Polarized Type 1 Cytokine Profile in Bronchoalveolar Lavage T Cells of Patients with Hypersensitivity Pneumonitis

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Hypersensitivity pneumonitis (HP) is a lung disease caused by sensitization to and repeated inhalation of various organic Ags. It is characterized by lymphocytosis as detected in the BAL fluid, inflammatory lymphocytic alveolitis, and loosely formed granuloma in the lung (1–5). Repeated exposure leads to a progression of inflammation and finally to end-stage lung disease with severe fibrosis. Several studies have indicated that immune complex-mediated immunity and cell-mediated immunity are sequentially involved in the pathogenesis of HP (6–8). Studies using animal models suggest that the latter process is more important (9–15); however, the precise mechanism for the pathogenesis of HP has still not been elucidated.

As mentioned above, one of the most striking pathological features of HP is a remarkable lymphocytosis in the BAL fluid, which is usually comprised predominantly of CD8+ T cells, and therefore, the CD4/CD8 ratio usually drops below 1.0 (16–19). However, there is an increase in absolute numbers of total T cells, not only CD8+ T cells but CD4+ T cells are also significantly higher in HP patients compared with control or other interstitial lung diseases (20, 21). These findings suggest that both CD4+ and CD8+ T cells found in the lung might contribute to the pathogenesis of HP.

CD4+ and CD8+ T cell subsets can be characterized by restricted cytokine secretion profiles (Ref. 22; reviewed in Ref. 23), whereby type 1 (e.g., CD4+ Th1) cells are defined by a preferential secretion of IFN-γ and IL-2, and type 2 (e.g., Th2) cells are defined by preferential secretion of IL-4, IL-5, and IL-10. Certain diseases are also characterized, based on the nature and proportion of cytokines present, as either Th1- or Th2-type diseases. In general, Th1 cytokines are present in cell-mediated immune responses and appear to be involved in chronic inflammatory conditions, whereas Th2 cytokines up-regulate IgE production and are prominent in the pathogenesis of allergic diseases. The in vivo cross-regulation and significance of the CD4+ Th1 and Th2 subsets in multiple disease models and clinical states (reviewed in Ref. 24) have been documented even in lung diseases such as sarcoidosis, characterized by a Th1-type cytokine-dominant profile (25), and bronchial asthma, characterized by a Th2-type (26) cytokine profile.

Recent data using animal models of HP have drawn increasing attention to the importance of Th1-type cytokines and IFN-γ in particular. Schuyler et al. (27) reported that a Th1 CD4+ cell line could adoptively transfer experimental HP in mice, and Cudmundsson et al. (28) reported, using IFN-γ knockout mice, that IFN-γ was essential for the expression of HP-like disease. While the animal models have clearly defined HP as a Th1 cell-type disease, clinical studies investigating cytokine profiles of HP patients have not been reported.

In this study we identify a predominance of IFN-γ-producing T cells obtained in the bronchoalveolar lavage fluid (BAL T), but not from peripheral blood-derived T cells (PB T), in HP patients. The potential mechanism for development of the Th1 profile in HP was investigated by addressing the endogenous generation of IL-10 and IL-12 cytokines as well as the effects of exogenous addition of IL-10 and IL-12 on IFN-γ production. The contributions of blood...
monocytes and alveolar macrophages to this process were also investigated. These studies indicate, for the first time, a polarized type 1 cytokine profile associated with the BAL T cells from HP patients and identify factors that probably contribute to development of this condition.

Materials and Methods

Hypersensitivity patients and normal controls

Six patients, all nonsmokers, were diagnosed with HP according to clinical symptoms, the positive environmental provocation test, the existence of specific Abs, characteristic manifestation in high-resolution computed graphic (HRCT) findings, the typical BAL findings, and the typical clinical course. Three patients were classified as having summer-type hypersensitivity pneumonitis induced by Trichosporon cutaneum, while the remaining three patients were diagnosed as having farmer’s lung. None of these patients was receiving steroid therapy at the time of examination.

