

Immunodeficiency and other clinical immunology

Defective interleukin-2 and interferon- γ gene expression in response to antigen in a subgroup of patients with common variable immunodeficiency

Michael B. Fischer, MD,^a Ilona Hauber, PhD,^a Erich Vogel, MSc,^{a, b}
Hermann M. Wolf, MD,^a Josef W. Mannhalter, PhD,^{a, b} and
Martha M. Eibl, MD^a Vienna, Austria

Patients with common variable immunodeficiency (CVID) are heterogeneous in the clinical manifestations of the disease and in the underlying mechanisms leading to the immunodeficiency. The impairment of B-cell function can be due to an intrinsic defect of the B cells, a deficiency in the function of the antigen-presenting cell, or a dysfunction in the course of T-cell activation. In the present report we have focused our attention on T-lymphocyte activation in three patients with CVID. Numbers of T and B cells, as well as CD4 and CD8 T cell subsets, were within the normal range. The patients' B cells secreted IgM but did not secrete IgG and IgA in response to B-cell stimuli in vitro. Addition of allogeneic T cells was followed by an increase in IgM production but had no effect on the other immunoglobulin isotypes. Examination of T-cell function revealed impaired proliferative response, interleukin-2 (IL-2) and interferon- γ gene expression and IL-2 release after antigenic stimulation, whereas T-cell proliferation, as well as IL-2 gene expression and release in response to stimulation via anti-CD3, were comparable to those of healthy control subjects. Anti-CD3-induced IFN- γ gene activation in T cells from two patients was comparable to that of control subjects, whereas T cells from the third patient showed reduced expression of IFN- γ mRNA. In contrast to the decreased IL-2 and IFN- γ mRNA levels, IL-2R transcripts examined in parallel were normal in CVID T cells on stimulation with antigen. The defect in IL-2 and IFN- γ mRNA expression after stimulation with antigen, but not with anti-CD3, suggests an abnormality confined to T-cell activation by the T-cell receptor. (J ALLERGY CLIN IMMUNOL 1993;92:340-52.)

Key words: Common variable immunodeficiency, antibody deficiency, cytokine gene expression, defect in T-cell activation

Common variable immunodeficiency (CVID) comprises a heterogeneous group of disorders characterized by defects in B-cell differentiation and/or antibody secretion.^{1, 2} Serum concentrations of some or all isotypes of immunoglobulins may be reduced or absent, while circulating B cells are present, albeit occasionally reduced in

Abbreviations used

CVID: Common variable immunodeficiency
MNCs: Mononuclear cells
mAb: Monoclonal antibody
PCR: Polymerase chain reaction
PMA: Phorbol 12-myristate 13-acetate
PPD: Purified protein derivative
TBE: Tickborne encephalitis virus
TCR: T-cell receptor

From ^athe Institute of Immunology, University of Vienna; and
^bthe Department of Immunological Research, Immuno AG,
Vienna.

Received for publication Apr. 20, 1992; revised Jan. 25, 1993;
accepted for publication Feb. 17, 1993.

Reprint requests: Martha M. Eibl, MD, Institute of Immunology,
Borschkegasse 8a, A-1090 Vienna, Austria.

Copyright © 1993 by Mosby-Year Book, Inc.
0091-6749/93 \$1.00 + .10 1/1/46655

numbers. The intrinsic antibody deficiency makes these patients unduly susceptible to infections caused by some ubiquitous bacteria and mycoplasmas. The deficiency can be due to functional impairment at any stage of the complicated inter-

actions among B cells, T cells, and antigen-presenting cells required for antibody production. On the basis of B-cell function, for example, several groups of patients could be identified according to the capacity of their B cells to secrete immunoglobulins of different isotypes on in vitro stimulation with anti-IgM and interleukin-2 (IL-2).³⁻⁵ In some patients a deficiency of antigen-presenting cell function has been reported.⁶⁻⁸ Defects at the T-cell level included abnormal T helper functions,^{2, 8} increased T suppressor activities,⁹ and abnormalities in T-cell activation caused by an impairment of lymphokine production.¹⁰⁻¹³ Recently, a subgroup of patients with CVID in whom T lymphocytes could not be adequately stimulated by mitogens has been described.¹⁰ This was accompanied by significantly decreased expression of IL-2, IL-4, IL-5, and interferon- γ in these patients' T cells, whereas their IL-2R expression was comparable to that of the control subjects.

In previous studies we identified patients whose T lymphocytes proliferated normally in response to mitogens (e.g., phytohemagglutinin) but were unable to mount an adequate proliferative response when stimulated with antigens.¹⁴ In the present study we have concentrated on this subgroup of patients and attempted to delineate further the nature of the underlying T-cell defect. We approached the question by analyzing lymphoproliferative responses, lymphokine secretion, and gene expression of IL-2, IL-2R, and interferon- γ in antigen- and mitogen-driven cultures. Lymphocytes of healthy control subjects and patients with CVID, as well as naive lymphocytes from umbilical cord blood, were investigated. The results of these studies indicate that T lymphocytes from this subgroup of patients with CVID did not respond to antigen with adequate expression of IL-2 and interferon- γ mRNA.

METHODS

Patients and control subjects

Criteria for the diagnosis of CVID include significantly decreased serum IgG levels and a consistent impairment of the production of specific antibody. Patients with CVID are heterogeneous regarding serum concentrations of IgA and IgM and with respect to B and T cell numbers and function and presumed pathogenesis.¹⁵ In the present article we report on three patients with CVID who had severe agammaglobulinemia (i.e., IgG level below 2 gm/L and IgA and IgM levels below 0.2 gm/L), normal numbers of T and B cells, and no lymphoproliferative response to recall antigens. Three other patients of 12 who were followed up at our institution at the time of the study were also

agammaglobulinemic and showed decreased cellular response to recall antigen, but two of them showed very low numbers of B cells (i.e., <2% of the peripheral blood lymphocytes), and one patient was not available for repeated examinations. The three patients described in this study—one woman, aged 49 and two men, aged 39 and 24, had received bacille Calmette-Guérin vaccinations at birth and had received tetanus toxoid and tickborne encephalitis virus (TBE) vaccinations or boosters within the last 12 months before testing. They were receiving regular gammaglobulin replacement therapy (intravenous application of 400 mg IgG per kilogram of body weight every 2 to 4 weeks), and their IgG trough levels ranged between 5 and 8 gm/L. None of them had acute infections or were receiving corticosteroids or antibiotics. Blood samples were taken 2 to 4 weeks after administration of immunoglobulins.

The patients had normal numbers of T cells and B cells, and their CD4- and CD8-expressing T lymphocytes were also within the normal range. The patients' T cells cocultured with autologous monocytes responded normally to mitogens (phytohemagglutinin, concanavalin A, and anti-CD3). However, the patients' T cells failed to respond to a number of different antigens (e.g., *Escherichia coli*, tetanus toxoid, purified protein derivative [PPD] and TBE).

Thirty healthy volunteers, who were investigated in parallel, served as control subjects. The control subjects included both men and women and ranged in age from 19 to 50 years. The vaccination histories of the control subjects were comparable to those of the patients.

