0023-6837/03/8308-1131$03.00/0
LABORATORY INVESTIGATION
Vol. 83, No. 8, p. 1131, 2003
Printed in U.S.A.

Cytokine Expression Pattern in Benign Prostatic Hyperplasia Infiltrating T Cells and Impact of Lymphocytic Infiltration on Cytokine mRNA Profile in Prostatic Tissue

Georg E. Steiner, Ursula Stix, Alessandra Handisurya, Martin Willheim, Andrea Haitel, Franz Reithmayr, Doris Paikl, Rupert C. Ecker, Kristian Hrachowitz, Gero Kramer, Chung Lee, and Michael Marberger

Departments of Urology (GES, US, AH, FR, DP, RCE, KH, GK, MM), Pathophysiology (MW), and Clinical Pathology (AH), University of Vienna Medical School, Vienna, Austria; and the Department of Urology (CL), Feinberg School of Medicine, Northwestern University, Chicago, Illinois

SUMMARY: The aim of the study is to characterize the type of immune response in benign prostatic hyperplasia (BPH) tissue. BPH tissue–derived T cells ($n = 10$) were isolated, activated (PMA + ionomycin), and analyzed for intracellular reactivity with anti–IFN-γ and IL-2, -4, -5, -6, -10, and -13, as well as TNF-α and -β by four-color flow cytometry. Lymphokine release was tested using Th1/Th2 cytokine bead arrays. The amount of IFN-γ and IL-2, -4, -13, and TGF-β mRNA expressed in normal prostate ($n = 5$) was compared with that in BPH tissue separated into segments with normal histology ($n = 5$), BPH histology with ($n = 10$) and without ($n = 10$) lymphocytic infiltration, and BPH nodules ($n = 10$). Expression of lymphokine receptors was analyzed by immunohistology, flow cytometry, and RT-PCR. We found that 28 ± 18% of BPH T helper cells were IFN-γ^+/IL-4$^-$ Th1 cells, 10 ± 2% were IFN-γ^-/IL-4$^+$ Th2, and 12 ± 6% were IFN-γ^+/IL-4$^+$ Th0 cells. In relation, cytotoxic and double-negative BPH T lymphocytes showed a slight decrease in Th1 and Th0 in favor of Th2. In double-positive BPH T lymphocytes, the trend toward Th2 (35 ± 15%) was significant (Th1: 12 ± 7%; Th0: 5 ± 4%). Lymphokine release upon stimulation was found in the case of IL-2, IL-5, IFN-γ, and TNF-$\alpha > 4$ μg; of IL-4 > 2 μg; and of IL-10 > 1 μg/ml. Expression of lymphokine mRNA in tissue was increased (2- to 10-fold) in infiltrated BPH specimens with and without BPH histology. The infiltrated BPH specimens with normal histology differed from those with BPH histology, most evident by the significant decrease in IFN-γ and the increase in TGF-β mRNA expression. Infiltrated BPH specimens with BPH histology expressed significantly more IFN-γ (5-fold), IL-2 (10-fold), and IL-13 (2.8-fold) when compared with noninfiltrated BPH specimens. BPH nodules, however, showed the highest level of expression of IL-4 and IL-13, with only intermediate levels of IFN-γ and very low levels of IL-2 mRNA. Immune response in histologically less transformed BPH specimens is primarily of type 1, whereas in chronically infiltrated nodular BPH and especially within BPH nodules, it is predominantly of type 0 or type 2. (*Lab Invest 2003, 83:1131–1146*).

Benign prostatic hyperplasia (BPH) is a nodular transformation, with the participation of both epithelial and fibromuscular stromal components (Franks, 1976). Presently BPH is considered a stromal disease (Bartsch et al, 1987). Further studies suggest a maturational sequence of stromal nodules, a possible significance of immunocompetent cells, and the notion that both stroma and epithelium respond with nodular hyperplasia to the stimulus, which causes BPH (Bierhoff et al, 1996, 1997).

DOI: 10.1097/01.LAB.0000081388.40145.65

Received September 30, 2002.

Sponsored in part by the Medizinisch-Wissenschaftlicher Fonds des Bürgermeisters der Bundeshauptstadt, Vienna, Austria (Grant No. 862), and by a research grant from Merck, Sharp & Dohm, Whitehouse Station, New Jersey.

Address reprint requests to: Dr. Georg E. Steiner, Department of Urology, University of Vienna, Währinger Gürtel 18–20, 1090 Vienna, Austria. E-mail: georg.steiner@akh-wien.ac.at

An association of BPH with inflammatory processes was noted as early as 1937 (Moore, 1937). A study by Kohnen and Drach (1979) reported that 98% of analyzed BPH specimens showed signs of an inflammatory process. We and others demonstrated that the largest portion of BPH tissue infiltrating leukocytes are T lymphocytes (Anim et al, 1998; Bierhoff et al, 1996; McClinton et al, 1990; Steiner et al, 1994; Theyer et al, 1992), in particular, activated CD4$^+$ memory T lymphocytes (Steiner et al, 1994). Furthermore, it could be shown that BPH tissues and T cell lines contain considerable amounts of IFN-γ, IL-2, and IL-4 mRNA, and proliferation experiments suggested that these lymphokines may alter the growth of prostatic stromal cells (Kramer et al, 2002). These results raise the question as to which kind of immune response is dominant.

Besides the phenotypic characteristics, the most important tool in defining a T cell immune response is the characterization of its cytokine expression pattern.

Type-1 T lymphocytes are negative for IL-4 and IL-13, are sources of IFN-γ or IL-2, and are involved in cell-mediated inflammatory reactions. They exhibit both cytotoxic and inflammatory functions (Del Prete et al, 1994; Mosmann and Coffman, 1989; Mosmann and Sad, 1996). Th1 clones induce delayed-type hypersensitivity reactions (Yamamura et al, 1991), and, at higher cell numbers, suppress B cell help (Del Prete et al, 1991). T lymphocytes belonging to the group of type-2 cells are negative for IFN-γ and IL-2, and positive for IL-4 or IL-13. They are commonly found in association with strong antibody and allergic responses (Mosmann and Coffman, 1989). A similar dichotomy has been reported for $CD8^+$ T cells and for T cells expressing TCR-γ/d (Ferrick et al, 1995; Romagnani, 1997; Sad et al, 1995).

This study, therefore, resorted to freshly isolated BPH T lymphocytes and intracellular lymphokine staining. Single CD4 or CD8 positive, double-positive, and double-negative subpopulations were analyzed for coexpression of IFN-γ in combination with IL-2, -4, -5, -6, -10, or -13, as well as TNF-α and -β. The effective amounts of lymphokines released by BPH T cells were analyzed by bead array assay.

The amounts of IFN-γ, IL-2, -4, and -13, and TGF-β mRNA expressed in prostatic tissue were analyzed quantitatively by TaqMan Real-Time PCR using tissue-derived cDNA and correlation with β-actin. Cytokine mRNA expressed in normal prostate was determined by using cDNA prepared from prostate specimens derived from trauma victims 18 to 25 years of age. To analyze the effect of lymphocytic infiltration on lymphokine expression, we chose pieces of BPH resection tissue with a typical hyperplastic glandular component that were either strongly infiltrated or showed no increased lymphocytic infiltration. The lymphokine pattern within BPH nodules was analyzed using a group of cDNAs that was prepared from macroscopically identified central areas of BPH nodules. Finally, for reasons of comparison, a group of BPH resection chips was selected that was without hyperplastic glandular component but showed signs of lymphocytic infiltration. The pattern of cytokine receptor expression was analyzed by immunoperoxidase staining, RT-PCR, and triple immunofluorescence.

Results

Intracellular Cytokine Profile of BPH T Lymphocytes

Fresh primary BPH T cells ($n = 10$) were stained using anti–CD4, -CD8, and -IFN-γ in combination with the above mentioned anticytokine antibodies. The percentages of anticytokine-reactive T cells of $CD4^+$/$CD8^-$ (Th), $CD4^-$/$CD8^+$ (Tc), $CD4^+$/$CD8^+$ (DP), and $CD4^-$/$CD8^-$ (DN) T cells and of cells coexpressing IFN-γ were calculated by setting gates and calculation of positive cells (Fig. 1).

Analysis of BPH T lymphocytes in 10 patients revealed that $CD4^+$ Th cells (mean: 55 $\pm$ 21%) were significantly more common than $CD8^+$ Tc (mean: 28 $\pm$ 15%; $p = 0.012$), DP (mean: 8 $\pm$ 7%; $p = 0.000$), and DN (mean: 10 $\pm$ 12%; $p = 0.001$) T cells and that Tc cells were more frequent than DP and DN T cells. In addition a significant negative correlation between the percentage of Th and Tc cells ($p = 0.01$) and between Th and DN cells ($p = 0.038$) was observed (Table 1).

