnormal and one with normal CD20/CD62L display (Fig. 2d). The inverse relationship between CD20 and CD62L expression can be clearly seen in this patient's flow histogram. In these well established CVID subjects, the $CD20^{bright}/CD62L^{dim}$ phenotype has been shown to be stable [16].

CD95 expression was dramatically increased on these patients' cells (Fig. 3) with essentially all their B cells being

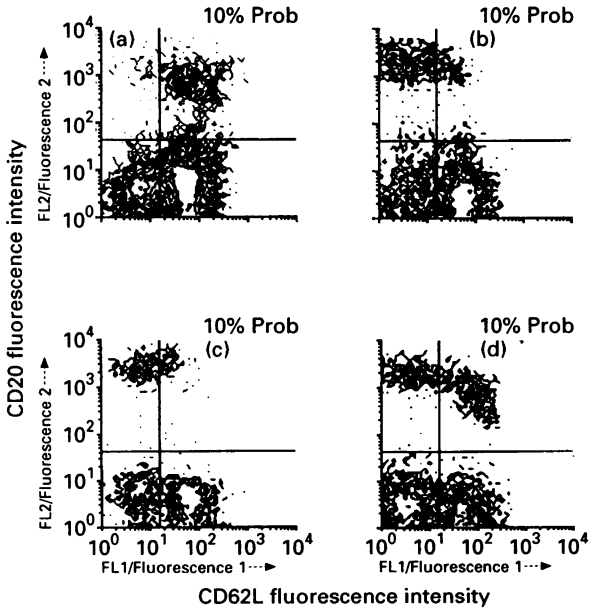

Fig. 2. Increased CD20 and decreased CD62L (L-selectin) display by CVID patients' B cells. Two-colour flow cytometric analysis of CD20/CD62L expression as log fluorescence intensity on patients' and normals' cells. A normal donor is shown in (a). (b,c) From CVID patient nos 109 and 175, respectively. (d) From patient no. 144, showing the presence of two distinct cell $CD20^+$ populations, one normal and one abnormal. A summary of the CD20 and CD62L data is given in Table 1.

Table 1. Summary of selected surface marker display showing differential display on CVID cells†

	CVID subjects ($n = 6$)	Normal ($n = 10$)	Disease controls ($n = 3$)
B cell display of:			
CD20	2370*	1091	1350
L-selectin (CD62L)	25*	73	73
CD95 (Apo-1)	286*	27	37
CD38	9*	28	19**
T cell display of:			
CD95 (Apo-1)	412*	125	268

†Numbers given are median channel fluorescence.

*$P < 0.005$ compared with normal; **$P < 0.05$ compared with normal.

$CD95^+$. The patients' level of B cell CD95 expression was 4·5–250-fold greater than on normals or disease controls ($P < 0.001$ and 0·01, respectively) (Table 1). The CVID patients' T cell populations also showed markedly increased CD95 display compared with normals ($P = 0.001$). Whereas about 50% of the CD3 or non-B cell population is $CD95^+$ in normals, in our defined subset of CVID subjects, essentially all the non-B lymphocytes expressed CD95 (Fig. 3). The increase in CD95 in the CVID patients was limited to lymphoid lineage cells. Myeloid/macrophage cells (determined by forward *versus* side scatter) did not show increased CD95 expression (data not shown), comprising a single negative/dim cell population.

CD38, an 'activation' marker whose ligation can block apoptosis of germinal centre cells, is increased on germinal centre cells [20,21]. The expression of CD38 on the CVID patients' B cells was clearly decreased compared with normals ($P = 0.001$) (Table 1, Fig. 3). In contrast, CD38 expression on the patients' non-$CD20^+$ cells was normal, both a CD38 negative and positive population being observed. Similar normal results for CD38 expression were obtained with natural killer (NK) cells ($CD56^+$) and monocytes (data not shown). This normal CD38 display on the patients' T, NK and monocyte cells provided an internal control underscoring the abnormality in the circulating B cell population. The disease control patients showed an intermediate level of CD38 display that was less than that in normals ($P = 0.02$). CD58 expression on CVID B cells was increased ($P < 0.05$) with a median channel fluorescence of 96 compared with 29 in normals [22]. Several other cell surface molecules potentially related to the failure of CVID patients' B cells to differentiate showed no change from normals: CD40 expression on B cells [23], CD2 expression on T cells, or induced CD40L display on T cells (data not shown).

