

Up-Regulation of CD40 Ligand and Induction of a Th2 Response in Children Immunized with Pneumococcal Polysaccharide Vaccines

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We wished to determine whether pneumococcal polysaccharide antigens induce mRNA expression of CD40 ligand (CD40L) and Th1 or Th2 cytokines in unimmunized individuals in vitro and whether immunization with the 23-valent pneumococcal polysaccharide vaccine induces changes in CD40L and cytokine mRNA expression. Children with recurrent respiratory infections were studied before and 4 to 6 weeks after receiving the pneumococcal vaccine. One patient who failed to respond to the polysaccharide vaccine subsequently received a single dose of the experimental 7-valent pneumococcal conjugate vaccine. Unimmunized healthy adults were included as controls. Quantification of mRNA expression of CD40L, interleukin-4 (IL-4), IL-12p40, and gamma interferon (IFN- γ) was performed by reverse transcription-PCR and enzyme-linked immunosorbent assay (ELISA)-PCR with resting and stimulated peripheral blood mononuclear cells. Serum immunoglobulin G (IgG) anti pneumococcal antibody levels were measured by ELISA. The results showed a significant increase in the expression of mRNAs for CD40L and IL-4, but not IL-12p40 or IFN- γ , in stimulated cultures from unimmunized individuals. CD40L and IL-4 mRNA expression was significantly higher in postimmunization than in preimmunization samples stimulated with the individual pneumococcal serotypes. These results suggest that pneumococcal polysaccharide antigens specifically up-regulate CD40L expression and induce a Th2 response in vitro which parallels the increase in IgG antipneumococcal antibody levels in serum.

The immune response to vaccines, including polysaccharide vaccines, is evaluated by measuring the production of antibodies against specific vaccine antigens in vivo. Polysaccharides are thymus-independent (TI) antigens, which, like thymus-dependent (TD) antigens, induce immunoglobulin secretion and immunoglobulin class switching. However, the induction and mechanisms regulating the response to the polysaccharides appear to be different. Antibody responses to TD antigens require antigen-specific T-cell help, while TI antigens are known for their ability to stimulate antibody production in T-cell-depleted mice in vivo and in T-cell-depleted cultures in vitro (3). TI antigens have been subdivided into type 1 (TI-1) antigens, which have no T-cell interaction, and type 2 (TI-2) antigens, which have some interaction with T cells. Recent studies have shown that pneumococcal polysaccharides are TI-2 antigens, which stimulate T-cell help in regulating antibody production (11, 14, 20, 21, 31). However, neither the way in which such stimulation occurs nor the regulatory mechanism of antibody production is well understood. Regulatory molecules, such as CD40 ligand (CD40L) and cytokines, may play an important role in the antibody response to pneumococcal polysaccharides.

CD40L is essential for the antibody response to TD antigens by inducing B-cell proliferation and isotype switching through the interaction with CD40 expressed on B cells (6, 7, 18, 19).

The role of CD40L in the immune response to TI-2 antigens is less clearly understood despite the observation that TI-2 antigens induce CD40L expression in vivo (32).

Cytokines secreted by T helper (Th) cells play a critical role in antibody-mediated immune responses. The Th1 subset produces interleukin-2 (IL-2) and gamma interferon (IFN γ), which promote delayed-type hypersensitivity, whereas the Th2 subset produces IL-4, IL-5, IL-10, and IL-13, which shift the immune response to immunoglobulin G (IgG) and IgE antibody production (16, 17, 22). The differentiation of naive T cells to either the Th1 or Th2 subset is regulated by cytokines present at the time of antigenic stimulation (12, 25). IL-12, a p70 heterodimer composed of 35- and 40-kDa subunits, is a regulatory cytokine produced by macrophages and B cells which stimulate Th1 differentiation in vitro and in vivo (4, 12).

Recent evidence indicates that TI-2 antigens are capable of inducing regulatory cytokines in the spleens of mice immunized with trinitrophenyl-Ficoll (5, 32). The role of CD40L and cytokines in the induction of an antibody response to pneumococcal vaccines has not been extensively studied in humans.

Polysaccharide antigens are known to be poor immunogens in humans under 2 years of age. Furthermore, some patients with recurrent respiratory infections fail to respond to polysaccharide antigens at any age (8, 23, 30, 35). This lack of response can be overcome by conjugating the polysaccharide to a protein carrier. Several experimental pneumococcal conjugate vaccines have recently been developed (1, 2, 9, 27). We have previously shown that the CRM197-heptavalent pneumococcal conjugate vaccine induced an IgG antibody response in pa-

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tients with recurrent infections who had failed to mount an adequate response to the polysaccharide vaccine (28).

In the present study, we wished to determine whether pneumococcal polysaccharide antigens are able to induce mRNA expression of CD40L and Th1 or Th2 cytokines in unimmunized individuals in vitro and whether immunization with the 23-valent polysaccharide vaccine (23-PV) or the conjugate vaccine induces changes in CD40L and cytokine mRNA expression 4 weeks after immunization.