Isolation of mononuclear cells and preparation of a T-enriched lymphocyte population

Mononuclear cells (MNCs) were isolated from the peripheral blood of patients and healthy human volunteers and from umbilical cord blood by buoyant density gradient centrifugation.¹⁶ Monocytes were prepared by adherence to plastic.¹⁷ Nonadherent cells were fractionated into T-enriched cells (>95% CD2⁺ and >90% CD3⁺) and non-T cells (>40% B cells as judged by CD19 expression) by rosetting with sheep erythrocytes treated with neuraminidase (Behring, Marburg, Germany) or 2-aminoethylisothiuronium bromide (Sigma Chemical Co., St. Louis, Mo.), as described elsewhere.^{17, 18}

Phenotypic analysis of MNCs

Monocytes, B cells, T cells, T-cell subsets, and T-cell activation markers were analyzed by direct two-color immunofluorescence staining with the following monoclonal antibodies (mAbs): Leu-2a (CD8), IL-2R (CD25), HLA-DR, Leu 18 (CD45RA), Leu 45RO (CD45RO), and WT-31 (T-cell receptor [TCR] α - β) (Becton Dickinson, San José, Calif.); T3 (CD3), T4 (CD4), Mo2 (CD14), B4 (CD19), and 2H4 (CD45RA)

(Coulter Immunology, Hialeah, Fla.); and BMA-031 (TCR- α - β) (Behring). Evaluation was performed with a cytofluorograph (Becton Dickinson FACS 440 or FACStar plus), and a total of 10,000 cells were analyzed.

Lymphocyte proliferation

Triplicate cultures that contained adherence-purified monocytes (1×10^4 per well) and T-enriched cells (1×10^5 per well) were set up in flat-bottomed microtiter plates (Falcon Microtest II; Becton Dickinson, Lincoln Park, N.J.). To assess the proliferative response to ubiquitous antigens, heat-inactivated *E. coli* 089:H10 (5×10^6 *E. coli* per milliliter) and tetanus toxoid (10 LF/ml, Swiss Serum and Vaccine Institute, Berne, Switzerland) were added. In addition, the cells were stimulated with mAbs specific for anti-CD3 (10 ng/ml OKT3, Ortho Diagnostic Systems Inc., Raritan, N.J.) and TCR α - β (WT-31, BMA-031, 2 μ g mAb per milliliter) coated on beads (Dynabeads M450, 4×10^8 per milliliter; Dynal, Oslo, Norway), as well as with a combination of the diacylglycerol analog phorbol 12-myristate 13-acetate (PMA) (10 ng/ml; Sigma) and the calcium ionophore ionomycin (0.1 μ g/ml; Calbiochem, San Diego, Calif.). The cell cultures were kept at 37° C in a CO₂ incubator (5% CO₂ in humidified air). After incubation with antigen for 7 days, PMA + ionomycin for 5 days, and the mAbs for 3 days, the cultures were pulsed with 0.2 μ Ci tritiated thymidine (Amersham, Buckinghamshire, England) and harvested 16 hours later onto glass fiber filter strips with a semiautomatic cell harvester (Skatron, Lier, Norway). Tritiated thymidine incorporation was determined by liquid scintillation counting (Packard Instruments Corp., Tri-Carb, Meriden, Conn.). Results are presented as mean disintegrations per minute $\pm$ SD. Statistical analysis was performed with Student's *t* test.

Induction of IL-2 release and assay of IL-2 activity

Macrophages (1×10^4 per well) and T-enriched cells (1×10^5 per well) were activated with the stimuli and culture conditions described above. Culture supernatants were removed once every 24 hours over a period of 7 days, sterile-filtered, and frozen at -20° C until analysis. IL-2 concentration in the culture supernatants was determined by a bioassay on the basis of the supernatants' capacity to induce proliferation of the murine cytotoxic lymphoid cell line CTLL-2 according to the method of Gillis and Smith.¹⁹ The linearity of the dose-response calibration curve allowed assessment of IL-2 concentrations that ranged from 0.1 to 100 U/ml.

Cytokines

Purified recombinant human IL-2 (Amersham), IL-1- α , IL-3, IL-4, IL-5, IL-6, TNF- α and interferon- γ (Genzyme, Boston, Mass.) were added to the cultures in concentrations of 100 IU/ml and 5 IU/ml.

Polymerase chain reaction

Reverse transcription²⁰ of total RNA, followed by polymerase chain reaction (PCR) analysis,²¹ was used to detect the activation levels of IL-2, IL-2R, and interferon- γ mRNA. For this purpose T lymphocytes isolated from three patients and three control subjects were stimulated with PMA + ionomycin, tetanus toxoid, or anti-CD3 mAbs (OKT3). At 6 hours and 24 hours after stimulation, the total cellular RNA was isolated from 5×10^6 T cells by the method of Chirgwin et al.²² and quantified by measurement of absorbance (optical density) at 260 nm. Equal amounts (2 to 5 μ g) of total RNA were reverse transcribed into cDNA by first and second strand synthesis with the use of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals, Mannheim, Germany). The cDNA copies were then amplified (30 cycles) with a BIO-MED Thermocycler 60 (BIO-MED Devices, Inc., Madison, Conn.) by Taq polymerase (USB Corp., Cleveland, Ohio) with oligonucleotide primer sets specific for IL-2, IL-2R, interferon- γ and, as an internal control, β -actin (MAPPING Amplimer, Clontech Laboratories, Inc., Palo Alto, Calif.). The amplification program consisted of denaturation at 94° C for 1 minute, primer annealing at 60° C for 2 minutes, and extension at 72° C for 3 minutes. Finally, the PCR-generated products were fractionated on 1.5% agarose gels and validated by the predicted size of the amplified cDNA fragments and by hybridization of the corresponding filters with specific cDNA probes (IL-2 and IL-2R), as well as by interferon- γ and β -actin-specific oligonucleotides.²³

DNA hybridization

The fractionated amplification products were blotted from agarose gels onto Hybond-N membranes (Amersham) according to the manufacturer's protocol and hybridized at 65° C in 5 $\times$ standard sodium citrate, 25 mmol/L NaHPO₄, pH 6.5, 5 $\times$ Denhardt's solution, 0.1% sodium dodecylsulfate, 10% dextran sulfate for 16 hours. pT-cell growth factor-11 (IL-2; ATCC No. 39673), pIL-2R2 (IL-2R- α)²⁴ plasmid DNA and an interferon- γ -specific (position 366-395)²⁵ or β -actin-specific (position 401-434)²⁵ oligonucleotide probe were used for hybridization. The excess radioactive label was removed by washing three times in 0.1 $\times$ standard sodium citrate/0.1% sodium dodecylsulfate at 65° C. The air-dried filters were exposed at -70° C to Kodak X-OMAT AR films (Eastman Kodak Co., Rochester, N.Y.) with intensifying screens.

Quantitative evaluation of PCR assays

The relative density of the signals obtained by Southern blot analysis of the PCR-generated products was determined by laser densitometry (LKB Ultrascan XL 2.1 Laser Densitometer; LKB, Bromma, Sweden). The densitometric reading of IL-2, interferon- γ , and IL-2R in a particular test was related to the corresponding

densitometric reading for β -actin, and the results for a given patient are expressed as percentage of the reading for the healthy control subject who was examined in parallel. In addition, quantitative analysis of the amplified mRNA specific for IL-2 was performed with radioactive labeled IL-2-specific anti-sense oligonucleotides. The anti-sense oligonucleotide was labeled with γ -phosphorous 32-adenosine triphosphate by using polynucleotide kinase, and unincorporated nucleotides were removed on a Pharmacia NAP-5 column, followed by ethanolic precipitation.