Induction of CD38 on CVID B cells

Freshly obtained cells from three test CVID subjects were incubated with all trans retinoic acid (RA) (10^{-6} M), and CD38 expression was analysed over the ensuing 3 days. The patients' B cells showed increased CD38 expression with RA. However, both the increase in the median intensity (mean 1·5-fold) and the absolute level of CD38 expression (mean median channel = 14) induced on the CVID $CD19^+$ B cells

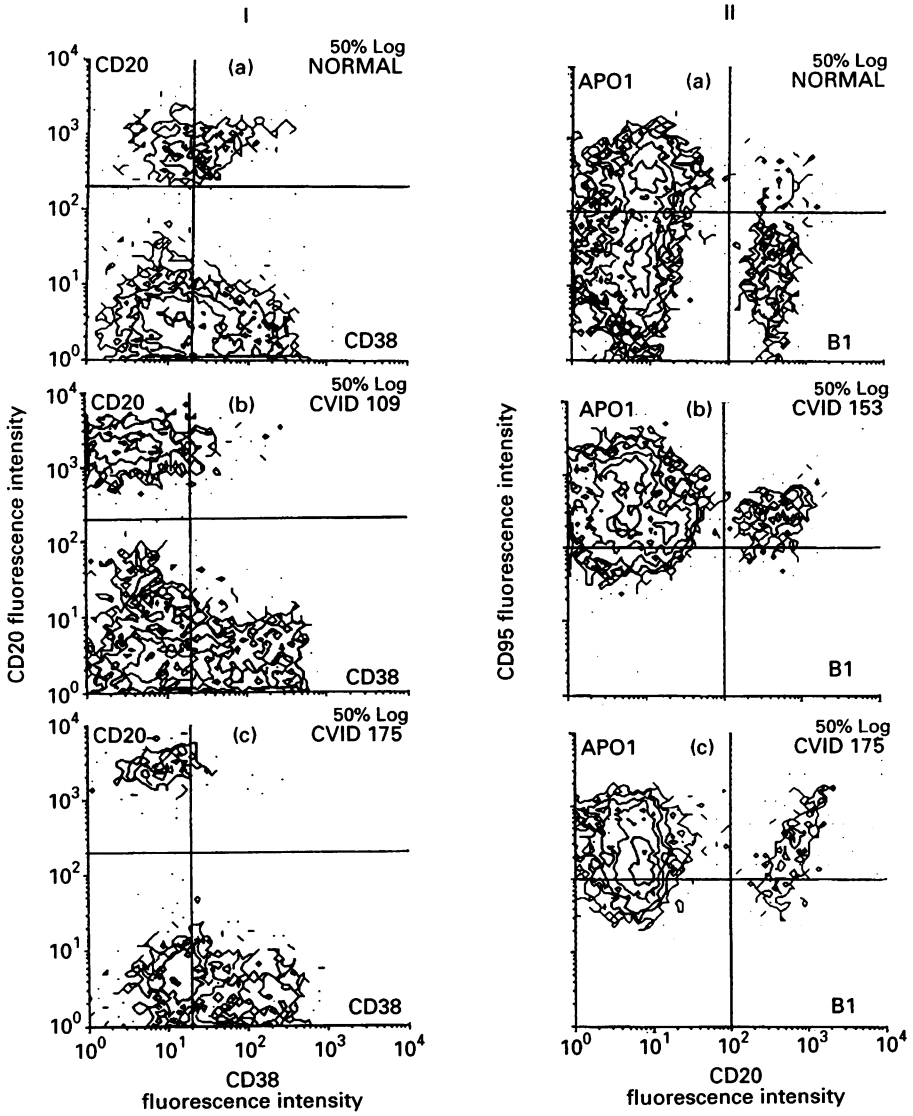

Fig. 3. CVID patients exhibit increased CD95 display on B and T cells (I) and decreased CD38 display on B cells (II). I. CD95 expression on patient and normal $CD20^+$ cells displayed as log fluorescence intensity. A normal donor is shown in (a). (b,c) From CVID patient numbers 153 and 175, respectively. There is increased CD95 expression by both the $CD20^+$ and $CD20^-$ populations. A summary of the CD95 data is given in Table 1. II. CD38 expression on patient and normal $CD20^+$ cells displayed as log of fluorescence intensity. A normal donor is shown in (a). (b,c) From CVID patient numbers 109 and 175, respectively. There is decreased CD38 expression by the $CD20^+$ population. A summary of the CD38 data is given in Table 1.

were less than that seen with normal B cells (mean 2·5-fold increase and mean median channel = 31) (n = 8). Enhancement of CD38 on T cells and monocytes was equivalent in patients and controls (data not shown). Additionally, Epstein–Barr virus (EBV)-transformed B cell lines from these CVID patients expressed CD38, albeit at a lower level than EBV lines from normals (Guo & Saxon, Cell Immunol, in press).

CVID patients' cells undergo increased apoptosis and their B cells are rescued by IL-4 plus CD40

To assess apoptosis in fresh cells, we utilized a flow cytometric method (detailed elsewhere) [17]. We employed unseparated cell populations because of concern that separation procedures might hasten the loss of cells already programmed for apoptosis. The identification of apoptotic cells was validated by sorting the live and apoptotic cell populations and analysis by microscopy and DNA laddering (Fig. 1).