Bronchoalveolar lavage and cell preparation

In all cases BAL was performed in the right middle lobe as previously reported (29), using three aliquots (50 ml each) of sterile physiologic saline solution. Immediately after the lavage, the lavage fluid was filtered through gauze into 50-ml conical tubes. The tubes were centrifuged at 150 × g for 5 min at 4°C, and the residual pellets were resuspended and washed three times in RPMI 1640 medium (Life Technologies, Grand Island, NY). The cells were finally suspended in RPMI 1640 supplemented with 10% FBS, 5 × 10^{-5} M 2-ME, 100 U/ml penicillin, and 100 ng/ml streptomycin. Cellular differentials were performed by cytocentrifugation and stained using a Diff Quick Stain (Hareco, Gibbstone, NJ).

To prepare the BAL T cells, they were placed in glass dishes at 37°C in 5% CO2 for 1 h. The nonadherent cells were gently collected and used as BAL T cells, and the adherent cells were collected after extensive pipetting and used as alveolar macrophages (AM). Over 95% of the nonadherent cells were CD3 positive as determined by FACScan (Becton Dickinson, Mountain View, CA), and >97% of the adherent cells were alveolar macrophages as determined by morphology and esterase staining. The total recovered BAL cells were 2.9 ± 1.2 × 10^{6} cells for HP patients compared with 0.3 ± 0.1 × 10^{6} cells obtained from healthy controls. The percent lymphocytes isolated from the BAL were 85.7 (psum) 5.2 in HP patients and 11.5 ± 3.8 for normal controls, with an average CD4/CD8 ratio of 0.72 for summer-type HP and 1.85 for farmer’s lung.

Preparation of peripheral blood T cells and monocytes

Heparinized peripheral blood was obtained from every subject before the BAL procedure. To prepare PB T cells, PBMC obtained by Ficoll-Hypaque density centrifugation were suspended in RPMI 1640 medium with 10% FCS and were cultured in a 24-well flat-bottom plate (Costar, Cambridge, MA). Each well contained 2 × 10^{5} T cells and 2 × 10^{5} AMs or monocytes in 1 ml of culture medium. The cells were stimulated with soluble OKT3 mAb (10 ng/ml) for both BAL T cells and PB T cells, in the presence or the absence of optimal doses of human rIL-10 (10 ng/ml; PharMingen, San Diego, CA), human rIL-12 (5 ng/ml; gift from M. Kobayashi, Institute of Environ. Health, Osaka University, Osaka, Japan), or IL-10 plus IL-12 for 24 or 48 h at 37°C in a humidified atmosphere containing 5% CO2. In some cases cell stimulation was also conducted using immobilized anti-CD3 and anti-CD28 Ab. For those cultures 1 µg of each Ab was added to each well for 2 h at room temperature. The wells were washed with medium before adding the cells. Results obtained using immobilized Ab was identical with those obtained from soluble Ab stimulation, and therefore only data from the soluble Ab experiments are shown. The cell culture supernatants were stored at −20°C before assessment.

Cytokine levels in the cell culture supernatants and BAL fluid

The cytokines in each cell culture supernatant and BAL fluid were measured by ELISA. Before analysis, BAL fluid was concentrated 10-fold using ultrafiltration through a Centricon-3 (m.w. cut-off, 3000 Da) membrane. ELISA kits for IL-2, IFN-γ, IL-12, IL-4, and IL-10 were purchased from R & D Systems (Minneapolis, MN) and PharMingen, respectively. The lower detection limits of the ELISA kit for each cytokine were as follows: IL-2, 0.6 pg/ml; IL-4, 10 ng/ml; IL-10, 14 pg/ml; IFN-γ, 10 pg/ml; and IL-12, 5 pg/ml.