3' ^{32}P -end-labeled IL-2-specific primer (100 $\mu\text{mol/L}$), 5' IL-2-specific primer (100 $\mu\text{mol/L}$) and deoxyribonucleic triphosphates (50 $\mu\text{mol/L}$) in $1 \times$ PCR buffer were mixed to produce a total volume of 50 μl . The amplification profile involved denaturation at 94°C for 1 minute, primer annealing at 60°C for 2 minutes, and extension at 72°C for 3 minutes for 25 cycles. Fifteen microliters of each PCR reaction mixture was electrophoresed on 1.5% agarose gels in $1 \times$ Tris/acetate/ethylenediaminetetraacetic acid buffer. Gels were exposed for 1 hour to Kodak X-OMAT AR film. Appropriate bands were cut out of the gel, and radioactivity was determined in a liquid scintillation counter. Results were expressed as counts per minute. Quantitation of changes in IL-2 mRNA levels in activated T cells by PCR can be problematic because of the exponential nature of this assay. Therefore we used an internal DNA standard (pJC) in a competitive PCR assay. pJC, a nonhomologous DNA fragment derived from the plasmid pBR 322, was cloned between the IL-2-specific primer pairs and thus will compete with the target cDNA in the PCR assay. In each reaction 3 μg of total RNA was used for the synthesis of double-stranded cDNA. Identical aliquots (5%) of these cDNA templates were amplified in the presence of twofold serial pJC dilutions ranging from 50 $\text{fg}/\mu\text{l}$ to 0.195 $\text{fg}/\mu\text{l}$. The PCR assay was performed as described above, and the amplified products were resolved on a 1.5% ethidium bromide-agarose gel. The size of the resulting pJC-specific DNA product was 300 bp, whereas that of the IL-2-specific sequence of the target cDNA was 458 bp. Because the quantities of the competitive pJC DNA that were used are known, the actual amount of IL-2 cDNA (in femtograms per microliter) added to the PCR reaction can be determined by estimating the dilution in which the pJC and the target PCR products appear to be equal.

Induction of B-cell Ig secretion by stimulation of patients' and control subjects' peripheral blood mononuclear cells with pokeweed mitogen

MNCs (1×10^6) of three patients and four control subjects were cultured in 24-well flat-bottomed culture plates (Multiwell Tissue Culture Plates, Falcon, Lincoln Park, N.J.). To assess Ig production, cultures were stimulated with pokeweed mitogen (Gibco, Grand Is-

land, N.Y.) at a final dilution of 1:500, anti-IgM mAbs (kindly provided by Dr. O. Majdic, Institute of Immunology, University of Vienna) coupled to Dynabeads M 450 at a final dilution of 1×10^6 beads per milliliter, anti-IgM-beads in combination with rIL-2 (Genzyme) at a concentration of 100 U/ml, or *Staphylococcus aureus* Cowan I (Calbiochem Corp., San Diego, Calif.) at a final concentration of 1:5000. On day 7 culture supernatants were harvested, sterile-filtered, and kept at -20°C until analysis of immunoglobulin concentrations by ELISA.

Immunoglobulin isotype-specific ELISA

Maxisorp Immuno plates (Nunc GmbH, Wiesbaden, Germany) were coated overnight at 4°C with 4 $\mu\text{g/ml}$ of sheep anti-human antibodies (Boehringer Mannheim) in a coating buffer (carbonate-bicarbonate buffer, Sigma, pH 9.6). The plates were washed three times and blocked with 1% bovine serum albumin. Culture supernatants or purified Ig of the respective isotype (N/T protein control serum [human], Behring) in different concentrations were added in order to obtain standard curves, and the plates were incubated overnight at 4°C . Then the plates were washed and incubated for 1 hour at room temperature with the appropriate alkaline phosphatase-labeled isotype-specific goat anti-human IgG, IgM, and IgA mAbs (Sigma). After this was done, the plates were washed once again and 1 mg/ml of p-nitrophenyl phosphate (Sigma) in 10% diethanolamine buffer, pH 9.8, containing 0.5 mmol/L MgCl_2 was added. Reaction was stopped by addition of 50 μl 3M NaOH per 200 μl of reaction mixture. The plates were assayed with an ELISA reader with a wavelength of 405 nm, and supernatant concentration of Ig was calculated with regression analysis. The linearity of the assay allowed determination of 0.01 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$.

RESULTS

Enumeration and phenotypic characterization of lymphocytes

Both patients and control subjects presented with normal numbers of circulating T cells, and the distribution of T-cell subsets was comparable in the two groups. Immunofluorescence analysis of patients' MNCs (freshly isolated) showed normal percentages of CD2 ($61\% \pm 7\%$), CD3 ($59\% \pm 6\%$), CD4 ($38\% \pm 3\%$), and CD8 ($27\% \pm 4\%$) positive cells, and the majority of the T cells coexpressed the heterodimer TCR- α - β ($97\% \pm 2\%$ of the CD3-positive cells were TCR-positive). T-cell subsets, defined as memory cells by the coexpression of CD4 and CD45RO and naive cells identified by CD4 and CD45RA expression, were comparable in patients and control subjects (data not shown). B cells and monocytes of all patients were normal in numbers and in

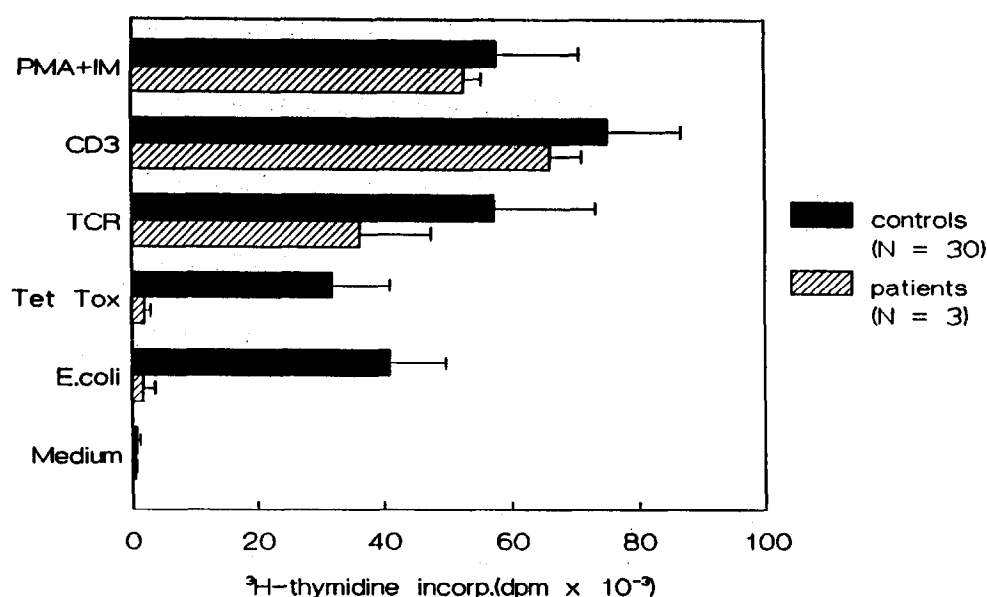


FIG. 1. T-cell proliferation in patients and control subjects. T cells isolated from the peripheral blood (1×10^5 per well) were cultured in triplicate with autologous monocytes (1×10^4 per well) and stimulated with a combination of PMA (10 ng/ml) + ionomycin (IM) (0.1 μ g/ml), mAbs (OKT3, 10 ng/ml, BMA031 coated on beads, 2 μ g mAbs per milliliter beads), or antigen (tetanus toxoid [Tet Tox], 10 Lf/ml, and *E. coli*, 5×10^6 /ml). Proliferative responses were determined by incorporation of tritiated thymidine (0.2 μ Ci). Results are expressed as mean dpm $\pm$ SD.

their expression of major histocompatibility complex class II molecules.

T lymphocytes obtained from cord blood differed from those of patients and control subjects in that the cord blood CD4 population expressed high levels of bright CD45RA but no bright CD45RO (data not shown), results that confirm data in the literature.²⁶

Lymphoproliferative response

T cells from 30 healthy control subjects responded with substantial proliferation to antigens such as *E. coli* or tetanus toxoid after 7 days of cultivation (Fig. 1), as well as to TBE and PPD.¹⁴ In contrast, the lymphoproliferative response of the patients' T cells to antigens was significantly decreased. A shift in the peak of the T-cell response seemed unlikely, because antigen-induced T-cell proliferation was found to be decreased in patients between day 3 and day 21 of the culture period.