When cultured for three days, $CD20^+$ cells from the CVID patients demonstrated increased apoptosis (Fig. 4, Table 2). The CVID patients' B cells showed a mean of 22·7% apoptotic cells at day 3 compared with 12·8% for normals (P = 0·01) and increased dead cells. Treatment with IL-4 plus αCD40 rescued the CVID patients' B cells, with only 13·2% apoptotic cells then being present (P = 0·025 compared with untreated B cells). The percentage decrease in apoptotic cells (45%) was almost identical to that seen with IL-4 plus CD40-treated normal cells (48%). However, the percentage of apoptotic cells was still statistically greater in the patients' cells than with normal cells stimulated with IL-4 plus αCD40 (13·2% *versus* 6·6%, P = 0·002). Changes in apoptotic cell percentage were directly

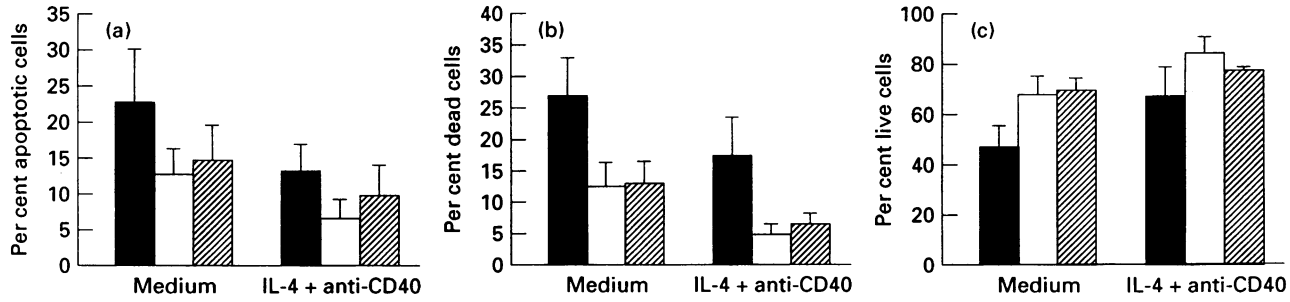

Fig. 4. CVID patients' B cells exhibit increased spontaneous apoptosis. The percentage of apoptotic (a), dead (b) and live (c) $CD19^+$ B cells following culture for 60 h is shown for selected CVID patients (■), normal controls (□) and disease controls (▨). The error bars represent 1 s.d. Cells cultured with medium alone are shown on the left, while cells cultured with IL-4 (100 U/ml) plus CD40 MoAb G28.5 (0·1 μg/ml) are shown on the right. The patients' B cells showed statistically more apoptotic and dead cells compared with normals. While IL-4 plus CD40 MoAb statistically decreased the apoptotic and dead cells in the patients, these percentages remained greater than in comparable controls. Complete statistical analysis of the data in this figure is given in Table 2.

reflected in the percentage of dead cells and reciprocally reflected in the percentage of live cells. The three disease control subjects' B cells had profiles of apoptotic, live and dead cells similar to normals.