Flow cytometric analysis of surface molecules on BAL cells and peripheral blood cells

Purified BAL T cells (1 × 10^{5}), PB T cells, monocytes, and AMs were incubated in 50 µl of staining buffer (PBS containing 2% BSA and 0.05% sodium azide) for 15 min at 4°C in the presence of 1 µg/ml normal human IgG to reduce background binding via Fc receptors expressed on the cell surface. Cells were then incubated with cell-specific mAbs-FITC: CD3, CD4, CD8, and CD14 (PharMingen) for monocytes and AM. To evaluate CD80 and CD86 Ag expression on monocytes and AM, an indirect staining method was performed using unlabeled anti-CD80 or anti-CD86 mAb (PharMingen) and FITC-conjugated goat anti-mouse IgG-specific F(ab’)2 Ab (PharMingen). Flow cytometric analysis of cell surface markers was performed using FACSscan (Becton Dickinson) with CellQuest software. A minimum of 10,000 events were collected for each sample. In all experiments, negative controls consisted of mouse IgG isotype-matched Abs.

Analysis of intracellular cytokine production by flow cytometry

Intracellular cytokines were detected by flow cytometry using a modification of the methods reported by Jung et al. (30). Briefly, for each sample, 2 × 10^{5} BAL T cells or PB T cells, in 1 ml of RPMI 1640 with 10% FBS, were stimulated with 12-O-tetradecanoylphorbol-13-acetate (TPA) (10 ng/ml) and A23187 (250 ng/ml) in the presence of brefeldin (2 µM). After 6–8 h of activation, cells were washed in PBS, and the cell surface was stained with anti-CD4 or anti-CD8-Quantum Red-conjugated Ab. Then the cells were fixed for 20 min with 2% formaldehyde, washed twice with PBS, and permeabilized by PBS supplemented with 2% FCS, 2 mM NaN3, and 0.5% saponin. In every case, incubation was conducted at room temperature. Permeabilized cells were incubated with anti-IL-4-PE mAb or anti-IL-12-PE mAb and anti-hIFN-γ-FITC mAb in PBS/FCS/Na3/saponin. After 30 min of incubation at room temperature, cells were washed twice with the same buffer, resuspended in staining buffer (PBS supplemented with 2% FCS and 0.05% NaN3), and analyzed by flow cytometry. The 6–8 h point for these studies was chosen to avoid any side effects due to long term exposure to monensin, which usually occur by 12 h. Although this time point may not represent maximal production of all cytokines, it is sufficient to determine relative production by peripheral vs lung-derived cells.

RNA analysis by RT-PCR

BAL T cells and PB T cells (2 × 10^{5}) were obtained before and after 24-h stimulation with TPA and A23187. The cells were washed, and total RNA was isolated using isopropylalcohol-PA (Promega, Madison, WI) as recommended by the manufacturer. Reverse transcriptase reactions to generate cDNA were performed using AMV reverse transcriptase (Promega). PCR were performed using 1 µg of cDNA, 0.5 µM oligonucleotide primers (each), 2 mM MgCl2, and 0.2 mM NTPs in a final reaction volume of 50 µl. Thirty amplification cycles were performed (1 min at 94°C; 1 min at 55°C; 1.5 min at 72°C). The PCR primers used in this study are listed below. Following amplification, a portion of the PCR reactions was electrophoresed on a 1% agarose gel and visualized using ethidium bromide: β-actin: 5’ primer, 5’-TCTGAAATGTGACGACATTCCGT; 3’ primer, 5’-CCCTAGAGACA TTTCGGGTGCACAGATG; IFN-γ: 5’ primer, 5’-GCTCTGCACTGTTT GGGTTCTCTTGCG; 3’ primer, 5’-CCTTCCTCCCGTCTC; IL-4: 5’ primer, 5’-CTAATGTTGTCTTTATAGCTACTTT; 3’ primer, 5’-AGCTAGTTGTAATTTTTT; IL-12: 5’ primer, 5’-GGATGTTGCTACCTCCACAACTGT; 3’ primer, 5’-TACGTCCTGAACACCTT GAAATTCTTTCTCAT; IL-10: 5’ primer, 5’-AAGCTGCGAAGCAAGA CAGACA T; 3’ primer, 5’-AGCTATCCCCAGACCCAGATCCCG; and
that there was no apparent difference in the intracellular IL-4 and of the amount of cytokine produced by individual cells indicated 5
performed. This is a representative experiment of four separate experiments performed.