Th1 cytokine-specific antibodies and anti–IFN-γ and –IL-2 reacted with 43 $\pm$ 18% and 38 $\pm$ 25% of $CD4^+$ Th cells, respectively. The percentages of IFN-γ and IL-2 expressing cells of Th were significantly above those of $CD8^+$ Tc and DN T cells (Fig. 2)($p <$ 0.01). Th cells also showed a significantly increased anti–IFN-γ ($p = 0.001$) reactivity when compared with DP T cells (40% versus 17%), while reactivity with anti–IL-2 was equally frequent (42 $\pm$ 31% of DP cells versus 38 $\pm$ 25% of Th cells).

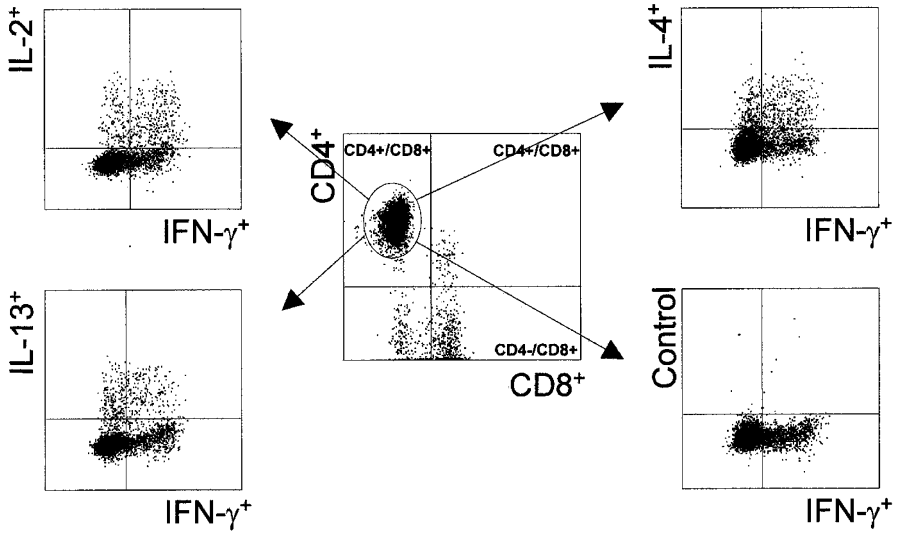

Figure 1.

Benign prostatic hyperplasia T cells were stained using conjugated anti-CD4 APC and anti-CD8 PerCP in combination with anti-IFN-γ FITC plus a second anti–PE-conjugated anticytokine antibody. T cells were divided into Th, Tc, DN, and DP T cell subsets, and the percentage of single or double anticytokine-reactive T cells was calculated.

Table 1. Distribution of Type-1, Type-2, and Type-0 Cytokine-Expressing T Cells in the Different BPH T Cell Subsets Analyzed for Intracellular Cytokine Staining and Four-Color Flow Cytometry

BPH T lymphocyte subsets		% Of BPH T Cells	Intracellular lymphokine coexpression pattern of BPH T cells		
			% Of type 1 IFN-γ^{pos} and IL-4^{neg}	% Of type 2 IL-4^{pos} and IFN-γ^{neg}	% Of type 0 IL-4^{pos} and IFN-γ^{pos}
CD4$^+$/CD8$^-$	Th cells	55.3 ± 20.6[a]	28.0 ± 17.8%	9.8 ± 2.2%	12.3 ± 5.6%
CD4$^-$/CD8$^+$	Tc cells	27.8 ± 15.3	19.8 ± 9.3%	13.0 ± 8.7%	4.8 ± 3.8%
CD4$^+$/CD8$^+$	DP T cells	7.8 ± 6.7	12.0 ± 7.3%	34.5 ± 15.4%	5.0 ± 3.5%
CD4$^-$/CD8$^-$	DN T cells	10.0 ± 11.5	16.8 ± 10.4%	13.0 ± 7.6%	5.0 ± 2.2%

BPH, benign prostatic hyperplasia.
[a] Mean percentage ± SD.

More importantly DP T cells were significantly more frequently anti–IL-4 (42 ± 17%) and IL-13 (41 ± 15%) reactive than all other BPH T cell subsets (mean range between 20% and 23%; $p < 0.01$). Thus DP T cells differed significantly from CD8$^+$ Tc cells and DN T cells by increased expression of IL-2, -4, and -13 ($p < 0.01$). They differed from single CD4$^+$ Th cells not only by significantly increased expression of IL-2 (in relation to IFN-γ) but also of IL-4 and IL-13. No significant differences between Tc and DP T cells were observed.

Coexpression analysis showed that Th cells consisted of significantly more IFN-γ^+/IL-4$^-$ type-1 (28%) than IL-4$^+$/IFN-γ^+ type-0 (12.3%, $p = 0.008$) and IL-4$^+$/IFN-γ^- type-2 (9.8%, $p = 0.024$) cells, with no significant difference between the percentage of type-0 and type-2 cells.

In relation to the Th1:Th2 ratio in Th cells, CD8$^+$ Tc showed a relative increase in Th2 cells (13%) when compared with Th1 (20%) and significantly less Th0 cells (5%). A similar balanced ratio between Th1 and Th2 cells was observed in DN T cells.

In DP T cells, type-2 cells were significantly more frequent when compared with all other T cell subsets (mean percentage of type-2 cells of DP cells: 35% versus 13% of both Tc and DN, as well as 10% of Th; $p > 0.001$). Significant differences between the percentages of type-1 and -0 cells were only seen within the CD8$^+$ Tc population.

The mean anti–IL-6 reactivity ranged between 0.9% in the case of Th and 4.8% of DP cells. Anti–IL-5 revealed a similar pattern of reactivity with 7.5 ± 5.4% anti–IL-5$^+$ DP cells followed by Tc and DN T cells (2.5% and 2%, respectively). Up to 4% of DP cells were anti–IL-10$^+$ followed by Tc (2.5 ± 1.7% IL-10$^+$ cells). Anti–TNF-α reactivity was very common and present in more than 59% of T cell subpopulations. Reactivity with anti–TNF-β, however, was restricted to only a small subset of less than a mean of 5.1% of BPH T cells (Table 2).

Cytokine Release by BPH-T-Lymphocytes

Release of cytokines by BPH T lymphocytes ($n = 6$) was analyzed using supernatants of BPH T cells that were kept either unstimulated or were stimulated with PMA or a combination of PMA and anti-CD3. Contamination by stromal and epithelial cells was avoided by culturing BPH T cells for three passages in suspension culture before use. The amount of lymphokines was analyzed using quantitative cytometric bead array assay.

In supernatants of unstimulated BPH T cell lines, four out of six (67%) BPH T cell lines (Fig. 3 and Table 3) released IL-5 (range between 27 and 185 pg/ml) and two out of six released IL-4 (33%); Lines 2 and 6 showed 577 and 426 pg, respectively. Interestingly, both IL-4$^+$ lines were IL-5$^-$. The amount of IFN-γ ranged between 15 and 596 pg/ml (66% above 190 pg). IL-2 was almost negative, and TNF-α could be shown in supernatants of four of the six lines (range between 37 and 99 pg/ml). Two out of six BPH T cell lines released some IL-10 (> 60 pg/ml).

In supernatants of PMA-stimulated BPH T cell lines, stimulation with PMA resulted in a multifold massive increase of cytokine release (Table 3). The release of IL-5, for example, was increased > 35-fold and that of IFN-γ as well as of TNF-α more than 29-fold. A heterogeneous pattern was observed in the case of IL-2, IL-4, and IL-10. An increase in the case of IL-2 was seen in only 33% of lines (> 5-fold). The mean increase in the case of IL-4 was more than 100-fold. However, 66% of lines showed almost no increase. Interestingly, the two lines that were IL-4$^+$ without stimulation (2 and 6) became IL-4$^-$ upon PMA activation (down to 11 and 17 pg, respectively). The IL-10 response to PMA ranged between a 10- and 400-fold increase.