To further characterize the defect in the course of T-cell activation, lymphocytes were stimulated with a combination of PMA and ionomycin. PMA, a diacylglycerol analog, is a direct activator of protein kinase C and can bypass membrane receptors. The calcium ionophore ionomycin mobilizes free calcium from internal and external

stores. The combination of these two agents mimics activation of the second messengers diacylglycerol and inositol triphosphate during T-cell activation.²⁷⁻³¹ Application of PMA + ionomycin resulted in comparable proliferation of lymphocytes in patients with CVID and control subjects (Fig. 1).

To determine whether the defect was located at the TCR level, the proliferative response of T lymphocytes after stimulation via CD3 (OKT3) and TCR (WT-31, BMA031) was measured. Proliferation in response to anti-CD3 mAbs was comparable in patients and control subjects, whereas the response to anti-TCR mAbs was decreased in patients' T cells (Fig. 1).

IL-2 production

To determine IL-2 release by lymphocytes of patients with CVID and control subjects, peripheral blood lymphocytes were subjected to antigenic and mitogenic stimuli. To study the time kinetics of IL-2 release, supernatants were assayed every 24 hours over a period of 7 days. When cells were activated with PMA + ionomycin or anti-CD3 mAbs, the time kinetics of IL-2 release and the amounts secreted were comparable in patients and control subjects (Fig. 2). Maximum levels of IL-2 could be observed in the

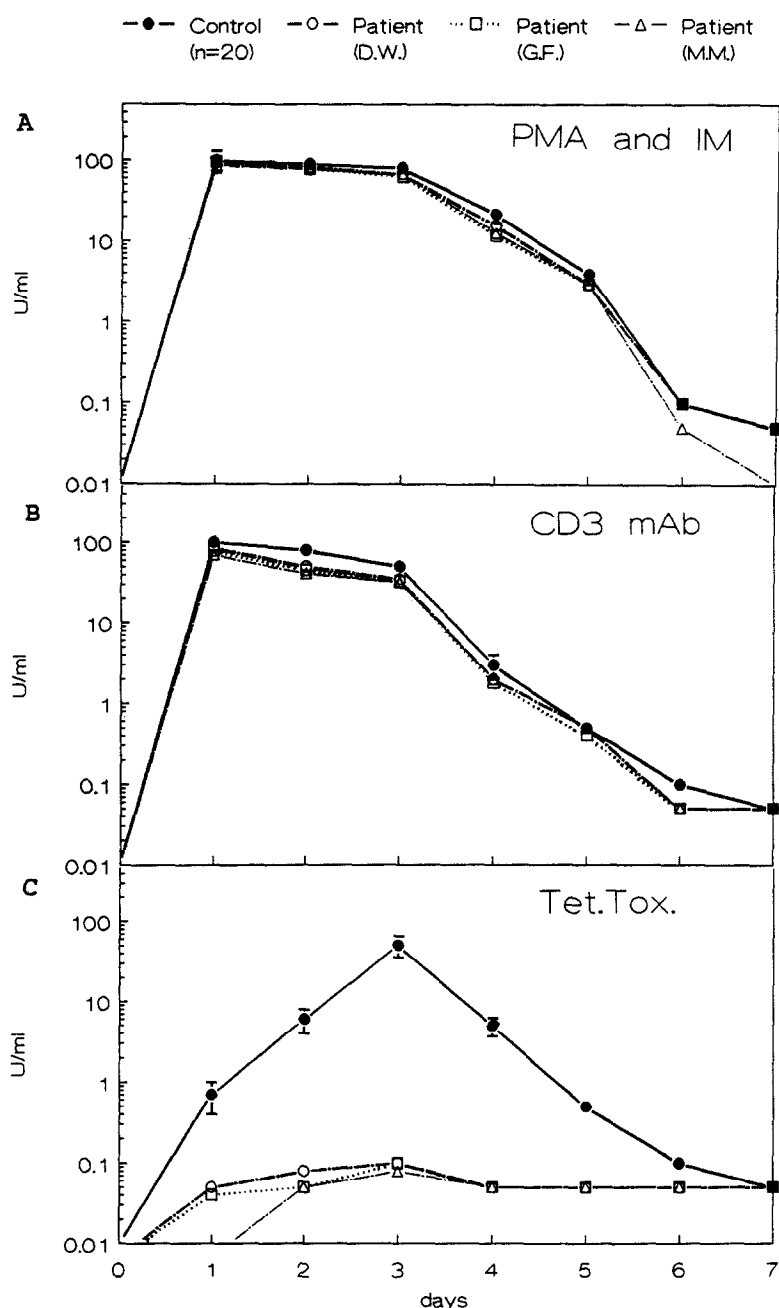


FIG. 2. IL-2 production in response to PMA + ionomycin, anti-CD3 mAbs, and antigen (tetanus toxoid) in T cells of healthy control subjects and patients with CVID. T cells and monocytes from control subjects and patients were cultured in the presence of PMA + ionomycin (IM) (A), anti-CD3 mAbs (B), and tetanus toxoid (C), respectively. Culture supernatants were harvested at the times indicated and assayed for IL-2 as described in the Methods section.

culture supernatants 24 hours after stimulation. In response to antigen, however, a marked difference between the two groups was observed. Although lymphocytes of the 30 control subjects showed peak IL-2 release on day 3 and secreted an average of 50 IU/ml on stimulation with tetanus toxoid, T lymphocytes of patients failed to respond (Fig. 2). The amounts of IL-2 detected in

the supernatant of cultures set up with the patients' cells were below 1% of control levels.

Lymphocyte proliferation in the presence of externally added lymphokines

Proliferation of patients' T lymphocytes in response to tetanus toxoid plus rIL-2 was significantly higher ($p < 0.05$) than the response to

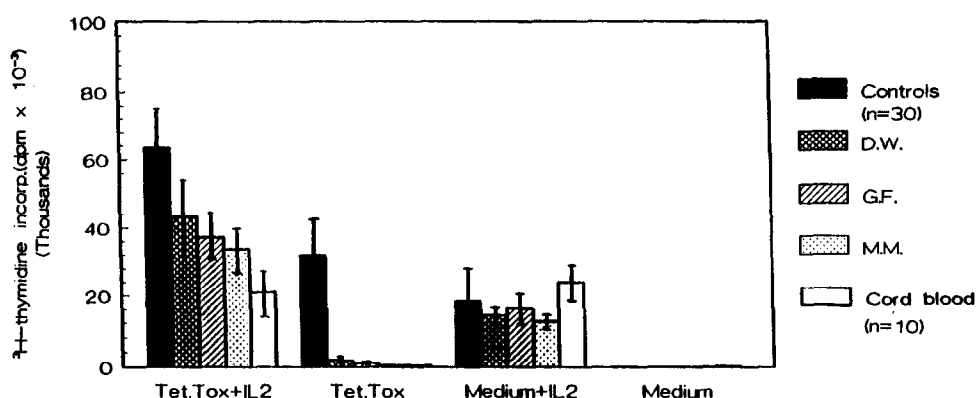


FIG. 3. T-cell proliferative response to antigen with and without addition of rIL-2 in healthy control subjects, cord blood, and patients with CVID. Control subjects', cord blood, and patients' T cells and monocytes were cultured in triplicate in the presence of antigen (tetanus toxoid, 10 U/ml) with and without 100 U rIL-2/ml added. Proliferation was determined by tritiated thymidine uptake. Results are expressed as mean dpm $\pm$ SD.

either rIL-2 or tetanus toxoid alone (Fig. 3). Other exogenously added lymphokines (rIL-1, rIL-3, rIL-4, rIL-6, recombinant interferon- γ , rTNF- α) had no effect (data not shown). Cord blood cells, which are naive with respect to antigenic stimulation and represent a population of truly unprimed cells, did not proliferate in response to antigenic stimulation but responded to externally added rIL-2. The lymphoproliferative response to rIL-2 was not increased by the addition of antigen to the culture (Fig. 3). Addition of rIL-2 resulted in comparable augmentation of the PMA + ionomycin and anti-CD3-induced responses in patients', control subjects', and cord blood lymphocytes (data not shown).