Coexpression of intracellular IL-2 and IFN-γ production in BAL CD4+ T cells and PB CD4+ T cells

In addition to IFN-γ, another type 1-defining cytokine is IL-2. We next compared intracellular IL-2 production in stimulated BAL vs

Intracellular analysis of IFN-γ and IL-4 in stimulated BAL and PB T cells. Isolated BAL and PB T cells from HP patients were stimulated for 6 – 8 h with TPA and A23187 in the presence of brefeldin. The cells were then labeled with conjugated Ab to detect intracellular IFN-γ and IL-4 cytokines. A. Representative flow cytometric staining pattern comparing IFN-γ-producing cells with IL-4-producing cells. B. Intracellular cytokine data, expressed as cytokine-positive CD4+ or CD8+ T cells relative to the percentage of total T cells, obtained from four different individuals. The asterisk denotes a significant difference in the percentage of positive cells comparing BAL and PB T cells.
IFN-γ protein, classified as Th0 cells (Fig. 3B and Table I). This suggests that the predominant CD4⁺ T cell type in HP BAL cells can be classified as highly polarized effector Th1 cells.

Endogenous IL-10 and IL-12 production by OKT3-stimulated BAL cells and PBMC of HP patients

Next we investigated what factor(s) may influence the development of the observed type 1 cytokine profile of BAL T cells in HP patients. We speculated that a type 1 cytokine profile might develop as a result of less BAL T cell production of intrinsic IL-10 and/or more production of IL-12 following stimulation compared with stimulated blood-derived T cells. To determine whether this...
IL-4 production and determined that IL-4 was produced by both BAL and PB T cells comparably, and in neither case was IL-4 production affected by the addition of IL-10 (data not shown). Taken together, these results demonstrate that BAL T cells of HP patients preferentially produce IFN-γ when stimulated with sOKT3 cocultured with AM even in the presence of IL-10. Conversely, IL-10 significantly suppressed the production of IFN-γ by sOKT3-stimulated PBT cells cocultured with monocytes.

Expression of CD80 (B7-1) and CD86 (B7-2) on AM and monocytes of HP patients

The cultures used to generate the previous data contained AM and blood-derived monocytes. These accessory cells have been shown to play a role in the effect of IL-10 on IL-12 production and therefore on IFN-γ production (31). The difference in the responses of BAL and PB T cells to IL-10 might indicate that the difference resides in the accessory cells. To investigate this possibility we first determined whether there was a phenotypic difference in the expression of either B7-1 (CD80) or B7-2 (CD86), membrane proteins shown to influence T cell cytokine development (22). Alveolar macrophages and blood monocytes were isolated from HP patients and assessed for surface expression of either CD80 or CD86. Relative surface expression was determined by FACS analysis. Ag expression by AM is denoted by the open bars, and expression by blood monocytes is shown by the shaded bars. The data represent the average relative expression (±SD) for each Ag for five different individuals.

Effect of AM and monocytes on the production of IFN-γ in sOKT3-stimulated PB T cells

With no difference in the expression of B7-1 and B7-2 molecules for AMs and monocytes, we next investigated whether there was a functional difference between the two and assessed the potential of AM to influence IFN-γ production by PB T cells. In these experiments PB T cells were purified to >97% by negative selection. Plastic-adherent enriched autologous AM or peripheral blood monocytes (2 × 10⁵/well) were added to the PB T cell cultures and stimulated with sOKT3 Ab for 24 h. Both the AM and monocytes were added at a ratio of 9:1 T cells to AM to duplicate the average ratio seen in peripheral blood. The cultures received either IL-10 (10 ng/ml) or IL-10 plus IL-12 (10 ng/ml). As shown in Fig. 7, IFN-γ production by sOKT3-stimulated PBT cocultured with AMs was suppressed with IL-10 to a similar degree as PB T cells cocultured with monocytes. The presence of IL-12 was unable to reverse the IL-10-induced suppression of IFN-γ production (Fig. 7). These results suggest that in this culture system AM from HP patients are not functionally different from blood-derived monocytes obtained from the same individual, and that the lack of a response by BAL T cells to IL-10 stimulation is not due to a phenotypic difference in the AM.