MATERIALS AND METHODS

Study population. We evaluated 15 patients (2 to 13 years of age) referred to our pediatric allergy-immunology clinic for evaluation of recurrent respiratory infections. None of these patients had immunoglobulin, IgG subclass, or other known primary or secondary immunodeficiencies. They had all received routine immunizations, including DPT, required for their ages (by history). As part of their evaluation, patients received one dose of a 23-valent pneumococcal polysaccharide vaccine (Pnu-Immune; Wyeth-Lederle, Pearl River, N.Y.). One patient who failed to respond to 23-PV received a single dose of an experimental 7-valent pneumococcal conjugate vaccine within 6 months of immunization with 23-PV after parental consent (28). Four unimmunized healthy adults were also included as controls. Serum concentrations of antipneumococcal polysaccharide antibodies and mRNAs for CD40L, IL-4, IL-12p40, and IFN- γ extracted from in vitro-stimulated peripheral blood mononuclear cells (PBMC) were measured before and 4 to 6 weeks after pneumococcal vaccination for patients and 4 weeks apart for the unimmunized controls.

Experimental heptavalent pneumococcal conjugate vaccine. The experimental pneumococcal conjugate vaccine (supplied by Wyeth-Lederle) contains polysaccharide conjugates of serotypes 4, 6B, 9V, 14, 19F, and 23F and an oligosaccharide conjugate of serotype 18C produced by reactive amination. The carrier protein for all conjugates is CRM197, a nontoxic variant of diphtheria toxin. This vaccine has recently been approved for use by the Food and Drug Administration (FDA).

Cell preparations and cultures. PBMC from 2 ml of blood were isolated by Ficoll-Hypaque (density, 1.077g/cm³; Sigma, St. Louis, Mo.) gradient centrifugation. After an overnight incubation in RPMI 1640 at 37°C in humidified air containing 5% CO₂, PBMC (5×10^5) were cultured for 5 h in the absence or presence of concanavalin A (ConA) (20 mg/ml; Miles Scientific, Naperville Ill.), tetanus toxoid (TT) adsorbed USP (1:500; Connaught Laboratories Inc., Swiftwater, Pa.), 23-PV (1:500; Pnu-Immune, Lederle-Praxis Biologicals), and pneumococcal polysaccharide serotype 3, 14, or 18C (20 μ g/ml; American Type Culture Collection, Manassas, Va.). Preliminary kinetic studies revealed that optimal mRNA expression of CD40L, IL-4, IL-12p40, and IFN- γ was observed at 4 to 6 after stimulation. For comparison purposes, equal numbers of cells were used for both pre- and postimmunization samples; all cultures were incubated for 5 h. After incubation, cells were washed twice with ice-cold phosphate-buffered saline and mRNA was extracted.

mRNA capture. mRNA was extracted from stimulated cells using the commercial mRNA Capture Kit (Roche, Indianapolis, Ind.) according to the manufacturer's instructions. Briefly, the cell pellet was lysed with 200 μ l of cell lysis buffer; DNA was sheared mechanically by passing the lysate through a 21-gauge needle six times. Four microliters of biotin-labeled oligo(dT)₂₀ (1:20 dilution from stock solution) was added to the cell lysate and incubated at 37°C for 5 min. After hybridization of the mRNA with biotin-oligo(dT)₂₀, 50 μ l of the solution was added to streptavidin-coated PCR tubes and incubated at 37°C for 3 min. The tubes were then washed three times with 250 μ l of washing buffer.

RT-PCR. Captured mRNA was used as substrate for the synthesis of cDNA by reverse transcription (RT). The RT reaction was performed using avian myeloblastis virus reverse transcriptase (Roche). The reaction was performed at 42°C for 120 min. The cDNA was then amplified using commercially available sequence specific primers for IL-4 (456 bp), IL-12p40 (373 bp), IFN- γ (501 bp), CD40L (293 bp), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (600 bp) (Stratagene, La Jolla, Calif.) by PCR. Direct incorporation of digoxigenin (DIG)-labeled nucleotides in the PCR mixture containing 0.2 mM dATP, dCTP, dGTP, 0.19 mM dTTP, and 0.01 mM DIG 11-dUTP (Roche) was used to measure the PCR product by enzyme-linked immunosorbent assay (ELISA). Cycling parameters were as follows: a 5-min denaturation at 95°C and 5-min annealing at 60°C, followed by the appropriate number of cycles of 1 min at 72°C, 45 s at 95°C, 45 s at 60°C, and a final 7 min at 72°C (Twin Block System, Ericomp, San Diego, Calif.). All PCR products were analyzed in preliminary studies within

the linear range of amplification as follows: IL-4, 34 cycles; IL-12p40, 30 cycles; CD40L, 26 cycles; IFN- γ , 22 cycles, and GAPDH, 18 cycles. In all cases, the resultant PCR products of 600 bp (GAPDH), 501 bp (IFN- γ), 456 bp (IL-4), 373 bp (IL-12p40), and 293 bp (CD40L) were visualized by ethidium bromide staining on 2% agarose gels under UV light.

Quantification of PCR amplicons by capillary electrophoresis. Capillary electrophoresis using the Gold-P/ACE System 5000 with laser-induced fluorescence detection (Beckman, Fullerton, Calif.) was performed as previously described (33). To quantify mRNA, the amount of each different amplicon in each PCR sample was measured by integrating the fluorescence peak areas.