In supernatants of anti-CD3 and PMA-stimulated BPH T cell lines, the increase in lymphokine production was enormous, and it was necessary to dilute the supernatants 100-fold for appropriate results. The combination of anti-CD3 and PMA stimulated the release of all analyzed lymphokines. Comparison of PMA with PMA + anti-CD3–stimulated groups revealed in the case of IL-2 a > 450-fold increase, of IFN-γ > 60-fold, of TNF-α and IL-5 > 20-fold, of IL-10 > 9-fold, and of IL-4 a > 6-fold increase of all analyzed lines (Table 3). BPH T cell Line 1, for example, released 2 and 3 times more IFN-γ and IL-2, respectively, when compared with BPH T cell Line 2 (Fig. 3). However, Line 1 also released twice as much IL-4, a typical Th2 cytokine. We also found a marked difference between the amount of IL-10 release (320 versus 5170 pg of IL-10) by the BPH T cell lines. A typical example of this heterogeneity is given in Figure 3.

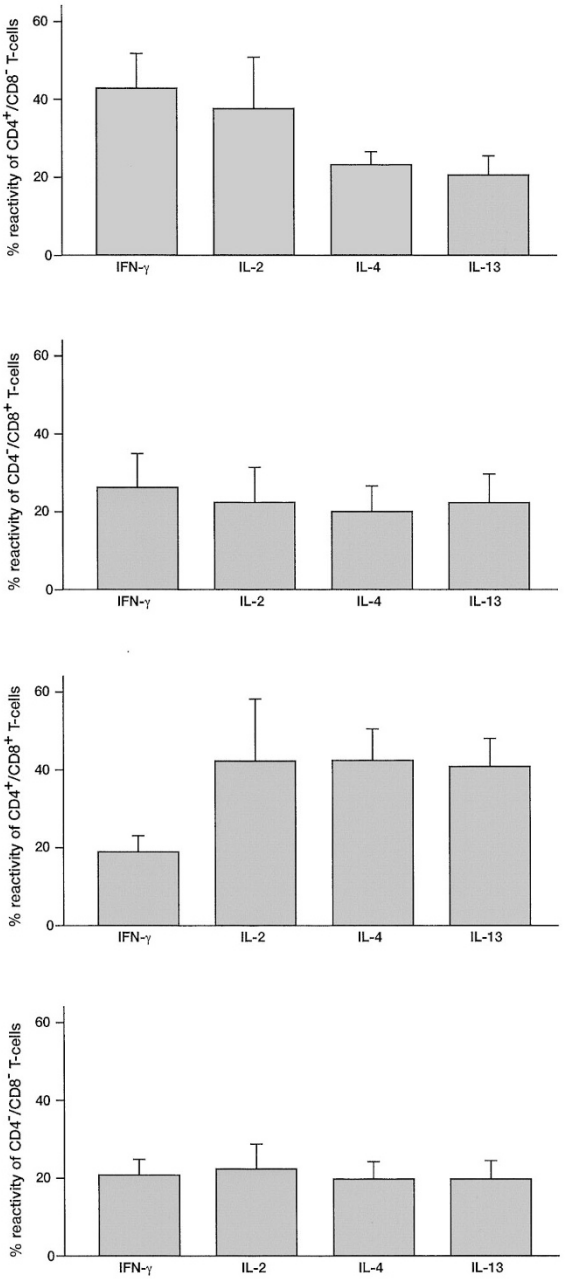

Figure 2.

Mean percentage and standard deviation of single anti–IFN-γ, IL-2, -4, and -13 reactive benign prostatic hyperplasia T cells ($n = 10$) of the four different subsets.

Comparison of the Amount of Lymphokine mRNA Expressed in Normal and BPH Tissue Using TaqMan Real-Time PCR

To determine cytokine mRNA expression quantitatively, TaqMan Real-Time PCR was performed using IFN-γ, IL-2, -4, and -13, and TGF-β–specific primers. The amount of cytokine mRNA was correlated with that of β-actin (Table 4). Normal prostatic cDNA was prepared from frozen specimens derived from trauma victims 18 to 25 years of age. BPH tissue–derived cDNA was prepared from transurethral resection (TUR) chips that have been histologically analyzed and categorized.

IFN-γ. All normal prostate specimens expressed intermediate amounts of IFN-γ mRNA (mean: 48), and all tested groups of cDNAs derived from BPH specimens expressed significantly increased amounts. Expression of IFN-γ mRNA was most abundant in infiltrated BPH specimens (mean: 437) followed by BPH nodules (mean: 270) and BPH samples without hyperplastic glandular components that were infiltrated (mean: 152). Noninfiltrated BPH samples showed an increase in IFN-γ mRNA expression that was < 2-fold (mean: 86) when compared with normal prostate ($p = 0.023$)(Table 5).

IL-2. In the normal prostate, noninfiltrated BPH specimens, and BPH nodules groups, expression of IL-2 mRNA was only marginal (mean: 18.4, 18.2, and 12.6, respectively). A 10-fold significantly increased expression of IL-2 mRNA ($p = 0.000$) was observed in infiltrated BPH specimens with BPH histology (mean: 205) and in the group of BPH samples with normal histology that were infiltrated (mean: 191). However, in the case of infiltrated BPH specimens with BPH histology, this 10-fold increased IL-2 mRNA expression was associated with 3 times more IFN-γ mRNA expression than in the group of infiltrated BPH tissue with normal histology.

IL-4. Compared with the very low levels of IL-4 mRNA expression observed in the group of normal prostate specimens (mean: 4.8), all other groups showed significantly increased expression of IL-4 mRNA. The increase was less pronounced in the cases of infiltrated BPH specimens with normal histology and of noninfiltrated BPH specimens (mean: 19.2 and 22, respectively), was intermediate in the group of infiltrated BPH specimens (mean: 55), and was strongest in the group of BPH nodules (mean: 116.9).

IL-13. In the groups of normal prostate specimens and of noninfiltrated BPH specimens uniform intermediate IL-13 mRNA expression was observed (mean: 29 and 36). In the remaining experimental groups, expression of IL-13 mRNA was significantly increased. Similar to the expression pattern of IL-4 mRNA, the most abundant IL-13 mRNA expression was observed in the groups of BPH nodules (mean: 188.2) and infiltrated BPH specimens (mean: 90.1).

TGF-β. In normal prostate specimens, TGF-β mRNA was massively expressed (mean: 178.2). Compared with these normal values, the only group that showed no significant increase in the expression of TGF-β mRNA was the infiltrated BPH specimens with BPH histology group (mean: 365.8). Expression of TGF-β mRNA was strongest in the group of infiltrated BPH specimens with normal histology (mean: 717.4) followed by the group of BPH nodules (mean: 501.1) and the group of noninfiltrated BPH specimens (mean: 398.1).

Highly significant correlation between the amount of TGF-β and IL-2 ($p = 0.007$) mRNA was found in the normal prostate specimens group. No significant correlation between any of the cytokines was observed in

Table 2. Profile of Additional Intercellular Lymphokine coexpression of the Four Different BPH T Cell Subsets

	% Reactivity of BPH T lymphocyte subpopulations			
Cytokines	$CD4^{pos}$ $CD8^{neg}$	$CD4^{neg}$ $CD8^{pos}$	$CD4^{pos}$ $CD8^{pos}$	$CD4^{neg}$ $CD8^{neg}$
IL-6	0.9 ± 0.2%[a]	1.4 ± 0.6%	4.8 ± 6.0%	1.1 ± 0.5%
IL-5	1.1 ± 0.5%	2.5 ± 1.1%	7.5 ± 5.4%	2.5 ± 0.5%
IL-10	0.8 ± 0.3%	2.5 ± 1.7%	4.3 ± 3.3%	2.0 ± 1.5%
TNF-α	69.8 ± 24.3%	60.0 ± 28.6%	64.0 ± 27.0%	62.3 ± 21.1%
TNF-β	2.3 ± 1.1%	2.1 ± 1.0%	5.1 ± 4.6%	1.4 ± 0.6%

BPH, benign prostatic hyperplasia.
[a] Mean percentage ± SD.