Detection of IL-2, IL-2R, and interferon- γ gene expression in stimulated T lymphocytes

With the use of PCR technology, the relative IL-2, IL-2R, and interferon- γ mRNA levels in cultured T cells from the three patients were determined. Three healthy individuals who were matched in vaccination history were tested in parallel, and results from one representative control subject are shown in Fig. 4. After stimulation with anti-CD3 mAb (OKT3) and antigen (tetanus toxoid), respectively, the total cellular RNA was isolated, reverse-transcribed into cDNA, and subjected to amplification by *Taq* polymerase with 30 reaction cycles (see Methods). To validate the PCR reaction conditions used, a series of reactions with IL-2R primers was performed by increasing the number of reaction cycles in increments of 5 cycles from 5 to 30. Below 20 reaction

cycles, no PCR product was detectable on the agarose gel. Between 20 and 30 cycles, however, a specific signal was easily detectable for the patients, as well as for the healthy individuals tested as control subjects. The ratio of the signals for the different cycles was constant, also indicating the validity of the reaction conditions used (data not shown). The PCR-generated products were visualized by agarose gel electrophoresis and subsequent hybridization. The results obtained for the activation of IL-2, IL-2R, and interferon- γ gene expression on stimulation with anti-CD3 mAbs are shown in Fig. 4, B. At 6 hours (Fig. 4, B) and 24 hours (Fig. 4, B) after stimulation, IL-2- IL-2R- and interferon- γ -specific gene expression in T cells from two patients (Fig. 4, B, lanes 2 and 3) was comparable to the results obtained from the healthy control (Fig. 4, B, lane 1). One patient (MM) expressed normal levels of IL-2 and IL-2R on stimulation with anti-CD3 mAbs but failed to produce interferon- γ mRNA 6 hours and 24 hours after stimulation (Fig. 4, B, lane 4). A negative control lacking the reverse-transcribed mRNA as primer substrate was run in parallel and indicated specificity of the amplification reaction (Fig. 4, A, B, and C, lane 5). Amplification of β -actin served as an internal control. The amplified cDNA fragments obtained in these and the following experiments displayed the predicted sizes; their specificity was confirmed by hybridization with specific IL-2, IL-2R cDNA, or specific internal interferon- γ and β -actin sequences (see Methods). In contrast to anti-CD3 stimulation, the response of all patients' T cells after stimulation with tetanus toxoid resulted in substantially

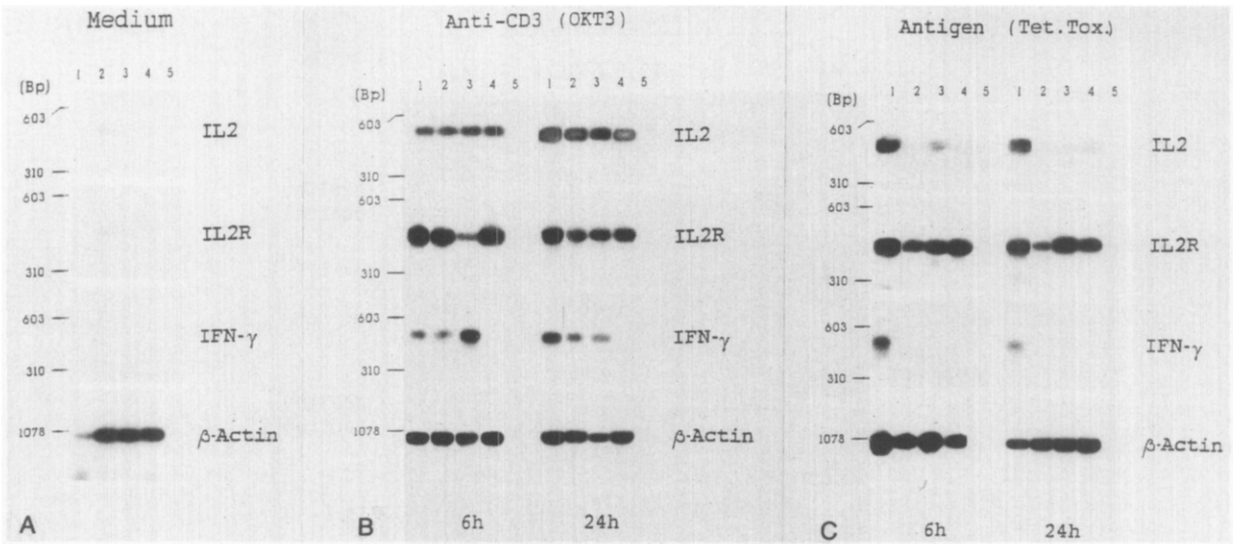


FIG. 4. Southern blot analysis of amplified cDNA from stimulated T cells. Total cellular RNA was isolated from T cells of three healthy control subjects (the level for one control subject is shown in lane 1 as representative of all three) and three patients with CVID (*lanes 2, 3 and 4*). T cells of patients and control subjects were incubated for 6 hours and 24 hours in the presence of anti-CD3 mAbs (**B**) or tetanus toxoid (**C**). Incubation of these cells for 24 hours in medium without stimulus was included as a control (**A**). After reverse transcription of the mRNA, the cDNA copies were amplified by PCR technology with the indicated specific primer sets (IL-2, IL-2R, interferon- γ and β -actin). PCR assayed without cDNA template served as a negative control (*lane 5*). PCR-generated products were fractionated on 1.5% agarose gels, blotted onto Hybond N membranes, and hybridized with specific ^{32}P -labeled cDNA and internal oligonucleotide probes (see Methods).

reduced activation of IL-2 and interferon- γ gene expression (Fig. 4, *C, lanes 2, 3, and 4*) as compared with the healthy control subject (Fig. 4, *C, lane 1*). The IL-2R signals obtained 6 hours and 24 hours after stimulation, however, did not differ in patients and control subjects (Fig. 4, *C, lanes 2, 3, and 4 vs lane 1*). Unstimulated T cells (medium) of the three patients and the control subjects expressed very low levels of IL-2 and IL-2R mRNA and completely lacked expression of the interferon- γ mRNA (Fig. 4, *A, lanes 1, 2, 3, and 4*).

Quantitative analysis of IL-2, interferon- γ , and IL-2R mRNA

Quantitative measurements of antigen-induced IL-2 and interferon- γ gene expression confirmed that the mRNA levels of both cytokines are significantly decreased in T cells of patients with CVID after antigenic stimulation. The data depicted in Table I show that levels of IL-2-specific mRNA as examined by quantitative PCR with radioactively labeled IL-2-specific anti-sense oligonucleotides were substantially lower in the three patients with CVID than in the three healthy control subjects. A significant reduction in

TABLE I. Quantitative PCR of IL-2-specific mRNA

Patients with CVID (cpm)		Healthy control subjects (cpm)	
D.W.	7 175	RC 1	39 525
G.F.	8 121	RC 2	40 740
M.M.	5 526	RC 3	38 690

IL-2-specific mRNA from T cells previously stimulated with antigen (tetanus toxoid, 10 Lf/ml) in the presence of autologous monocytes for 24 hours was obtained and quantitated as described in the Methods section, with [γ - ^{32}P]ATP-labeled IL-2-specific anti-sense oligonucleotides. The radioactivity of the IL-2 transcripts was determined in a liquid scintillation counter and is expressed as counts per minutes (cpm).