T cells from the CVID patients also demonstrated increased apoptosis in culture, showing a mean of 9·9% apoptotic T cells at day 3 compared with 2·3% for normals ($P = 0·001$) (Table 3). The three disease control subjects also showed increased T cell apoptosis compared with normals ($P = 0·01$), which is not surprising in view of the increased CD95 display by two of these subjects. As expected, IL-4 plus CD40 treatment had no effect on apoptosis by either patients' or controls' T cells (data not shown).

Immunoglobulin production by the CVID patients remains quantitatively and qualitatively abnormal after IL-4 plus αCD40 or IL-4, αCD40 plus IL-10 stimulation

We have previously shown that these CVID patients' B cells can be driven to produce IgE by stimulation with IL-4 plus αCD40, a result confirmed and extended by others to include production of IgG and IgM following IL-4 plus αCD40 plus IL-10 [12–14]. Yet we found that IL-4 plus αCD40-driven IgE production per B cell from CVID patients was less than that produced by normal subjects [12,22]. This quantitative failure of IgE production was confirmed in the present study in both selected patients and disease controls (Table 4). In corroboration, the total ε mRNA from the CVID patients stimulated with αCD40 plus IL-4 was 31% of that seen in cells from normals. Stimulation with IL-4 + αCD40 + IL-10 induced IgG production in the CVID patients (Table 4); however, the amount of IgG fell far short of the IgG produced by normal cells.

To determine if IL-4 plus αCD40 stimulation of CVID B cells resulted in a qualitatively, if not quantitatively, normal immunoglobulin response, we analysed the level of individual ε mRNA isoforms in cultures driven with IL-4 plus αCD40 or IL-4 + αCD40 + IL-10 [19,20]. The selected CVID patients showed a pattern of ε mRNAs distinct from normals under both conditions (Table 5). The patients' IL-4 plus αCD40-driven ε mRNA pattern was distinguished by less ε mRNA resulting from the CH4′-CH5 splice [19], it being 5·5-fold less in patients compared with normals ($P < 0·002$). In the presence of IL-10, patients showed an increase in the percentage of the CH4-M2′ mRNA and no change in the CH4-M2′ splice, while normals demonstrated a decrease in the former and a large increase in the latter. The disease control subjects showed an intermediate pattern (data not shown).

DISCUSSION

We investigated CVID subjects with a $CD20^{bright}$,$CD62L^{dim}$ B cell phenotype for B cell phenotype and function as related to apoptosis. The frequent occurrence of lymphopenia in the face of lymphadenopathy and splenomegaly with follicular hyperplasia in long-standing disease suggested that the patients' cells were entering the secondary lymphoid tissue but were unable to complete their maturation programme. Many of the circulating B cells in these patients appeared to represent recent bone

Table 2. Increased apoptosis by CVID B cells and inhibition of apoptosis by IL-4 plus αCD40: statistical comparisons for data in Fig. 4.

Comparison	IL-4/CD40	Apoptotic cells	Live cells	Dead cells
CVID *versus* normals	−	$P = 0·01$	$P = 0·.001$	$P = 0·002$
CVID *versus* normals	+	$P = 0·002$	$P = 0·003$	$P = 0·001$
CVID	− *versus* +	$P = 0·025$	$P = 0·012$	$P = 0·033$
Normal	− *versus* +	$P = 0·001$	$P < 0·001$	$P < 0·001$
DC	− *versus* +	$P =$ NS	$P =$ NS	$P =$ NS

NS, Not significant ($P > 0.05$); DC, CVID disease controls.