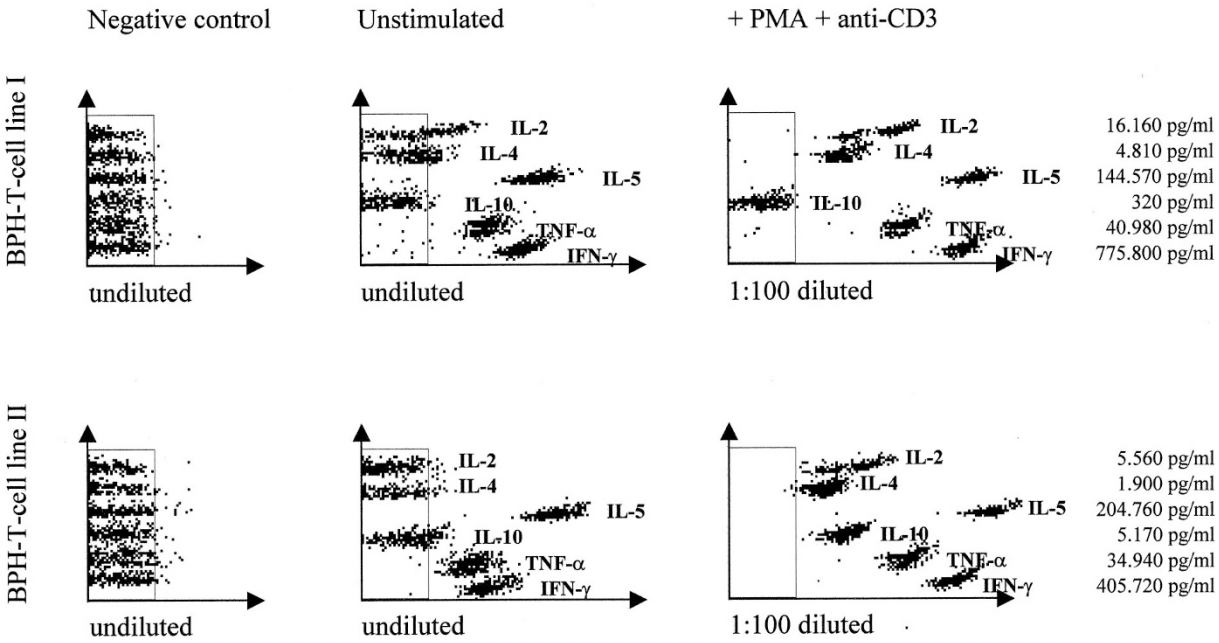

Figure 3.
Representative result of bead array analysis in culture supernatants of unstimulated and stimulated benign prostatic hyperplasia (*BPH*) T cells. Each of the six horizontal *dot clouds* represents the staining intensity for anticytokine-reactive beads for one cytokine. From top to bottom: IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ. *X-axis* represents the staining intensity, corresponding to the amount of cytokine.

Table 3. Cytokine Release of primary BPH T Lymphocytes Analyzed by Bead Array

	BPH T cells		
	Unstimulated (pg/ml)	+PMA (pg/ml)	+PMA + anti-CD3 (pg/ml)
IFN-γ	268 ± 298	10,520 ± 4,519	594,780 ± 185,171
IL-2	4 ± 6	23 ± 37	10,477 ± 5,341
IL-4	3 ± 3	454 ± 766	3,077 ± 1,533
IL-5	196 ± 151	7,034 ± 428	159,507 ± 39,938
IL-10	2 ± 2	303 ± 435	2,950 ± 2,451
TNF-α	53 ± 49	2,120 ± 2,248	43,343 ± 9,801

BPH, benign prostatic hyperplasia.

the group of infiltrated BPH specimens with normal histology. In the group of noninfiltrated BPH specimens, significant correlations were found between the amounts of IL-2 and IL-4 ($p = 0.006$) and between IFN-γ and TGF-β ($p = 0.019$) mRNA expression. In the infiltrated BPH specimens group, the amount of IFN-γ mRNA showed a highly significant correlation with the amount of IL-4 mRNA ($p = 0.001$) and a negative correlation with the amount of TGF-β ($p = 0.001$) mRNA. In addition this group also showed significant

Table 4. Effect of Lymphocytic Infiltration and Histologic Alteration on Cytokine mRNA Expression Pattern in Prostate Tissue

	Normal prostate		TUR/P-derived BPH specimens		Suprapubic prostatectomy-derived BPH specimens
	Normal histology	Normal histology	BPH histology		Central area of BPH nodules
Infiltration[a]	Grade 0 (n = 5)	Grades 1 and 2 (n = 5)	Grade 0 (n = 10)	Grades 2 and 3 (n = 10)	Not analyzed (n = 10)
IFN-γ	48.2 ± 15.7[b]	152.4 ± 46.2	86.3 ± 62.7	437.4 ± 188.6	270.1 ± 173.2
IL-2	18.4 ± 5.0	191.6 ± 40.5	18.2 ± 10.5	205.3 ± 95.3	12.6 ± 3.9
IL-4	4.8 ± 1.6	19.2 ± 5.1	22.0 ± 12.4	55.0 ± 39.1	116.9 ± 143.4
IL-13	29.6 ± 6.5	45.8 ± 9.3	36.1 ± 23.0	90.1 ± 44.3	188.2 ± 70.7
TGF-β	178.2 ± 57.7	717.4 ± 98.7	398.1 ± 141.5	365.8 ± 243.8	501.1 ± 317.9

BPH, benign prostatic hyperplasia; TVR, transurethral resection.

[a] Grading of leucocytic infiltration: 0 = No infiltration; 1 = scattered infiltration (without clusters), 2 = single foci of intense infiltration, 3 = multifocal intense infiltration.

[b] Mean ± SD 0.002.

Table 5. *P* Values of the Differences in Cytokine mRNA Expression

		IFN-γ	IL-2	IL-4	IL-13	TGF-β
Normal prostate	vs BPH, normal histology	**0.009**[a]	**0.009**	**0.009**	**0.021**	**0.009**
	vs BPH noninfiltrated	**0.023**	0.759	**0.007**	0.901	**0.003**
	vs BPH infiltrated	**0.002**	**0.002**	**0.002**	**0.032**	0.327
	vs BPH nodules	**0.002**	**0.048**	**0.002**	**0.002**	**0.006**
BPH	vs BPH noninfiltrated	**0.010**	**0.002**	0.713	0.056	**0.003**
Normal histology	vs BPH infiltrated	**0.003**	0.806	0.326	0.066	**0.014**
	vs BPH nodules	**0.098**	**0.002**	0.540	**0.002**	0.220
BPH noninfiltrated	vs. BPH infiltrated	**0.000**	**0.000**	0.112	**0.017**	0.880
	vs BPH nodules	**0.000**	0.256	0.173	**0.000**	0.650
BPH infiltrated	vs Central area of nodules	**0.009**	**0.000**	0.762	**0.005**	0.545

BPH, benign prostatic hyperplasia.

[a] Mann-Whitney test, asymptotic significance; $p < 0.05$ is significant.

negative correlations between TGF-β and IL-2 ($p = 0.025$) as well as IL-4 ($p = 0.039$), and a positive correlation between IL-2 and IL-4 ($p = 0.036$) mRNA. In the BPH nodules group, the amount of IFN-γ, IL-4, and IL-13 mRNA correlated significantly ($p = 0.001$).

Cytokine Receptor Expression of Prostatic Epithelial and Stromal Cells

cDNA of prostate carcinoma cell lines (DU145, LNCaP, and PC3), BPH prostatic stromal cell (PSC) clones ($n = 13$), and six highly enriched BPH epithelial cell (EC) preparations were analyzed, and representative results are shown in (Fig. 4, A and B). BPH-EC and carcinoma of the prostate (CaP) cells showed abundant expression of IFN-γ (except LNCaP), IL-4 (except BPH-EC 2), and IL-13R α-chain mRNA. Expression of the three members of the IL-2R family in CaP cell lines was restricted to the IL-2R γ-chain (Fig. 4A), whereas in five out of six BPH-EC preparations, transcripts of IL-2R α- and β-chain mRNA were found in addition.

PSC lines and clones showed significant expression of IFN-γ, IL-4, and IL-13 receptor α-chain mRNA but were lacking IL-2R α-chain mRNA transcripts. Expression of IL-2R γ- and β-chain mRNA in PSCs was heterogeneous and therefore analyzed in more detail using additional polyclonal PSC lines ($n = 9$) and BPH-PSC clones (Fig. 4B). Two of the PSC lines are derived from normal prostate specimens and seven from BPH tissues. RT-PCR analysis revealed that all polyclonal normal PSC and BPH-PSC lines and 6 out of the 15 BPH-PSC clones expressed mRNA for IL-2R γ-chain. In the case of the IL-2R β-chain mRNA, the two normal PSC lines were negative, whereas 4 out of the 7 polyclonal BPH-PSC lines and 12 out of 15 BPH PSC clones weakly expressed IL-2R β-chain mRNA (Fig. 4, A and B).