IL-2 mRNA of antigen-activated CVID T cells was also found in a competitive PCR analysis with a nonhomologous DNA fragment (pJC) as an internal standard (Fig. 5). Although between 9 and 11 fg/ μl of IL-2 mRNA could be detected in two healthy control subjects, between 1 and 3 fg/ μl were found in the three patients. Furthermore, quantitative evaluation of PCR analysis by laser densitometry showed that in the three patients tested both IL-2 and interferon- γ gene

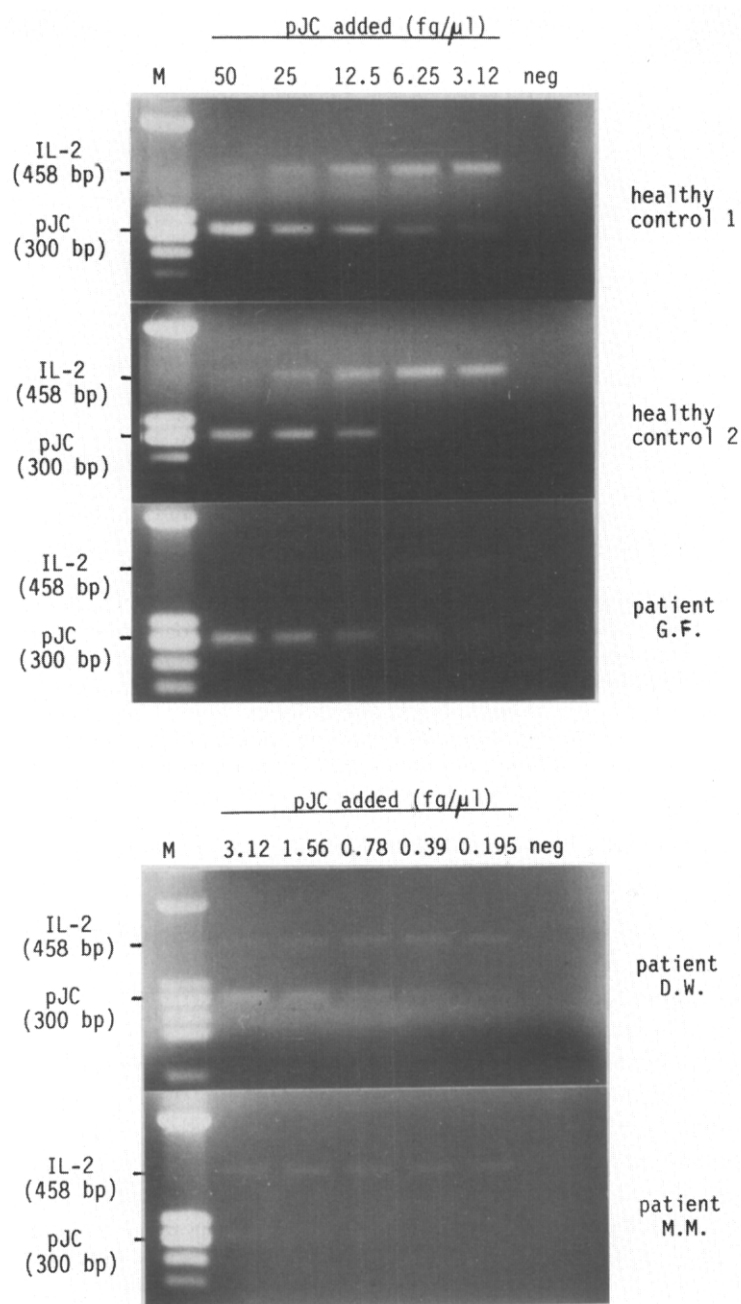


FIG. 5. Quantitation of IL-2 mRNA levels by competitive PCR analysis. Analysis of IL-2 mRNA levels in T cells from patients (G.F., D.W., M.M.) and healthy control subjects (1,2) after stimulation with tetanus toxoid for 24 hours was performed by a competitive PCR. Target cDNA was amplified in the presence of an internal DNA standard (pJC) as described in Methods. pJC was employed at twofold serial dilutions ranging from 50 fg/ml to 3.12 fg/ml for the healthy control subjects and the patient G.F., and between 3.12 fg/ml and 0.195 fg/ml of pJC was used for the patients D.W. and M.M. The PCR-generated products were analyzed on a 1.5% ethidium bromide-agarose gel. The size of the target cDNA is 458 bp; the size of the pJC product is 300 bp.

expression in antigen-activated T cells were significantly reduced as compared with a representative healthy control subject. The values for relative density of IL-2, interferon- γ , and IL-2R signals were calculated by relating the densito-

metric reading of the cytokine/cytokine receptor to the β -actin signal, and the results are given as percentage of the control value (Fig. 6). Reduced IL-2 and interferon- γ gene expression were found both 6 hours and 24 hours after activation (Fig.

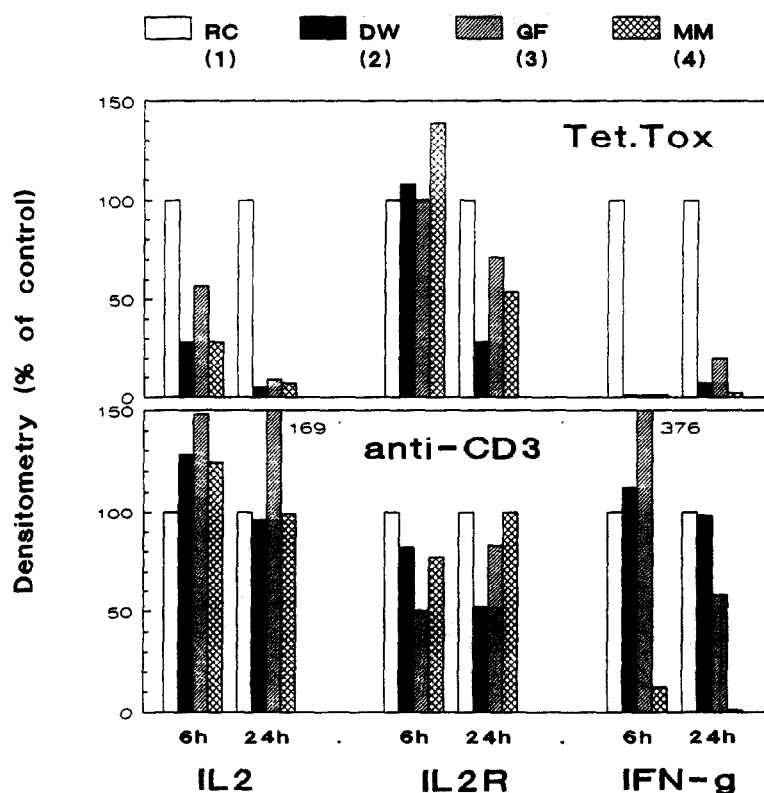


FIG. 6. Densitometric quantitation of IL-2, interferon- γ , and IL-2R signals obtained from patients' and control subjects' T lymphocytes after stimulation with antigen (tetanus toxoid) and anti-CD3 (OKT3). The relative density of the signal obtained by Southern blot analysis of the PCR-generated products (depicted in Fig. 4) was measured by laser densitometry. In each of the three patients (D.W., G.F., M.M.) and in one representative healthy control subject the relative density of the IL-2, interferon- γ , and IL-2R signals was calculated by relating the densitometric reading of the cytokine/cytokine receptor signal to the β -actin signal. Relative levels of IL-2, interferon- γ , and IL-2R mRNA in the three patients are presented as percentages of the levels found in the healthy control subject examined in parallel. The numbers 1 to 4 correspond to the respective lanes in Fig. 4.