Quantification of PCR amplicons by ELISA-PCR. A commercial ELISA-PCR system (Roche) was used for the semiquantitative detection of DIG-labeled PCR products by a hybridization-based microtiter plate assay. Wells of a streptavidin-coated microtiter plate were filled with 10 μ l of PCR product, followed by denaturation with 25 μ l of 0.2 N NaOH for 5 min and hybridization with 2 pmol of the respective 3'-biotinylated capture probe (Integrated DNA Technologies, Coralville, Iowa). The capture probes were specific for the following amplified target sequences: 5'-GAC AAC TTT GGT ATC GTG GAA GGA-3' for GAPDH, 5'-GGC ATT TTT AAG AAT TGG AAA GAG GAG-3' for IFN- γ , 5'-TGC GTT CAG GTC CAG GGC AAG AGC-3' for IL-12p40, 5'-TTC ACA GGA CAG GAA TTC AAG CCC-3' for IL-4, and 5'-AAT CCA TTC ACT TGG GAG GAG-3' for CD40L. Capture was allowed to proceed for 3 h at 45°C. Afterwards, the wells were washed three times with washing solution (Roche). To each well was added 200 μ l of anti-DIG-peroxidase (10 U/ml; Roche) diluted 1:1,000 in a buffer containing 100 mM Tris-HCl and 150 mM NaCl (pH 7.5). The plates were incubated at 37°C for 30 min and then washed as before. The reaction was visualized by adding 200 μ l of ABTS [2,2'-azinobis(3-ethylbenzthiazinesulfonic acid)] substrate solution (Roche) to each well and incubating the plates at 37°C for 30 min in the dark. The optical density (OD) was read at 405 nm with a reference at 490 nm using a Bio-Rad (Hercules, Calif.) reader. The run was considered valid if all negative control values were less than 0.2 OD unit and the positive control value was greater than 1.0 OD unit. ODs were converted into logs of copy numbers of initial cDNAs in the patient samples. Quantification of PCR products was performed by comparison to a standard curve. The standard curve for each amplicon was created using serial dilutions of known concentrations (logs of copy numbers of initial cDNAs) of plasmids coding for GAPDH, IFN- γ , IL-12p40, IL-4, or CD40L (American Type Culture Collection). Plasmids were isolated, serially diluted, PCR amplified, and detected as described above. OD units for patient samples were then converted to copy numbers based on their respective standard curves.

ELISA for antipneumococcal IgG. IgG antipneumococcal antibody levels against serotypes 1, 3, 4, 6B, 9V, 14, 18C, 19F, and 23F were determined by an ELISA protocol calibrated against the FDA 89-SF reference sample (CBER; FDA, Washington, D.C.) as previously described (29).

Statistical analysis. Comparisons between pre- and postimmunization paired data were performed using the Wilcoxon test (one tailed). A *P* value of <0.05 was considered significant.

RESULTS

The geometric means of IgG antibody concentrations specific to pneumococcal polysaccharide serotypes 3, 14, and 18C before and 4 to 6 weeks after immunization with 23-PV are shown in Table 1. Significant differences in antibody concentrations between pre- and postimmunization samples were observed for each serotype studied. One patient, who failed to respond to the polysaccharide vaccine, showed an adequate response, according to published criteria (28), 4 weeks after immunization with an experimental heptavalent pneumococcal conjugate vaccine (data not shown). Nonimmunized controls showed no increase in antibody concentrations from one sample to the other (data not shown).

A representative agarose gel with pre- and postimmunization samples from a patient who had a significant antibody response to 23-PV shows the ethidium bromide-stained PCR products from both unstimulated cells and cells stimulated (Fig. 1). GAPDH mRNA, the product of a housekeeping gene used as control, can be visualized as a 600-bp band showing the same level of intensity in every lane. In contrast, no CD40L or

TABLE 1. Geometric mean concentrations of IgG antibodies against pneumococcal polysaccharides in 14 children immunized with the 23-PV

Serotype	Geometric mean concn (range) ^a of IgG antibodies:		P
	Preimmunization	Postimmunization	
3	0.35 (0.15–0.86)	1.25 (0.41–5.01)	0.001
14	0.46 (0.20–1.28)	1.24 (0.11–8.44)	0.04
18C	0.26 (0.05–1.10)	0.90 (0.11–4.03)	0.006

^a In micrograms per milliliter.

cytokine message was detected in unstimulated cultures. In pre- and postimmunization samples, ConA stimulated detectable mRNA expression of CD40L, IFN- γ , IL-4, and IL-12p40, while TT induced some mRNA expression of IFN- γ but weak expression of IL-4 and IL-12p40. All pneumococcal antigens induced detectable IL-4 mRNA levels in postimmunization samples and CD40L mRNA levels in pre- and, even more intensively, postimmunization samples. These initial observations led us to use more sensitive methods to quantify the PCR products in order to test the possibility that pneumococcal immunization may up-regulate CD40L and IL-4 expression.