Expression of Cytokine Receptors in BPH Tissue

Acetone-fixed frozen sections of BPH tissue were stained using antigen affinity purified antisera directed against the three chains of the IL-2 receptor (α, β, and γ) and the α-chain of the IL-4 receptor and IL-13. Reactivity with anti–IL-2R α-chain antisera was significant on infiltrating T cells (Fig. 5A), but also a discrete

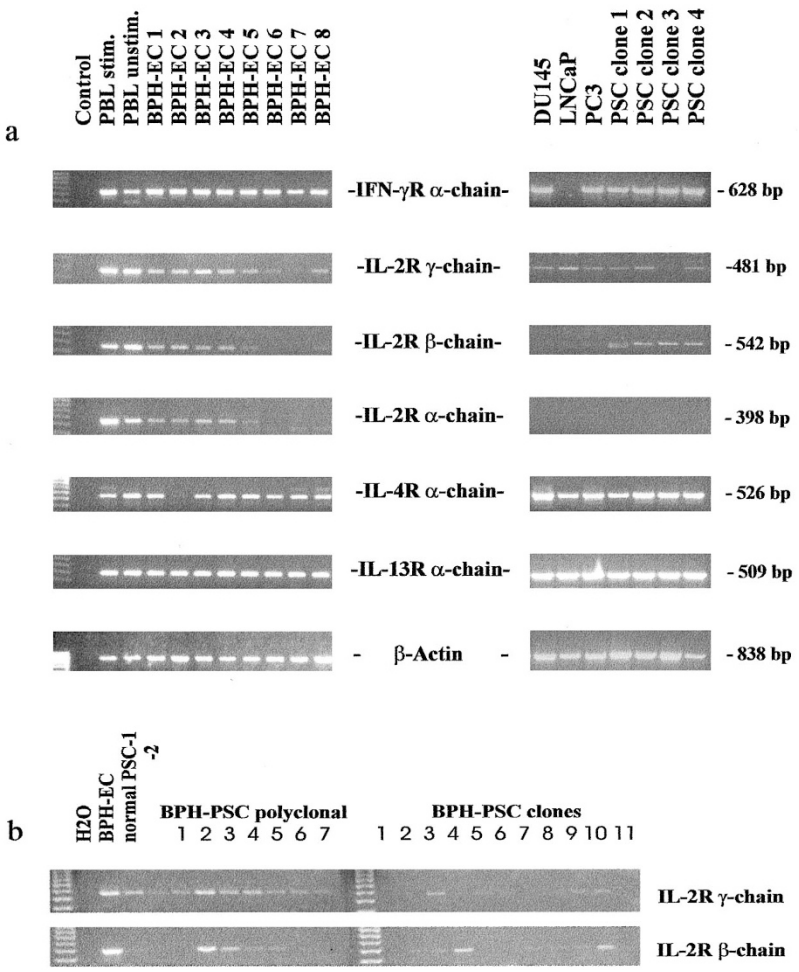

Figure 4.
RT-PCR analysis of prostatic epithelial and stromal cells and carcinoma cell lines using lymphokine receptor-specific primer. cDNA of 15 PSC clones, six benign prostatic hyperplasia (*BPH*) epithelial cell (*EC*) preparations, DU-145, LNCaP, and PC3 were analyzed using 35 cycles of RT-PCR and intron-spanning primer.

membranous reactivity of basal cells was observed. Reactivity with anti–IL-2R β-chain was also intense on infiltrating lymphocytes but showed a weak diffuse pattern of reactivity on subsets of PSCs and prostatic epithelial cells (PECs) (Fig. 5B). IL-2R-γ–specific antiserum reacted diffuse with a slight accumulation on lymphocytes and on basal epithelial cells (Fig. 5C).

Staining using anti–IL-4R α-chain antiserum and monoclonal antibody (mAb) revealed a very strong reactivity of basal PECs and a significant but much lower reactivity of a subset of prostatic interstitial cells (the majority of them showed the typical bundle-like histology of smooth muscle cells). Secretory PECs were weakly anti–IL-4R α-chain reactive (Fig. 5E).

A similarly intense basal cell reactivity was observed with antigen affinity purified goat anti–IL-13 (Fig. 5D). The obvious restriction of this reactivity to only a subset of basal cells prompted us to use triple staining experiments, including cell type–specific antibodies and confocal laser microscopy.

Anti–IL-4R α-chain reactivity was strongest on CK-18 negative and on weakly positive basal prostatic epithelial cells. Company expression of IL-4R α-chain and IL-13 was frequently observed (pink positive cells) (Fig. 6A). The fact that strong anti–IL-4R α-chain expression of basal PECs is not uniform is best indicated in Figure 6C, in which only a small subset of anti–CK-13 reactive cells were red or blue positive. An example of uniform IL-4R α-chain expression of basal PECs is shown in Figure 6D. This example also shows, however, that the staining intensity of anti–IL-13 was often heterogeneous, indicated by only one-half of the cells being pink. The specificity of the individual antibodies is indicated on the one hand by the selective distribution of reactivity pattern and on the other hand, in the case of anti–IL-4R and anti-13 reactivity, by the lack of reactivity of identical cell subsets within a gland (Fig. 6, C and D).

Using anti–IL-2R γ-chain (blue) and IL-4R α-chain (red) in combination with either anti-cytokeratin-(ck)-18 (data not shown) or anti–ck-13 (Fig. 6E) revealed that IL-4R$^+$ basal PECs also reacted weakly with anti–IL-2R γ-chain antibody. The single anti–IL-2R γ-chain reactivity observed on secretory PECs was very weak and predominantly luminal (data not shown).

Colocalization of IL-4 and IL-13 was analyzed using two direct conjugated monoclonal antilymphokine an-

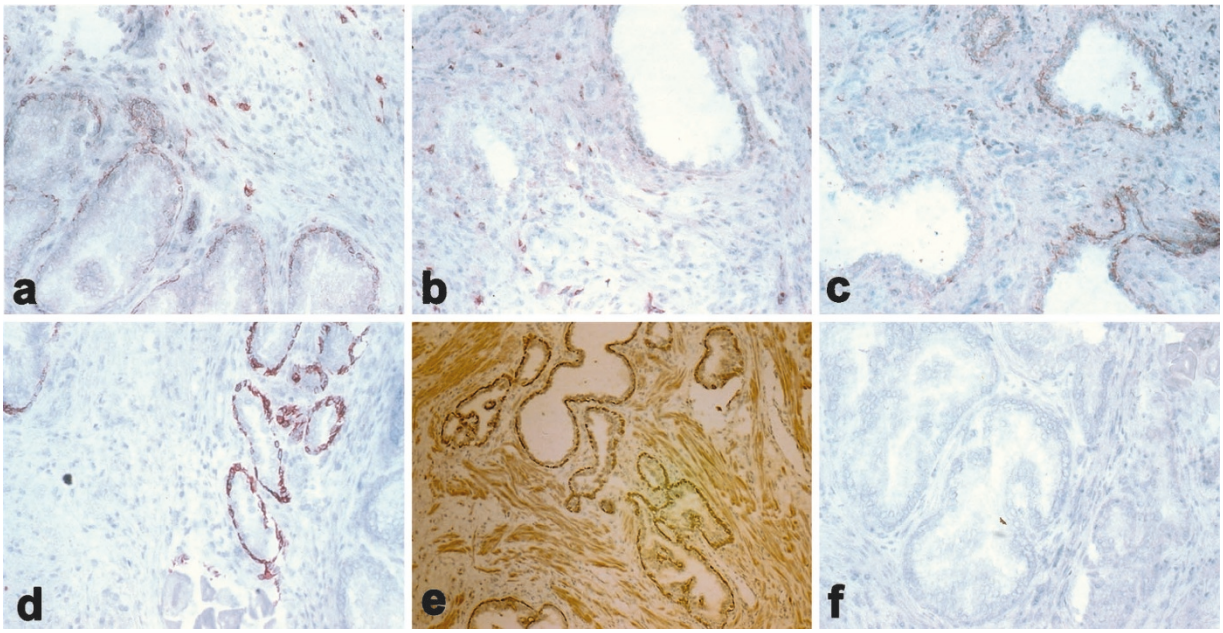

Figure 5.
Immunohistochemistry of frozen sections using IL-2R α-chain (A), IL-2R β-chain (B), IL-2R γ-chain (C), and IL-4R α-chain (E) specific antisera, as well as negative control sera (F). Reactivity with monoclonal anti–IL-13 is shown in D.

tibodies. Anti–IL-4 reactivity (red) was strongest on PECs and was more intense and not so restricted to the basal epithelial subset as it was the case for anti–IL-13 (Fig. 6F). Reactivity of stromal cells and infiltrating leukocytes was by far less intense when compared with epithelial cells with both anti–IL-4 and -13. Anti–IL-2 reactivity could not be shown. Immunohistochemical staining for IFN-γ (mAb and antiserum) revealed a strong membranous reactivity of all prostatic cell types. PEC and endothelial cells (data not shown) were slightly more positive, and focally increased reactivity was detected at sites of chronic inflammation, focal inflammatory atrophy, and glandular hyperplasia. Staining for IFN-γ receptor (R) α-chain (antiserum) revealed a similar uniform reactivity, with a tendency toward a stronger reactivity of basal PEC. Anti–IFN-γ R β-chain–specific antiserum also showed a uniform, yet only minor, reactivity of all prostatic cell types and seemed to accumulate on luminal secretory PECs (data not shown).