Table 3. CVID T cells display increased spontaneous apoptosis*

	Per cent apoptotic	Per cent live	Per cent dead
CVID ($n = 6$)			
Mean	9·9 ± 5·4†	78 ± 7·1	9·8 ± 4·8
Normals ($n = 9$)			
Mean	2·3 ± 0·9	95 ± 1·9	2·4 ± 0·9
Disease controls (*DC*) ($n = 3$)			
Mean	5·7 ± 3·1	89 ± 4·5	3·7 ± 1·5
Statistical comparisons			
CVID *versus* normals	$P = 0·001$	$P < 0·001$	$P = 0·001$
CVID *versus* DC	$P =$ NS	$P = 0·015$	$P = 0·007$
DC *versus* normals	$P = 0·001$	$P = 0·005$	$P = 0·001$

*Cells were cultured with complete medium alone (including serum) for 60 h.
†Mean (±1 s.d.).
NS, Not significant, $P > 0·05$.

marrow emigrants on the basis of their phenotype and fetal V_H gene expression [10,11]. Finally, IL-4 plus CD40, stimuli that are potent at preventing apoptosis in germinal centre cells [23,24], induced *in vitro* differentiation of CVID B cells [12–14].

In this study, patients' B and T cells revealed a striking increase in CD95, a member of the TNF/NGFR superfamily [25]. This degree of enhanced *in vivo* CD95 expression has not been reported in any other condition. It is expressed on most follicular centre cells, cells that undergo extensive proliferation and apoptosis, it is absent or very dim on marginal zone cells [26,27] and is preferentially displayed on circulating IgD^- rather than IgD^+ B cells [28]. In systemic lupus erythematosus (SLE), where increased spontaneous apoptosis has been reported [29], the level of CD95 on circulating cells was normal or only modestly increased. A secreted form of CD95 is generated by alternative RNA splicing. Injection of the soluble CD95 into mice induced an increase of spleen B cells and a shift away from $CD4/CD8^{++}$ T cells to more mature single-positive CD4 or CD8 phenotypes [30]. This increase in tissue B cells and more mature T lymphocyte pattern is reminiscent of the cell alterations seen in our CVID patients.

Expression of CD38 was very low on our subjects' B cells only. CD38 is an 'activation marker' highly expressed by normal germinal centre B cells. Thus, the failure of CVID B cells to signal via CD38 may contribute to the loss of this lymphocyte population and humoral immunodeficiency seen in our CVID patients. We employed RA to determine if the CVID B cells could express CD38 [31,32]. Following RA treatment, CD38 expression increased on patients' B cells, although the magnitude and absolute amount of CD38 did not achieve levels seen with normal B cells. As such, CVID B cells appear to have a diminished capacity for generating CD38 surface expression as well as potentially lacking *in vivo* influences which may be necessary for normal CD38 expression.

In agreement with the phenotype of the patients' cells, their T and B cells underwent increased *in vitro* spontaneous cell death (Fig. 4, Table 3). This increased apoptosis probably represents the outcome of *in vivo* events. Treatment with IL-4 plus αCD40, stimuli shown to induce CVID patients' B cells to

Table 4. IgE and IgG production by CVID and normal cells in response to combinations of IL-4, IL-10 and CD40*

	Stimulation		
	IL-4 + CD40	IL-4 + CD40 + IL-10	
	IgE	IgE	IgG
CVID subjects ($n = 6$)			
Mean	1·2 ± 3·3†	6·3 ± 3·3	329 ± 326
Disease controls ($n = 3$)			
Mean	2·9 ± 2·5	10·5 ± 6·7	371 ± 238
Normals ($n = 10$)			
Mean	23·6 ± 10·6	43·1 ± 18·8	5909 ± 3258

*IgE and IgG levels are given as ng/ml. Cells were cultured at 1×10^6/ml 12 days in complete RPMI plus stimuli.
†Mean (±1 s.d.)