FIGURE 5. Effect of IL-10 and IL-12 on IFN-γ production by OKT3-stimulated BAL and PB T cells. Soluble OKT3 Ab (10 ng/ml) was added to cell cultures containing BAL T cells with AM (9:1 ratio) or PB T cell blood monocytes (9:1 ratio) for 48 h. A, Amount of IFN-γ production as determined by ELISA quantitation. Parallel cultures also were cultured in the presence of 10 ng/ml of IL-10, IL-12, or a combination of the two. B, The relative amounts of IFN-γ produced under these culture conditions compared with those produced in cultures receiving no cytokine, normalized to 100%. For both panels IFN-γ production by PB T cells is represented by open bars, while IFN-γ production by BAL T cells is represented by shaded bars. The asterisk denotes significantly different amounts of IFN-γ between BAL and PB T cells (p < 0.05). The data are expressed as the average values of IFN-γ ± SD obtained from four separate experiments.

FIGURE 6. Expression of CD80 (B7-1) and CD86 (B7-2) on AM and monocytes derived from HP patients. Plastic-adherent AM or blood monocytes were isolated and labeled for surface expression of either CD80 or CD86. Relative surface expression was determined by FACS analysis. Ag expression by AM is denoted by the open bars, and expression by blood monocytes is shown by the shaded bars. The data represent the average relative expression (±SD) for each Ag for five different individuals.
High affinity IL-12R expression in BAL T cells

Regulation of IFN-γ production by T cells is facilitated in part by the relative amounts of IL-10 and IL-12. To date we have observed that BAL T cells generate less IL-10 than PB T cells under the same stimulating conditions. We have also observed that PB T and BAL T cell generate comparable levels of IL-12. Interestingly, IFN-γ production by PB T cells is not affected by stimulation with exogenously added IL-12, while BAL T cells demonstrate significant increases. To investigate this difference we next determined the expression level of the IL-12R. Optimal binding of IL-12 requires expression of the β chain, the high affinity component of the IL-12R (IL-12Rβ2). IL-12Rβ2 levels were examined in unstimulated PB and BAL T cells by RT-PCR. As shown in Fig. 8, BAL T cells contained message for IL-12Rβ2, while PB T cells had no detectable message. This finding would support the observed difference in PB vs BAL T cells in generating IFN-γ following IL-12 stimulation and, in combination with the findings that BAL T cells produce less IL-10, could provide a mechanism by which BAL T cells in HP are influenced to develop a Th1 profile.

Discussion

There is increasing evidence to suggest that HP is predominantly mediated by Th1 cytokines (27). Using an animal model of HP, Hunninghake et al. (28) have reported that IFN-γ is required for development of HP, as IFN-γ knockout mice are resistant to development of the disease. In vitro studies have demonstrated that modulation of IFN-γ is accomplished primarily by a change in the ratio of IL-10 and IL-12 present in the culture system. Stimulation by IL-10, through several different mechanisms (31–33) acts to reduce production of IFN-γ. Conversely, stimulation by IL-12 results in augmentation of IFN-γ production by T cells (34). Consistent with these findings, HP animal models have demonstrated that increases in IL-12 result in an amplification of the severity of HP, while the presence of IL-10 ameliorates the disease (35, 36). It is unclear at present whether this same paradigm exists in humans and what mechanism(s) contributes to development of the predominantly Th1 profile.