Discussion

This study examined for the first time the intracellular lymphokine expression pattern of T cells derived from BPH specimens. To avoid a switching in the cytokine pattern or over-proportional outgrowth of subpopulations, BPH T cells were analyzed immediately after the isolation procedure. Among the $CD4^+$ Th subset, 43 $\pm$ 18% and 37 $\pm$ 25% reacted with mAb directed against the type-1 cytokines IFN-γ and IL-2, respectively. However, 14% of IFN-γ^+ Th were also IL-4^+/IL-13^+. Therefore, the percentage of classic type-1 Th cells based on the anti–IFN-γ reactivity was 29%, and almost all of them coexpressed IL-2 (26% IFN-γ^+/IL-2^+ Th cells). Within the Th subset, the second most common cytokine expression pattern was that of type-0 cells characterized by the coexpression of type-1 and -2 cytokines (mean IL-4^+/IFN-γ^+ and IL-13^+/IFN-γ^+ cells: 14%), while classic type-2 cells lacking type-1 cytokines constitute only 10% of the Th cell population. This stood in sharp contrast to all other analyzed BPH T cell subpopulations, ie, single $CD8^+$, DP, and DN T cells, which were found to express predominantly a type-2 cytokine pattern followed by significantly fewer type-1 and type-0 cells. This trend toward a predominance of a type-2 cytokine expression pattern was most pronounced in DP T cells in which 36% were of type 2 as compared with 9% of type 1 and 7% of type-0 cells. Thus, DP T cells differed significantly from $CD8^+$ Tc cells and DN T cells by increased expression of IL-2, -4, and -13 ($p < 0.01$). They differed from single $CD4^+$ Th cells not only by significantly increased expression of IL-2 (in relation to IFN-γ) but also of IL-4 and IL-13.

Intracellular cytokine staining of freshly isolated T cells is the method of choice in characterizing T cell populations (Jung et al, 1993; Willheim et al, 1997, 1999). The cells are characterized according to their cytokine pattern. However, this method does not offer any insight into the amount of cytokines released. To detect locally released cytokines, which might exert an influence over adjacent cells, we deemed it necessary to perform further tests in addition to intracellular staining and PCR.

Considerable amounts of cytokines were detected in supernatants both without activation (due to 24-hour growth arrest using IL-2 withdrawal) or less than optimal activation using PMA alone. One-hundred percent of all supernatants from unstimulated BPH T cells contained IL-5 ($>$ 51 ng), 60% IFN-γ ($>$ 194 ng), and TNF-α ($>$ 57

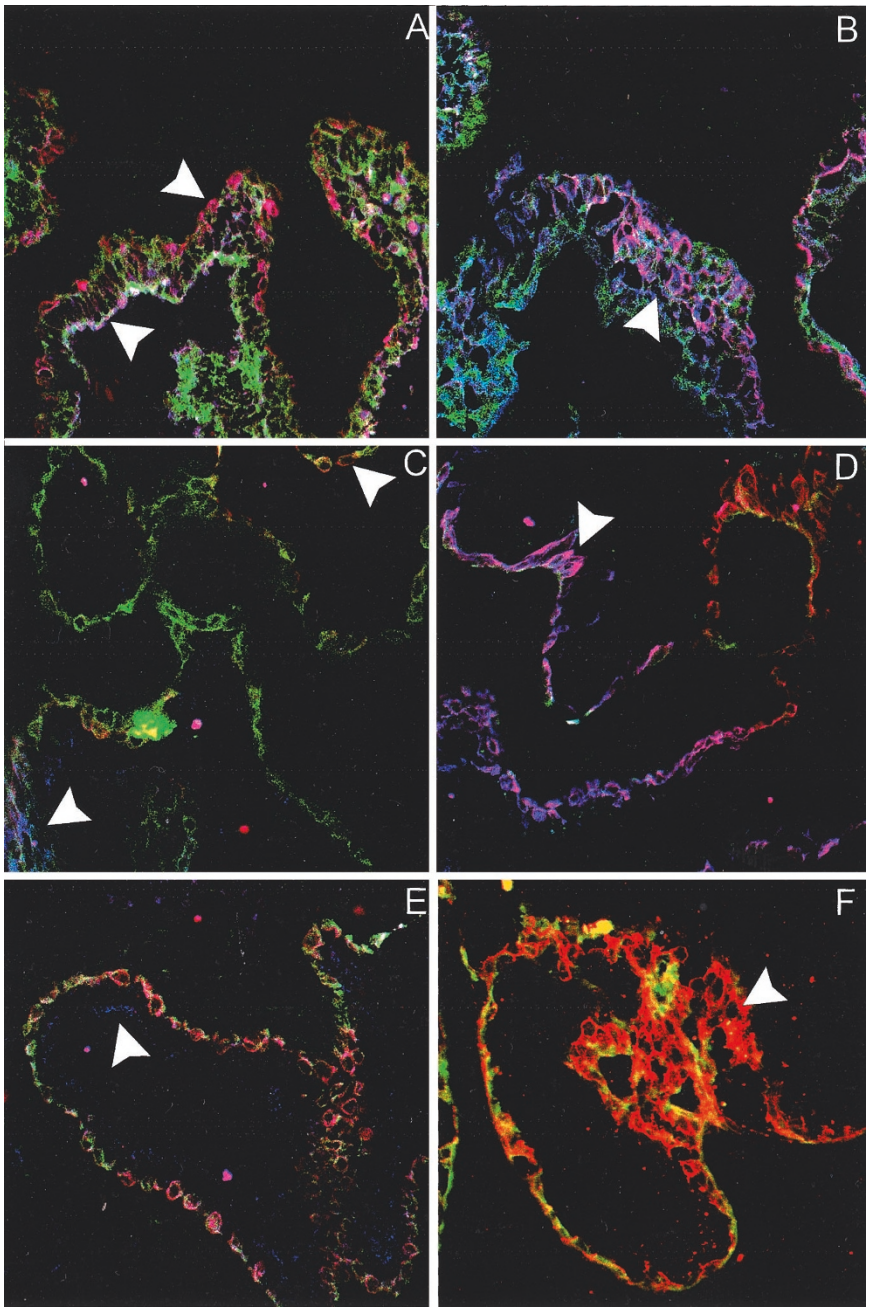

Figure 6.

Reactivity with anti-cytokeratin (ck)-18 (A, B, and E) and with anti–ck-13 (C, D, and F) is indicated in green. Reactivity with anti–IL-4R α-chain is indicated in red. Blue stainings represent reactivity with IL-13 (A to D) and IL-2R γ-chain (E and F).

ng), yet none contained relevant detectable amounts of IL-2, -4, or -10. Stimulation of BPH T cells with anti-CD3 and PMA (in place of antigen and antigen presenting cell) resulted in production of all analyzed cytokines by 100% of the examined cell lines. The largest variances were detected in the case of IL-10 (17-fold). Cell lines with the high amounts of single $CD4^+$ T cells and the low amounts of DP and DN T cells, released at least twice as much IL-10 as all other analyzed cells lines. However, no direct correlation was found to exist between CD4 Th cells and IL-10, as cell lines showing almost identical percentages of CD4, CD8, DN, and DP showed up to 11-fold differences in the amount of IL-10 released.

Although this method is not suited to determine the type of immune response, it became evident that only the secretion of IL-2 and IL-4 was anti-CD3 dependent and that four out of six cell lines did not respond to PMA stimulation. Furthermore, it struck our attention that the cell line that produced the largest amounts of IL-10 released the smallest amounts of IL-2 and IL-4. When comparing the total concentrations, it becomes apparent that the amount of released IFN-γ (mean: 0.6 μg/ml) surpasses all, followed by IL-5 (mean: 0.16 μg/ml). At quite a distance, TNF-α (mean: 0.04 μg/ml) follows in third place. IL-2, IL-4, and IL-10 constitute a distinct category and are released at relatively low

levels. However, it has been taken into consideration that what is measured in the supernatant is a steady state of production and uptake/degradation. Thus it would be conceivable that, for example, the two T cell growth factors IL-2 and -4 are produced at similarly high levels and are massively used up.