The initial PCR results visualized by ethidium bromide staining for one patient were confirmed in the entire study population. The amount of PCR product in each sample was initially measured by two different methods: capillary electrophoresis and ELISA-PCR. Both methods are semiquantitative and generated comparable results. Here we present results obtained with ELISA-PCR.

Unstimulated cells from pre- and postimmunization samples produced small amounts of CD40L mRNA expression. In ConA- and antigen-stimulated cultures, CD40L mRNA expression increased over that of unstimulated cultures in both pre- and postimmunization samples. The intensity of CD40L message induced by the individual polysaccharide serotypes 3, 14, and 18C was significantly higher ($P < 0.05$) in post- than in preimmunization samples (Fig. 2A). The response to 23-PV was also increased, but the difference did not reach statistical significance.

Analysis of the cytokine profiles induced in vitro in these patients shows differences in the intensity of mRNA expression for different stimuli. ConA induced higher expression of IL-4, IFN- γ , and IL-12p40 mRNAs in pre- and postimmunization samples in comparison to unstimulated cells (Fig. 2B to D). Similarly, the intensity of mRNA expression induced by TT was significantly higher than that of unstimulated cells for IL-4 and IL-12p40. There was no change in IFN- γ mRNA from pre- to postimmunization in unstimulated and stimulated samples (Fig. 2C). Pneumococcal polysaccharide antigens induced higher mRNA expression of IL-4, but not IFN- γ or IL-12p40, mRNA compared to unstimulated cultures. The differences in intensity of IL-4 mRNA expression between pre- and postimmunization samples were significant ($P < 0.05$) in cells stimulated with the individual serotypes 3, 14, and 18C (Fig. 2B). The response to 23-PV was also increased, but the difference did not reach statistical significance. mRNAs for CD40L and cytokines did not change in the control group (data not shown).

The individual responses shown in Fig. 3 and 4 demonstrate that cells stimulated with pneumococcal serotypes 3, 14, and 18C had significantly increased ($P < 0.05$) CD40L and IL-4 mRNA expression in post-compared to preimmunization samples. These increases were present in all of the patients studied. In contrast, no significant changes in the level of CD40L or IL-4 mRNA expression were observed in the control group.

The intensity of mRNA expression of CD40L and cytokines was studied in preimmunization, post-23-PV, and postconjugate samples from a patient who received the pneumococcal conjugate vaccine after failing to respond to 23-PV. Higher levels of mRNA expression of CD40L and IL-4 were observed in ConA- and TT-stimulated cultures from postconjugate samples in comparison to pre- or post-23-PV samples (Fig. 5, top panel). Cultures stimulated with 23-PV or with the individual serotypes showed a marked increase in mRNA expression of CD40L and, more significantly, of IL-4 in postconjugate samples in comparison to post-23-PV or preimmunization samples (Fig. 5, bottom panel). The conjugate vaccine did not increase IL-12p40 or IFN- γ mRNA (data not shown).

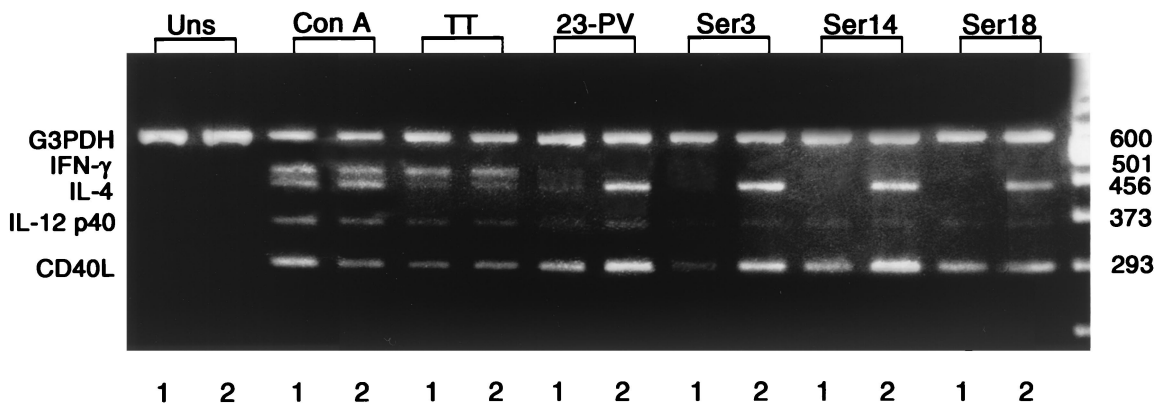


FIG. 1. Agarose gel electrophoresis of RT-PCR products. Lanes show RT-PCR products from preimmunization (lanes 1) and postimmunization (lanes 2) samples unstimulated (Uns) or stimulated for 5 h with the optimal concentration of ConA, TT, 23-PV, or serotype (Ser) 3, 14, or 18C. The sizes of the RT-PCR products were compared to those of DNA fragments of the markers.