In this study we examined the production of Th1 and Th2 cytokines by BAL T cells and PB T cells of HP patients. The results of intracellular cytokine detection at the single-cell level yielded several interesting findings. Intracellular cytokine analysis by flow cytometry clearly demonstrated a Th1-dominant profile in BAL CD4⁺ T cells and CD8⁺ T cells. Although Th1 cytokines (IFN-γ) were present in PB T cells, the percentage of positive cells was only 30% of that seen in BAL cells. When a correlation between IFN-γ-producing cells and cells producing IL-2 was examined, it was determined that there was a significantly lower percentage of BAL CD4⁺ T cells producing IL-2 compared with PB CD4⁺ T cells. Our data would suggest that while development of HP appears to be dependent on the Th1 cytokine IFN-γ, it does not correlate with the production of another Th1 cytokine, IL-2. These findings are consistent with studies by Semenzato and coworkers (19), who reported that no IL-2 mRNA was expressed in resting BAL T cells from HP patients and, in addition, with previous work from this laboratory (29), indicating that both BAL CD4⁺ and CD8⁺ T cells had decreased expression of IL-2 mRNA and produced significantly lower IL-2 when these cells were stimulated with TPA and A23187 compared with PB T cells. It has been suggested that IL-2 is more appropriately regarded as a marker of a precursor Th cell rather than a Th1 cell, and that cells that primarily produce IFN-γ or IL-4 which have developed from precursor Th CD4⁺ T cells should be designated effector Th1 or Th2 cells, respectively (37). Therefore, our data would suggest that PB T cells of HP patients are comprised of Th0, Th1, and Th2 cell types but are predominantly Th2-like, while BAL T cells from the same patients are Th1-like and, further, that the Th1-like CD4⁺ BAL T cells of HP patients can be considered polarized effector Th1 cells.

With regard to IL-4 production, the percentage of IL-4-producing CD4⁺ and CD8⁺ T cells was significantly lower than that seen in PB T cells, however, in absolute numbers, a significant number of IL-4-producing T cells was found in the lungs of HP patients. If IL-4-producing T cells, in addition to other IL-4-producing cells such as mast cells (38), persist in the lung for a sufficient period of time, they may contribute to the fibrotic scarring of the lung of HP patients.
A major factor that usually influences the development of either a Th1 or a Th2 cytokine profile is the local cytokine environment, in particular the ratio of IL-10 to IL-12. A predominance of IL-10 favors Th2 development, while IL-12 favors Th1 expansion (34). In BAL cells stimulated with anti-CD3 Ab, we observed a significant decrease in IL-10 production compared with stimulated PMCs. The decrease in IL-10 production could be a result of a variety of factors. Monocytic cells cannot only serve as a major source of IL-10 (39), but they can stimulate the production of IL-10 by T cells. As monocytic cells were present in the cultures, their contribution to IL-10 production was investigated. There was no difference in expression of either costimulatory molecules CD80 (B7-1) or CD86 (B7-2) on AMs of HP patients compared with blood-derived monocytes, indicating that the difference in production was not due to the ability of the monocytic cells to stimulate T cell IL-10 production. Monocytic cells also did not appear to be a source of IL-10, as culturing AMs with PB T cells resulted in comparable levels of IL-10 as those seen with monocytes and PB T cells. Therefore, it appears that the lack of IL-10 production seen in the BAL cell cultures could not be attributed to a difference in the monocytic cells, but, rather, was due to a difference in the T cells’ ability to generate the cytokine. These cells demonstrated an inability to generate as much IL-10 as PB T cells even when stimulated with TPA and ionomycin. The lack of IL-10 production was not the result of a generalized suppression, as production of IL-12 was comparable to that in PB T cells.

The source of increased T cells in the lung, in general, is due to recruitment of peripheral T cells rather than to clonal expansion within the lung (40). However, this has not been addressed specifically in HP-related inflammation, although it raises the possibility that once peripheral T cells are recruited to the lung, regulatory mechanisms associated with HP exist within the lung that reduce the capacity of the recruited cell to produce IL-10. The reduction in IL-10 production by the BAL T cells probably contributes to the development of the Th1-dominant cytokine profile. Unfortunately, direct ELISA measurement of IL-10 contained data demonstrating a Th1-type response were evident for all HP patients studied, we were limited in the number of patients we could recruit, and therefore, further studies are required to confirm our findings.

References