The enormous variance in the levels of IL-5 detected with the two different techniques (Bead array and flow cytometry) could be explained by the fact that the epitope recognized by anti–IL-5 antibody is structurally altered by the paraformaldehyde fixation. From our point of view, the speed of IL-5 release plays only a subordinate role.

Whether infiltration has an impact on the extent of cytokine expression in situ was analyzed by comparing the amount of cytokine mRNA expressed in BPH specimens with and without lymphocytic infiltration. The significantly increased expression of IFN-γ (5-fold), IL-2 (10-fold), and IL-13 (3-fold) mRNA in the group of infiltrated BPH specimens clearly indicates that lymphocytic infiltration of BPH is associated with increased cytokine mRNA expression. However, comparison of noninfiltrated BPH specimens with normal prostate specimens showed that these BPH specimens also revealed a higher expression of IFN-γ (1.8-fold), TGF-β (2.2-fold), and IL-4 (4.6-fold) mRNA. To analyze to which extent the benign hyperplastic growth process interferes with the cytokine mRNA expression pattern, a group of TUR/P-derived BPH chips exhibiting a normal histology were included in the study. All of the chips showed either a Grade-1 or -2 lymphocytic infiltration. Comparison of the cytokine mRNA expression pattern in this BPH group (normal histology) with noninfiltrated BPH specimens (BPH histology) revealed a significantly increased expression of IL-2 and IFN-γ but not of IL-4 and -13 mRNA. These data suggest that in histologically normal parts of BPH specimens, a type-1 immune response prevails. The over-proportional increase of IFN-γ (in relation to IL-2) together with a 2-fold increase of IL-4 and -13 mRNA expression in infiltrated BPH with BPH histology, when compared with BPH chips with normal histology, suggests a shift toward a type-0 immune response in hyperplastic transformed tissue. Theoretically, increased participation of Th0 cells is associated with advanced BPH, and TUR/P chips with normal histology (prevailing type 1) are representatives of the initial phase of BPH development. Increased IL-4 and IL-13 mRNA in the group of macroscopically identified central areas of BPH nodules associated with only very weak IL-2 but intermediate IFN-γ mRNA expression might confirm this hypothesis. As a further indication for a predominant type-0 immune response in BPH nodules, the highly significant correlation among levels of IL-4 and -13, and IFN-γ mRNA expression might be used ($p > 0.001$).

It is remarkable that increased lymphokine mRNA expression in infiltrated BPH chips exhibiting normal histology was associated with a significant 4.2-fold increase of TGF-β mRNA expression ($p = 0.05$) when compared with the group of normal specimens. No such significant increase was observed in infiltrated BPH specimens with BPH histology. These specimens expressed significantly less TGF-β mRNA than the group of BPH specimens with normal histology and noninfiltrated BPH specimens with BPH histology.

In the prostate TGF-β has been shown to inhibit prostatic stromal cell growth by induction of differentiation (Lee et al, 1999) and to play a key role in the regulation of cell death by the induction of apoptosis of prostatic epithelial cells in the proximal region (Griffiths et al, 1993, 1996; Lee et al, 2001). In addition TGF-β has evolved into a key molecular mediator stimulating extracellular matrix accumulation (Chung et al, 1996; Ignotz et al, 1987; Inagaki et al, 1994; Jimenez et al, 1994). TGF-β functions as a major anti-inflammatory mediator in lung and skin fibrosis and has also been recognized as a major mediator of the immune response in the lung where it prevents persistent inflammation (McCormick et al, 1999). Recently, it has been shown that IFN-γ inhibits the transcription of collagen in fibroblasts and abrogates its stimulation induced by TGF-β (Chung et al, 1996; Ignotz et al, 1987; Inagaki et al, 1994; Jimenez et al, 1994). Thus, TGF-β and IFN-γ may exert opposite effects on collagen synthesis in the prostate. Because inflammatory T cells secrete both cytokines, their antagonistic interactions regulating collagen synthesis are likely to be of great importance in the maintenance of connective tissue homeostasis (Higashi et al, 1998; Kahari et al, 1990; Varga et al, 1990; Yuan et al, 1999; Yufit et al, 1995). IFN-γ also abrogates other TGF-β responses such as collagenase-3 expression in epithelial cells (Ala-Aho et al, 2000), as well as fibronectin and laminin receptor expression in monocytes (Bauvois et al, 1992). This antagonistic function might also account for the significant negative correlation obtained between the amount of IFN-γ and TGF-β ($p = 0.001$) mRNA expression in the group of infiltrated BPH tissues with BPH histology. Notably, this was the group with the most abundant IFN-γ mRNA expression and the only group showing no significant increase in the expression of TGF-β mRNA. In theory the combination of TGF-β and IFN-γ also inhibits differentiation of T cells and keeps $CD4^+$ T cells in a proliferating Th0 state (Sad and Mosmann, 1994), with a memory phenotype similar to that described for BPH T cells (Steiner et al, 1994).

In BPH tissue, Th0 cells showed decreased IL-2 expression and were predominantly IL-4/IL-13 and IFN-γ positive T cells. A non-Th1/Th2 representative cytokine pattern has been shown in autoimmune thyroiditis (Paschke et al, 1994), rheumatoid arthritis (Steiner et al, 1999; van der Graaff et al, 1999), and chronic hepatitis B and C virus infection (Bertoletti et al, 1997). IFN-γ mRNA expression in nonlymphoid tissue has also been linked to intense lymphocyte infiltration in autoimmune thyroiditis (Paschke et al, 1994) and in colorectal carcinoma by Matsushita et al (1996).

Significant expression of IL-13 in normal prostate and increased expression of both IL-13 and IL-4 in BPH, especially within BPH nodules, might upregulate the production of 3β-hydroxydehydroge-

nase/isomerase type 1 (3β-HSD) by PECs (Gingras and Simard, 1999). 3β-HSD is known to catalyze an essential step in the formation of active androgens and estrogens from dehydroepiandrosterone (Simard et al, 1996). Previously it has been shown that the major product of IL-4– and IL-13–treated normal PECs is 4-DIONE. It arises from a transient formation of testosterone (catalyzed by IL-4/IL-13–induced 3β-HSD) and dehydroepiandrosterone (catalyzed by 17β-HSD). Expression of 17β-HSD type 5 in human prostate (El-Alfy et al, 1999; Lin et al, 1997) and enzyme activity has been shown previously (Dufort et al, 1999; Labrie et al, 1997). Thus up-regulation of 3β-HSD activity by increased IL-4 and or IL-13 expression may lead to increased synthesis of testosterone and dehydrotestosterone, respectively (Labrie et al, 1985, 1994, 1997). In addition IL-4 and -13 have been shown to regulate gene expression of the tissue inhibitor of metalloproteinase-1 and -2 action in immortalized PECs (Wang et al, 1996). Expression of high affinity IL-13 receptors by the prostate carcinoma cell lines PC3, DU145, and LNCaP cells has already been demonstrated (Maini et al, 1997). It has also been established that IL-13 stimulates the proliferation of the prostate carcinoma cell lines (growth stimulation ranged between 21% and 39% of the three cell lines) (Maini et al, 1997). However, the expression pattern of both the IL-4 and IL-13 receptors in normal human prostate and in BPH tissue has not yet been investigated. Looking at our data, we concluded that PECs and PSCs express receptor chains involved in IL-4/IL-13 binding (ie, α-chains of the IL-4 and IL-13 receptors and γ-chain of the IL-2 receptor) and that IL-13 is the predominant cytokine that is bound to this receptor in normal and benign hyperplastic prostate.