6). In contrast, levels of IL-2R mRNA determined in parallel were only slightly reduced. Furthermore, stimulation of CVID T cells with anti-CD3 mAbs (OKT3) resulted in normal activation of the IL-2 gene in all three patients and of the interferon- γ gene in two patients, whereas in one patient (MM) interferon- γ mRNA was reduced on anti-CD3 activation of T cells as well (Fig. 6).

IgG, IgA, and IgM production by B cells from patients and control subjects after T-cell-dependent and T-cell-independent B-cell stimulation

After stimulation with pokeweed mitogen, a T-cell-dependent B-cell stimulus, MNCs from all three patients showed adequate mitogenic responses as determined by proliferation, but production of IgG and IgA by their B cells was almost absent (IgG, patients: 0.15 ± 0.08 μ g/ml,

control subjects: 47.55 ± 10.01 μ g/ml; IgA, patients: 0.57 ± 0.16 μ g/ml, control subjects: 25.41 ± 12.34 μ g/ml). In contrast, patients' B cells were capable of secreting IgM, but the amounts observed were reduced in comparison with those of control subjects (patients: 2.56 ± 0.38 μ g/ml, control subjects: 25.56 ± 8.98 μ g/ml). When a T-cell-independent stimulus such as *S. aureus* Cowan I or a combination of anti-IgM and IL-2 was used, patients' B cells could be activated to proliferate and produce IgM but not IgG and IgA. With the same stimuli, control subjects' B cells secrete IgM, IgG, and IgA. Addition of allogeneic T cells to patients' B-cell cultures driven by pokeweed mitogen, *S. aureus* Cowan I, or IgM + IL-2 led to a two- to three-fold increase in IgM production but did not affect IgG or IgA production (data not shown).

DISCUSSION

IL-2 is an important growth factor involved in T-cell activation. A complete defect in IL-2 gene expression leads to the clinical manifestation of severe combined immunodeficiency. In two patients with severe combined immunodeficiency who were described recently,^{32, 33} the defect in IL-2 gene expression was due to absence of a transacting regulatory factor involved in the initiation of transcription of the affected lymphokine gene. Defects in IL-2 gene expression and/or IL-2 secretion have also been described in patients with CVID. In these patients, however, lymphocytes had the basic potential for IL-2 transcription but failed to express the IL-2 mRNA when activated via certain pathways, resulting in impairment of the dialogue between T cells and B cells in these syndromes.^{9-12, 34-36} Kruger et al.³⁷ demonstrated defects in response to mitogens such as phytohemagglutinin and OKT3, as expressed by reduced IL-2 production in some patients. In their hands, the response could be normalized by addition of exogenous IL-2, whereas other authors³⁸ did not succeed in reconstituting the depressed response by adding exogenous cytokines. This indicates that patients with CVID are heterogeneous in this context. Pastorelli et al.¹² described a subgroup of patients with CVID whose lymphocytes reacted to mitogen with adequate proliferation but produced reduced levels of IL-2, IL-4, and interferon- γ protein. Yet another subgroup has been described by North et al.¹¹ These authors recognized that the patients whose lymphocytes were poorly responsive to phytohemagglutinin comprised two distinct entities: one group whose T cells responded normally when stimulated via the antigen receptor-independent pathway with PMA + ionomycin and a second group in whom this response was impaired as well. Sneller et al.¹⁰ studied patients who were likewise unable to mount an adequate response to mitogenic stimulation. They demonstrated that the defect in mitogen-induced T-cell activation was due to an impairment in the course of lymphokine gene activation in these patients. This resulted in decreased expression of IL-2, IL-4, and interferon- γ mRNA, whereas IL-2R gene expression was normal.

In the present study we describe three patients with CVID whose lymphocytes could be stimulated by mitogens but were incapable of responding to antigenic stimulation. When activated with antigens such as tetanus toxoid, TBE, and PPD, the patients' T cells failed to proliferate, although

the patients had previously been vaccinated. The response to the ubiquitous antigen *E. coli* was also impaired.¹⁴ The defect after activation by antigens could not be attributed to a shift in the peak of the T-cell response. Time kinetic studies showed that the antigen-induced response was decreased during culture periods up to day 21. Transwell experiments indicated that the release of soluble suppressor factors was not responsible for the deficiency seen in patients' antigen-driven cultures (data not shown). A defect in the patients' antigen-presenting cells seems unlikely, because these cells were capable of presenting the antigen tetanus toxoid to tetanus toxoid-specific cell lines of histoidential or major histocompatibility complex class II-compatible subjects (manuscript in preparation).

Impairment of cytokine gene expression was limited to activation by antigen. Although IL-2 gene expression in patients' antigen-driven cultures was significantly reduced or absent, expression of IL-2R mRNA was comparable in patients and control subjects. Anti-CD3 mAb stimulation, however, led to IL-2 mRNA levels comparable to those of control subjects. In one of the patients, however, no interferon- γ mRNA was detectable after anti-CD3 mAb stimulation. Because lymphocytes of this patient produce IL-2 in response to anti-CD3 mAbs, a lack of this cytokine could not be the reason for impaired interferon- γ expression.³⁹ The results observed in these experiments were comparable to data reported by Lewis et al.⁴⁰ on cord blood lymphocytes. When stimulated with anti-CD3 mAbs, T lymphocytes isolated from cord blood expressed IL-2 and IL-2R mRNA but no interferon- γ mRNA. Unlike cord blood cells, however, our patients' T cells had been primed in vivo, as indicated by surface expression of putative markers for memory T cells (CD29 and CD45RO expressed on CD4-positive lymphocytes). The presence of memory markers on the patients' CD4-positive lymphocytes made it unlikely that impaired recognition of antigen by the T-cell receptor was responsible for the defect observed. This was also demonstrated by experiments in which exogenous rIL-2 was added to tetanus toxoid-driven cultures, resulting in significantly higher proliferation than was observed when rIL-2 or tetanus toxoid was used alone. This assumption is furthermore confirmed by the results obtained with cord blood cells, a truly unprimed population of lymphocytes. These cells could not be activated by antigen, and proliferation with antigen plus exogenously added rIL-2

did not differ from proliferation in response to rIL-2 alone.

T cells play an important role in inducing B cells to proliferate and produce IgM, IgG, and IgA by delivering signals mediated via both T-cell and B-cell contact and lymphokines.⁴¹ IL-2 and interferon- γ are essential for B cells to produce certain immunoglobulin isotypes *in vivo*.⁴² A defect in antibody production has been shown to be a feature in mice rendered IL-2-deficient by gene targeting.⁴³ Thus it seems likely that the impaired IL-2 and interferon- γ gene expression seen in these patients' T cells after antigenic stimulation may contribute to the pathogenesis of the immunodeficiency in these patients. However, further studies are needed to define whether the T-cell defect described in the present study is confined to a small segment of patients with CVID or can be identified in a larger subgroup of patients with this disease.