Table 5. Relative amounts of selected ε mRNAs produced by CVID patients and control cells

	Stimulation	
ε splice variants in selected CVID ($n = 5$)	IL-4 + αCD40 (%)	IL-4 + αCD40 + IL-10 %
CH4-M2′	3·2 ± 1·8†	2·5 ± 1·8*
CH4-M2″	20·2 ± 12·1	30·4 ± 12·6*
CH4′-CH5	1·3 ± 1·1*	1·5 ± 1·2
Normals ($n = 5$)		
CH4-M2′	2·5 ± 1·8	8·8 ± 4·7
CH4-M2″	17·4 ± 6·4	11·2 ± 5·8
CH4′-CH5	7·2 ± 2·1	1·0 ± 1·0

*$P < 0·02$, normal *versus* CVID.
†Mean ± 1 s.d.

differentiate, rescued the B cells from apoptosis. However, this rescue did not return the percentage of apoptotic and dead B cells to normal (Fig. 4). Attempts to induce apoptosis with αCD95 and further discriminate between patients' *versus* normals' cells were unsuccessful (data not shown). While ligation of CD95 can induce apoptosis of specifically sensitive cells [27,28,33], expression of CD95 is not tightly linked to ligation-induced apoptosis. Fresh or recently activated cells which express CD95 are resistant to CD95-induced apoptosis, and may only become sensitive after long-term culture [28,33,34]. Indeed, the CD95 on the CVID B cells can be functional, as we have derived EBV-transformed cell lines from these patients—cell lines that over express CD95 and which do show enhanced apoptosis in response to CD95 ligation (Guo & Saxon, Cell Immunol, in press).

The data that IL-4 plus αCD40 did not fully rescue the CVID B cells agree with our previous results showing that such stimulation, while driving IgE production, did not induce normal immunoglobulin levels [12,22]. We extended these findings to IgG production with cells stimulated by IL-10 + IL-4 + αCD40; again patients' B cells produced subnormal amounts of IgG and IgE [12]. These results agree with those of Eisenstein *et al.* [35], where a variety of stimuli were shown to only partially restore immunoglobulin production from CVID subjects. In contrast, Nonoyama *et al.* reported normal mean IgE production from CVID patients [13]. This difference may be more apparent than real, as our patients represent the more severely deficient group from Nonayama's study (12 of the 22) who did not make normal IgG and IgM and for whom individual IgE production levels were not reported. Alternatively, differences in IgE production may be due to the use by Nonoyama *et al.* of CDw32-transfected mouse L cells to cross-link the CD40 MoAb, as this line has now been observed to produce novel cytokines.

To extend these studies to the qualitative immunoglobulin production driven by IL-4 plus CD40, we took advantage of our description of developmentally regulated ε mRNA isoforms [19,20]. Our selected patients showed an immature pattern of ε mRNAs that was distinct from normals. Furthermore, the ε mRNA response to IL-10 in the patients was different from that in normals. Taken together, the immunoglobulin protein and ε mRNA data support the view that IL-4 plus CD40, while preventing apoptosis and driving CVID B cell differentiation, did not normalize the B cells.

This study defines in novel ways the nature of the B and T cells found in a carefully identified subgroup of adult patients with CVID. We do not propose that increased CD95 and CD20 or decreased CD38 display by CVID B cells is the 'primary' defect in these patients. Rather, such changes probably reflect the immature nature of the B cells found in the patients' circulation. The circulating cells in our patients are reminiscent of the recently described earliest stage of B cells in the human germinal centre [36]. Similarly, T cell alterations described in CVID including the inability to trigger normally through the T cell receptor [37–39] are probably a reflection of the cells' maturational state.

Our data provide strong phenotypic and functional evidence that B and T cells in a defined group of CVID patients undergo enhanced apoptosis. This can explain why most of these patients show an absolute lymphopenia involving multiple cell populations, have circulating immature B cells which show fluctuations in V_H gene repertoire, and have an absence of more mature circulating B cells required to make a normal immunoglobulin response [10–12,15,16]. Whether *in vivo* the CVID B cells fail to receive positive signals required to prevent apoptosis or are subject to increased negative signals that induce enhanced susceptibility to apoptosis remains unknown.

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