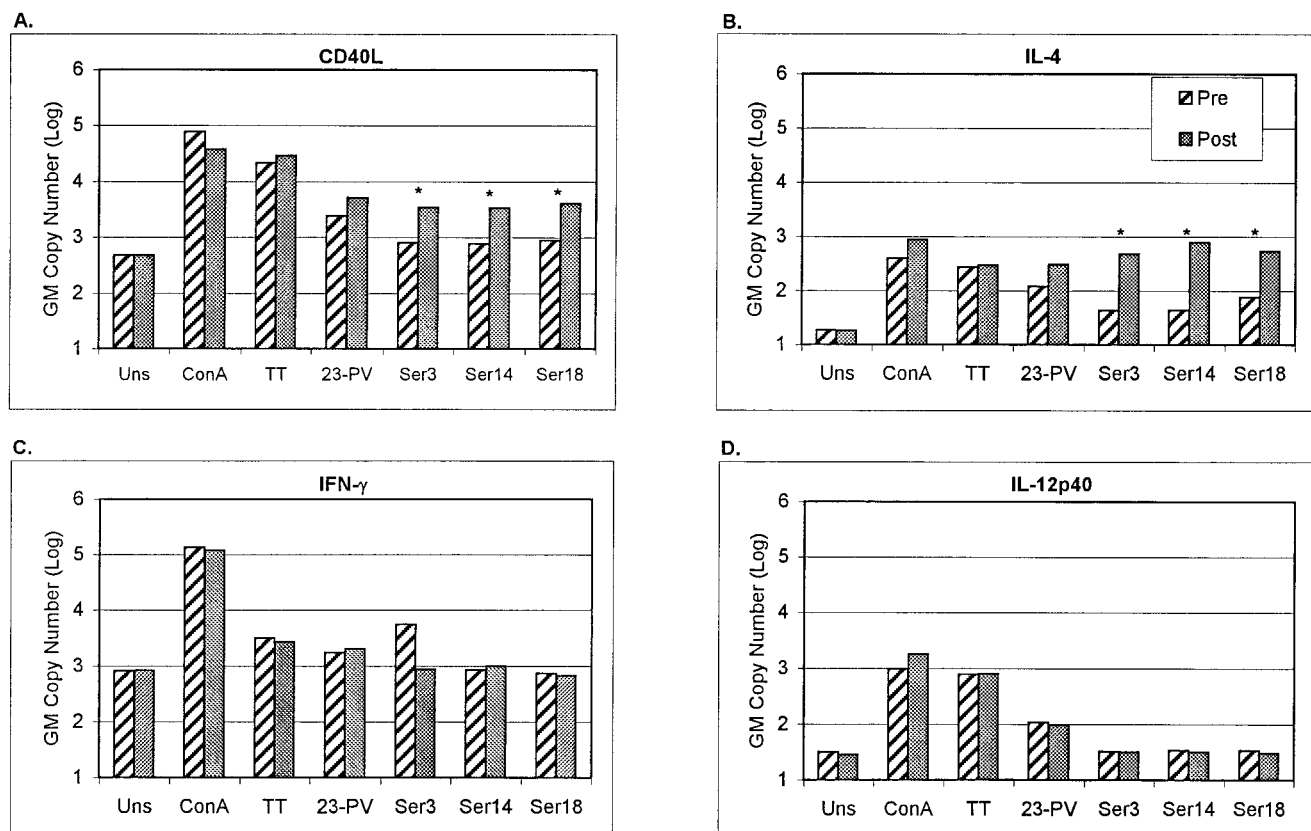


FIG. 2. mRNA expression of CD40L and cytokines in PBMC before and 4 to 6 weeks after immunization with 23-PV in unstimulated cells (Uns) or in cells stimulated for 5 h with the optimal concentration of ConA, TT, 23-PV, or serotype (Ser) 3, 14, or 18C. RT-PCR products were quantified by ELISA-PCR as described in Materials and Methods. Results are expressed as the geometric mean (GM) of the logs of copy numbers in pre- and post-immunization samples (*, $P < 0.05$).

DISCUSSION

Our observation that CD40L mRNA expression in PBMC is increased in vitro by polysaccharide antigens is consistent with the observation that TI-2 antigens induce CD40L expression in vivo (32). CD40L is expressed on activated CD4⁺ T cells, monocytes, B cells, and NK cells (7). The small blood sample that we could obtain from children precluded separation and identification of different cell types or measurement of cytokine secretion in our study. However, since T cells are the largest proportion of cells in the PBMC preparations used in our studies, our results are suggestive of T-cell activation by polysaccharide antigens. This is also supported by the observation of a significant increase in IL-4 mRNA in response to stimulation with pneumococcal polysaccharide antigens. On the other hand, the lack of IL-12 stimulation by pneumococcal polysaccharides suggests that monocytes, another possible source of CD40L, are not major contributors to the observed increase in CD40L mRNA (4, 12).

A role for NK cells in the increase of CD40L and IL-4 mRNAs observed in our experiments cannot be ruled out. Several reports have suggested that induction of an antibody response to TI-2 antigens does not require the participation of T cells but requires cytokines from cells other than T cells, such as NK cells (13, 14, 26, 34). On the other hand, our hypothesis that T cells do respond to pneumococcal polysaccharides is in

agreement with numerous studies that have questioned the T-cell independence of the response to TI-2 antigens (10, 13–15, 21, 31). Studies using flow cytometric methods to identify the cells expressing cytokines in cultures stimulated with pneumococcal polysaccharide antigens are in progress in our laboratory.