REFERENCES

- Cunningham-Rundles S, Cunningham-Rundles C, Ma DI, et al. Impaired proliferative response to B-lymphocyte activators in common variable immunodeficiency. *Scand J Immunol* 1982;15:279-86.
- Spickett GP, Webster ADB, Farrant J. Cellular abnormalities in common variable immunodeficiency. *Immunodef Rev* 1990;2:199-219.
- Saiki O, Ralph P, Cunningham-Rundles C, Good RA. Three distinct stages of B-cell defects in common varied immunodeficiency. *Proc Natl Acad Sci U S A* 1982;79:6008-12.
- Bryant A, Calver NC, Toubi E, Webster ADB, Farrant J. Classification of patients with common variable immunodeficiency by B-cell secretion of IgM and IgG in response to anti-IgM and interleukin-2. *Clin Immunol Immunopathol* 1990;56:239-48.
- Farrant J, Bryant A, Almandoz F, Spickett G, Evans SW, Webster ADB. B-cell function in acquired "common variable" hypogammaglobulinaemia: proliferative responses to lymphokines. *Clin Immunol Immunopathol* 1989;51:196-204.
- Eibl MM, Mannhalter JW, Zlabinger G, et al. Defective macrophage function in a patient with common variable immunodeficiency. *N Engl J Med* 1982;307:803-6.
- Eibl MM, Mannhalter JW, Zielinski CC, Ahmad R. Defective macrophage-T-cell interaction in common varied immunodeficiency. *Clin Immunol Immunopathol* 1982;22:316-22.
- Rozynska KE, Spickett GP, Millrain M, et al. Accessory and T-cell defects in acquired and inherited hypogammaglobulinaemia. *Clin Exp Immunol* 1989;79:1-6.
- Waldmann TA, Broder S, Blaese RM, Durm M, Blackman M, Strober W. Role of suppressor T-cells in pathogenesis of common variable hypogammaglobulinaemia. *Lancet* 1974;2:609-13.
- Sneller MC, Strober W. Abnormalities of lymphokine gene expression in patients with common variable immunodeficiency. *J Immunol* 1990;144:3762-9.
- North ME, Webster ADB, Farrant J. Role of interleukin-2 and interleukin-6 in the mitogen responsiveness of T-cells from patients with 'common variable' hypogammaglobulinaemia. *Clin Exp Immunol* 1990;81:412-6.
- Pastorelli G, Roncarolo MG, Touraine JL, Peronne G, Tovo PA, De Vries JE. Peripheral blood lymphocytes of patients with common variable immunodeficiency (CVI) produce reduced levels of interleukin-4, interleukin-2 and interferon-gamma, but proliferate normally upon activation by mitogens. *Clin Exp Immunol* 1989;78:334-40.
- Spickett GP, Farrant J. The role of lymphokines in common variable hypogammaglobulinemia. *Immunol Today* 1989;10:192-4.
- Fischer MB, Vogel E, Wolf HM, Mannhalter JW, Eibl MM. Further studies on the impairment of antigen-induced T-cell activation in CVID patients. In: Chapel HM, Levinsky RJ, Webster ADB, eds. *Progress in Immune Deficiency III. International Congress & Symposium Series No. 173*. London: Royal Society of Medicine Services, 1991;III:67-9.
- Report of a WHO Sponsored Meeting. 1989. Primary immunodeficiency diseases. *Immunodef Rev* 1:173-205.
- Bøyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 1968;21(suppl. 97):77-89.
- Eibl M, Mannhalter JW, Ahmad R. Macrophage-lymphocyte interaction in response to a bacterial antigen (*E. coli*). *Clin Exp Immunol* 1982;47:260-8.
- Pellegrino MA, Ferrone S, Theofilopoulos AN. Isolation of human T and B lymphocytes by rosette formation with 2-aminoethylisothiuronium bromide (AET)-treated sheep red blood cells and with monkey red blood cells. *J Immunol Methods* 1976;11:273-9.
- Gillis S, Smith KA. Long-term culture of tumor-specific cytotoxic T-cells. *Nature* 1977;268:154-6.
- Gubler U, Hoffman B. A simple and very efficient method for generating cDNA libraries. *Gene* 1983;25:263-9.
- Mullis KB, Faloona FA. Specific synthesis of DNA *in vitro* via a polymerase catalysed chain reaction. *Methods Enzymol* 1987;155:335-50.
- Chirgwin JM, Przybyla AE, MacDonald J, Rutter WJ. Isolation of biological active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 1979;18:5294-9.
- Ponte P, Ng S-Y, Engel J, Gunning P, Kedes L. Evolutionary conservation in the untranslated regions of active mRNAs: DNA sequence of a human beta-actin cDNA. *Nucleic Acids Res* 1983;12:1687-91.
- Nikaido T, Shimizu A, Ishida N, et al. Molecular cloning of cDNA encoding human interleukin-2 receptor. *Nature* 1984;311:626-35.
- Gray PW, Leung DW, Pennica D, et al. Expression of human interferon cDNA *E. coli* and monkey cells. *Nature* 1982;295:503-8.
- Ehlers S, Smith KA. Differentiation of T cell lymphokine gene expression: the *in vitro* acquisition of T cell memory. *J Exp Med* 1991;173:25-36.
- Merida I, Pratt JC, Gaulton GN. Regulation of interleukin-2-dependent growth responses by glycosylphosphatidylinositol molecules. *Proc Natl Acad Sci U S A* 1990;87:9421-5.
- June CH, Ledbetter JA, Lindsten T, Thompson CB. Evidence for the involvement of three distinct signals in the induction of IL-2 gene expression in human T-lymphocytes. *J Immunol* 1989;143:153-61.
- Kumagai N, Benedict SH, Mills GB, Gelfand EW. Com-

- parison of phorbol ester/calcium ionophore and phytohemagglutinin-induced signaling in human T-lymphocytes. *J Immunol* 1988;140:37-43.
30. Hadden JW. Transmembrane signals in the activation of T-lymphocytes by mitogenic antigens. *Immunol Today* 1988;9:235-9.
31. Desai DM, Newton ME, Kadlecsek T, Weiss A. Stimulation of the phosphatidylinositol pathway can induce T-cell activation. *Nature* 1990;348:66-9.
32. Disanto JP, Keever CA, Small TN, Nichols GL, O'Reilly RJ, Flomenberg N. Absence of interleukin-2 production in a severe combined immunodeficiency disease syndrome with T-cells. *J Exp Med* 1990;171:1697-1704.
33. Weinberg K, Parkman R. Severe combined immunodeficiency due to a specific defect in the production of interleukin-2. *N Engl J Med* 1990;322:1718-23.
34. Reinherz EL, Rubinstein A, Geha RS, Strelkauskas AJ, Rosen FS, Schlossman SF. Abnormalities of immunoregulatory T-cells in disorders of immune function. *N Engl J Med* 1979;301:1018-22.
35. Lebranchu Y, Thibault G, Degenne D, Bardos P. Deficiency of CD4+CD5+ T-lymphocytes in common variable immunodeficiency. *N Engl J Med* 1990;323:276-7.
36. North ME, Spickett GP, Webster ADB, Farrant J. Defective DNA synthesis by T-cells in acquired 'common variable' hypogammaglobulinaemia on stimulation with mitogens. *Clin Exp Immunol* 1989;76:19-23.
37. Kruger G, Welte K, Ciobanu N, et al. Interleukin-2 correction of defective in vitro T-cell mitogenesis in patients with common varied immunodeficiency. *J Clin Immunol* 1984;4:295-300.
38. Lopez-Botet M, Fontan G, Garcia-Rodriguez MC, de Landazuri MO. Relationship between IL-2 synthesis and the proliferative response to PHA in different primary immunodeficiencies. *J Immunol* 1982;128:679-83.
39. Grabstein K, Dower S, Gillis S, Urdal D, Larsen A. Expression of interleukin 2, interferon-gamma, and the IL 2 receptor by human peripheral blood lymphocytes. *J Immunol* 1986;136:4503-8.
40. Lewis DB, Larsen A, Wilson CB. Reduced interferon-gamma mRNA levels in human neonates. *J Exp Med* 1986;163:1018-23.
41. Noelle RJ, Snow EC. T helper cell-dependent B cell activation. *FASEB J* 1991;5:2770-6.
42. Nakagawa T, Hirano T, Nagakawa N, Yoahizaki K, Kishimoto T. Effect of recombinant IL-2 and gamma-IFN on proliferation and differentiation of human B cells. *J Immunol* 1985;134:959-66.
43. Schorle H, Holtschke T, Hünig R, Schimpl A, Horak I. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature* 1991;352:621-4.