The increase in IL-4 mRNA suggests that pneumococcal polysaccharides induce a Th2 response, characterized by increased secretion of IL-4, which shifts the immune response to IgG and IgE antibody production (16, 17, 22). Our results are in agreement with the observation of an IL-4 regulation of in vivo antibody production in response to TI-2 antigens in experimental animals (11). Th1 cells do not appear to be involved in the response to pneumococcal polysaccharides, since neither IL-12 nor IFN- γ mRNA increased in our experiments. An increase in these cytokine messages is observed in Th1 responses, where IL-12 promotes the differentiation of naive T cells to Th1 cells and activation of these cells results in IFN- γ production (12, 25).

Increased CD40L and IL-4 mRNAs in samples obtained 4 weeks after immunization confirm the same observations made prior to immunization. These results suggest that pneumococcal polysaccharide immunization may produce an expansion of memory T cells that persists for several weeks after immunization. The specificity of this response to pneumococcal anti-

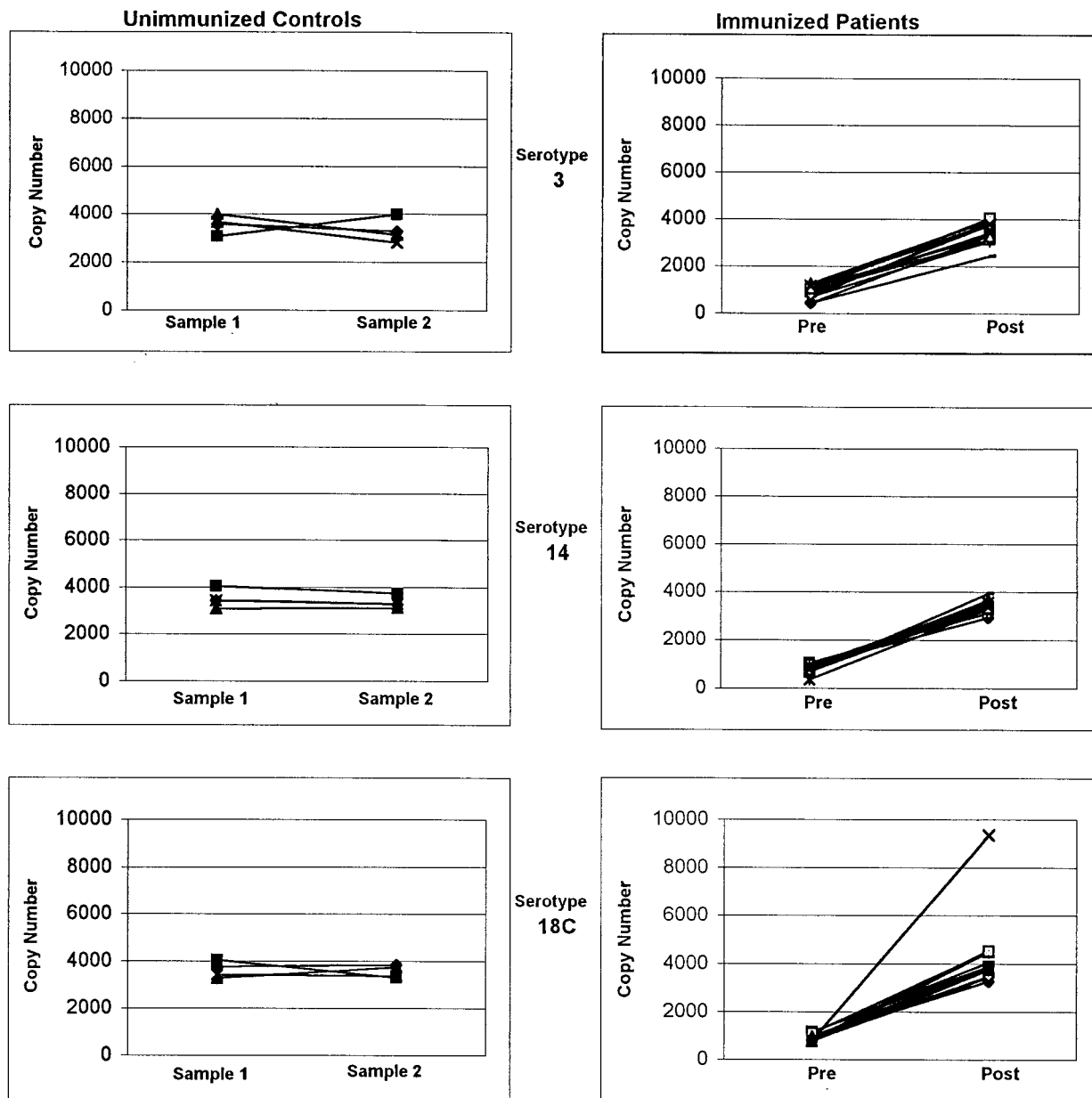


FIG. 3. mRNA expression of CD40L in PBMC cultures from 14 patients before and 4 to 6 weeks after immunization with 23-PV compared to mRNA expression in PBMC cultures from four unimmunized controls. Several patient values are very similar, so some lines are superimposed. Cells were stimulated for 5 h with the optimal concentration of pneumococcal polysaccharide serotype 3, 14 or 18C. ELISA-PCR results are expressed as copy numbers.

gens was supported by the contrasting results observed in cultures stimulated with ConA, a nonspecific stimulator, or with TT, a recall protein antigen, which induced a similar increase in CD40L and IL-4 mRNAs in pre- and postimmunization samples. These observation suggest that the in vitro response to TT was a secondary response in patients, who had all been immunized with vaccines containing TT in the past. The lack of significant differences in CD40L or IL-4 mRNA expression between two separate samples in the unimmunized control group further supports the idea that observed differences in

pre- and postimmunization samples were due to an immune response to pneumococcal polysaccharides in vivo.

Our observation for one patient who failed to mount an adequate antibody response to the pneumococcal polysaccharide vaccine is of interest. This patient showed a significant increase in antibodies to serotypes 3, 14, and 18C after vaccination with the CRM197 pneumococcal conjugate vaccine (28). Immunization with this conjugate vaccine induced not only greater expression of CD40L and IL-4 mRNAs in response to specific stimulation with pneumococcal polysaccha-

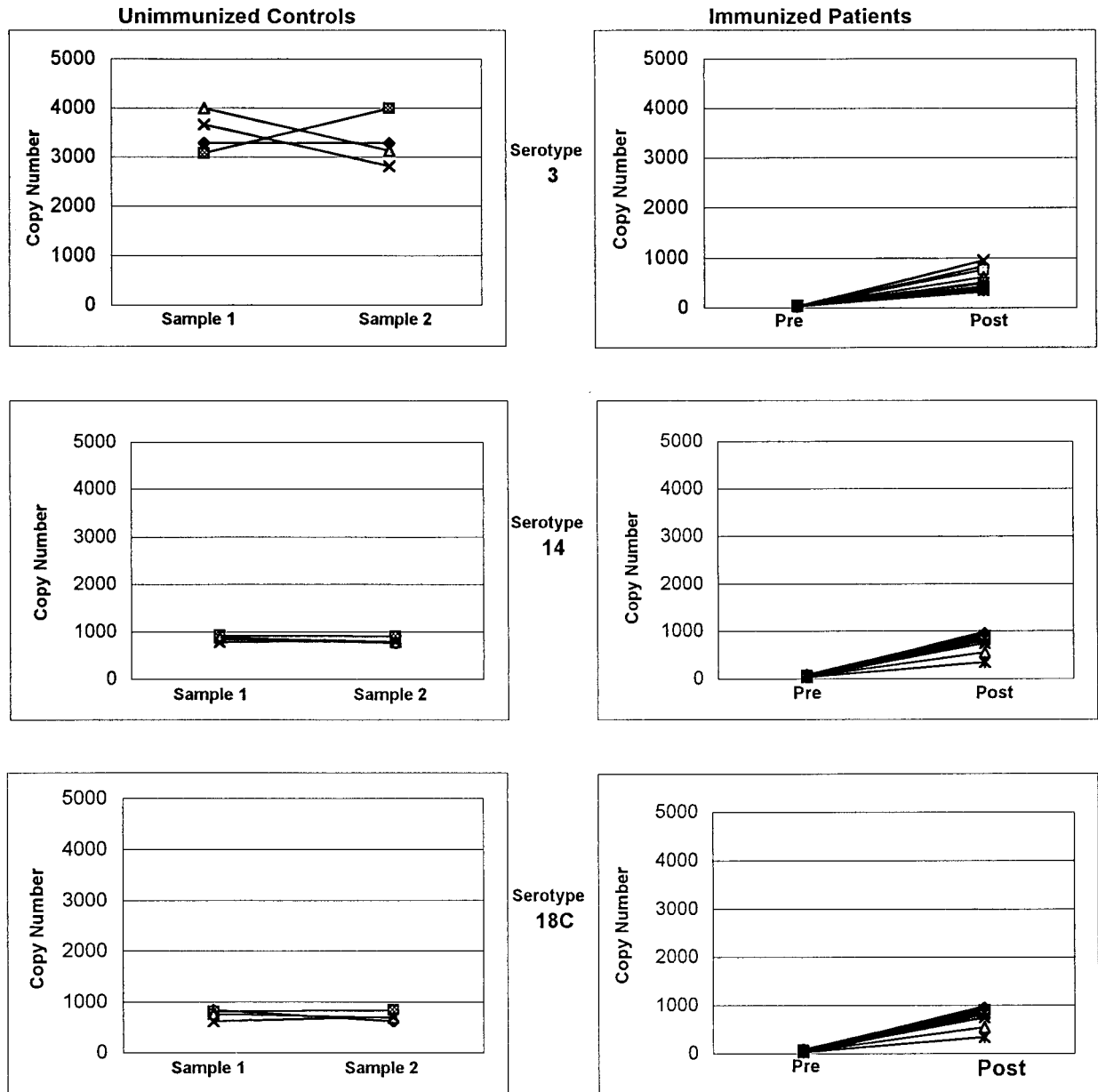


FIG. 4. mRNA expression of IL-4 in PBMC cultures from 14 patients before and 4 to 6 weeks after immunization with 23-PV compared to mRNA expression in PBMC cultures from 4 unimmunized controls. Several patient values are very similar, so some lines are superimposed. Cells were stimulated for 5 h with the optimal concentration of pneumococcal polysaccharide serotype 3, 14, or 18C. ELISA-PCR results are expressed as copy numbers.

ride antigens but also an increase in mRNA expression in response to ConA and TT. If similar observations can be confirmed for a larger number of individuals, it may reflect the adjuvant effect of the carrier protein in the conjugate vaccine.

The immune response to allergens has been shown to be a Th2 response, and allergen immunotherapy is thought to shift Th2- type to Th1-type responses (24). Now that the pneumococcal conjugate vaccine is available and has been recommended for generalized use in infants at 2, 4, 6, and 12 to 15 months of age, it will be important to study the effect of this

vaccine on the Th1-Th2 balance in large numbers of individuals.

In summary, this study shows that pneumococcal polysaccharide antigens induce the expression of CD40L and a Th2 response and that these responses are up-regulated by immunization with pneumococcal vaccines paralleling the increase in circulating IgG antipneumococcal antibody concentrations. Further studies aimed at identifying the cells regulating the response to pneumococcal polysaccharide antigens and investigating the role of pneumococcal immunization in the Th1-Th2 balance should expand the observations reported here.

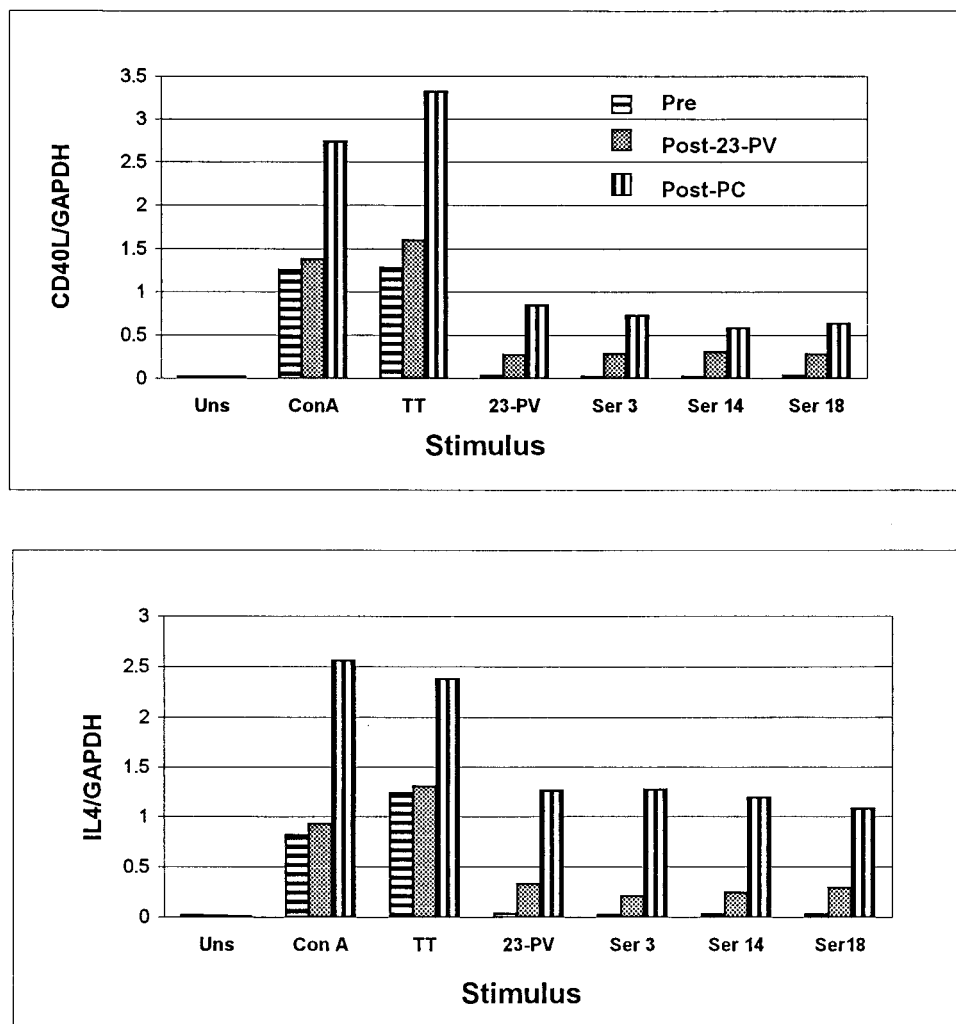


FIG. 5. Relative quantities of CD40L and IL-4 mRNA in PBMC from one patient before and 4 to 6 weeks after immunization with 23-PV and 4 weeks after a second immunization with the 7-valent pneumococcal conjugate vaccine (PC). mRNA was quantified by RT-PCR and capillary electrophoresis. Results are expressed as the ratio of the peak area of subunit mRNA to that of the mRNA of the housekeeping gene for GAPDH. Ser, serotype.

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