IL-13 binding of epithelial cells has been shown for glomerular visceral epithelial cells (van den Berg et al, 2000), intestinal epithelial cells (Ceponis et al, 2000) and keratinocytes (Vita et al, 1995). The overexpression of IL-13 stimulates stromal cells to increase collagen, fibronectin, and tenascin synthesis or deposition as shown for the human skin (Oriente et al, 2000; Postlethwaite et al, 1992; Schmidt-Graeff et al, 1994), the lung (Doucet et al, 1998), and keloid fibroblasts (Oriente et al, 2000). Constitutive IL-13 expression in lungs of mice can cause inflammation and subepithelial fibrosis (Zhu et al, 1999), and administration of IL-13 inhibitor has been shown to block hepatic fibrosis during Th2-dominated inflammatory response (Chiaramonte et al, 1999). In Th1-dominated responses such as in rheumatoid arthritis, IL-13 functions as an inhibitor of the production of proinflammatory mediators. It has been shown that IL-13 transfected into fibroblasts of human synovial tissue explants blocks production of activated monocyte/macrophage products IL-1β, TNF-α, and IL-8 (Woods et al, 2000). A similar anti-inflammatory activity paired with a down-regulation of nitrite levels in aqueous humor and enhancement of TNF- and IL-6 synthesis in ocular tissue achieved by systemic administration of IL-13 was described in endotoxin-induced uveitis in a rat model (Marie et al, 1999). Unlike IL-4, IL-13 was shown to lack the ability to induce Th2 cell differentiation due to a lack of expression of functional IL-13 receptors by T cells (Zurawski and de Vries, 1994). Analysis of the role of both cytokines in inflammation suggested that both display common and disparate roles in regulating type-1 and type-2 immune responses. In type-2 responses they are generally proinflammatory and promote local chemokine production. In addition IL-4 promotes Th2 cell development in regional lymph nodes during type-2 responses, whereas IL-13 has a tempering effect. In type-1 responses, low levels of endogenous IL-4 and -13 have a net anti-inflammatory effect by inhibiting local expression of TNF-α, IFN-γ, and MCP chemokines (Ruth et al, 2000).

IL-10 might also play an important role in controlling epithelial and tumor cell growth and is expressed in prostate cancer tissue, but also by infiltrating T lymphocytes in BPH. It has been demonstrated that transfection of IL-10 into prostate cancer cell lines blocks tumor cell growth, angiogenesis, and metastasis (Stearns et al, 1999a, 1999b).

TNF-α is released in substantial amounts by BPH T cells and has been shown to be capable of inducing cell cytotoxicity (Sintich et al, 1999) and prostate necrosis when injected locally (Kramer et al, 2001).

The consequences of increased or massive IL-5 expression have not yet been studied. In preliminary investigations using PCR, we were able to detect production of IL-5 in BPH tissue. It has not yet been shown which cells of the prostate express receptors for IL-5. In any case IL-5 is thought to be a type-2 cytokine.

In conclusion a considerable amount of data suggests that immune response in histologic BPH is of TH0 and TH2. We were also able to show that especially PECs massively bind the TH2 lymphokines IL-4 and IL-13 and express the respective receptors. In addition we showed that PECs produce IL-13 and, as already previously suggested, IL-4 (Kramer et al, 2002).

In light of the important roles played by these cytokines in hormone production, we consider these results highly interesting. To the best of our knowledge, classifying histologic alterations in BPH to a corresponding cytokine pattern has not yet been attempted.

We were astonished to find that in all BPH tissues, the production of TGF-β was significantly elevated. The extent of the increase in TGF-β production has to be seen in context with the release of its natural antagonist (IFN-γ). Only then does it become evident that prostatic cells, especially in infiltrated tissue, seem to counter-balance TGF-β production. Although we did not include these data in this study, one should not fail to mention that BPH T cells also release considerable amounts of TGF-β and FGF-2, thereby directly disrupting the balance.

It is highly unlikely that the increased production of TGF-β is caused only by $CD4^+$ suppressor T cells. While the isolated T cell population produces TGF-β (by far less than stromal and epithelial cells), it also

produces IFN-γ, IL-2, and so forth, as was shown in this study on the cellular level (FACS). Suppressor cells are not capable of producing these growth factors. However, their phenotypic characteristics ($CD4^+$ and $CD25^+$) correspond with those described for suppressor T cells, and it cannot be excluded that a small percentage of suppressor T cells is among the infiltrating cells.

In our working hypothesis, we postulated the existence of a specific suppression mechanism, or a population of suppressor cells that actively suppresses the recognition of prostatic antigens. Sustained suppression is necessary because a whole array of secretory products of the prostate have antigenic properties. This becomes especially important with regard to the fact that production of these proteins reaches its full extent only in puberty, a period of time at which the immune system has already fully matured. It is highly likely that hormones play a crucial role in this. That is to say, age-dependent weakening of the immune system, coupled with modified hormonal secretion, leads to the deterioration of this barrier and to gradual infiltration of the prostate by lymphocytes. These infiltrating cells are responsible for the production of the indicated cytokines, which alter the micro-environment. Once initiated, this process leads to continual immigration of T cells into the tissue. The cells seem to find favorable conditions, as they do not become apoptotic. Chronic activation leads to continual attraction of ever more T cells, attracted by increased production of proinflammatory cytokines such as IL-6, IL-8, IL-15, and IL-17 (Steiner et al, 2003), thus perpetuating chronic inflammation. When local accumulation of lymphocytes reaches a certain threshold, surrounding cells become targets and are killed either specifically or in bystander reactions, leaving behind vacant spaces, which are invaded by further leukocytes. These leukocyte clusters are detectable and are very often seen in close proximity to epithelium. A possible outcome of this could be the development of scar tissue. Formation of nodules and large amounts of connective tissue might be a consequence (Eickelberg, 2001).

Materials and Methods

Specimen Collection

BPH resection tissues from 30 patients were obtained by TUR immediately after surgery of noncatheterized patients. Trauma victims aged 18 to 25 years ($n = 5$) were used as normal controls. BPH tissue segments of the central region of BPH nodules ($n = 8$) were derived from suprapubic prostate specimens, and the central regions were identified macroscopically ($n = 8$). TUR/P-BPH resection chips used for RNA preparation were analyzed histologically on an individual basis by the pathologist using frozen sections stained with hematoxylin and eosin. Segments of TUR/P-BPH resection chips with remaining normal histology were marked under the microscope and were used for mRNA preparation in the BPH tissue with normal histology group. In the BPH tissue with BPH histology group, only BPH resection chips with a typical nodular hyperplastic glandular component were selected. This group of BPH specimens with BPH histology was then further divided into a group showing typical signs of significant lymphocytic infiltration and cluster formation and a group without such signs of an inflammatory process.

Pathologic Classification

All specimens were separated into several parts for immunohistology or cell culture. The bulk of the material was embedded in paraffin, sectioned at 2 μm, and stained with hematoxylin and eosin. Histologic classification was performed according to Dohm (1991) and Franks (1976).

Tissue Preparation

BPH tissue was minced into $< 3\text{-mm}^3$ fragments and extensively washed with PBS containing heparin (Novo Nordisk A/S, Bagsvaerd, Denmark) to prevent potential peripheral blood contamination.

Enzymatic Dissociation

Fragments of prostatic tissue were incubated with 200 U/ml type-1 collagenase (Sigma Chemical Company, St. Louis, Missouri) and 100 μg/ml DNAse type-1 (Sigma) in RPMI 1640 medium plus 10% FCS and 6% penicillin/streptomycin solution (Gibco BRL Life Technologies, Gaithersburg, Maryland). The tissue was digested overnight at 37° C. Digested tissue fragments were dissociated using a magnetic stirring bar, resulting in a heterogeneous cell suspension.

Intracellular Cytokine Detection by Flow Cytometry

Flow cytometric assessment of cytokine production was performed according to the technique described by Jung et al (1993) and modified by Willheim et al (1997, 1999). The primary BPH T cells were generated by the method described for primary BPH T cell enrichment (Steiner et al, 1994). Semi-pure BPH T cells were stimulated for 4 hours with phorbol 12-myristate 13-acetate (10 ng/ml; Sigma) and ionomycin (1.25 μM; Sigma) in the presence of monensin (1 μM; Sigma) for 4 hours at 37° C in 5% CO_2. The cells were then harvested on ice, washed twice in PBS (PBS), and fixed with 2% formaldehyde (1 ml per 2×10^6 cells; Merck) for 20 minutes. After two additional washes in PBS, the cells were re-suspended in Hank's balanced salt solution (supplemented with 0.3% BSA and 0.1% sodium-azide) and stored at 4° C in the dark until they were stained. The fixed cells were washed twice with PBS and made permeable with saponin (0.1%; Sigma), re-suspended with 50 μl of saponin buffer-diluted antibodies, and incubated for 25 minutes at room temperature in the dark. The following monoclonal antibodies (MAbs) were used: cytokine-specific mouse anti-human MAb (fluorescein-isothiocyanate [FITC]-labeled anti–IFN-γ [clone B27])

and rat antihuman MAb (phycoerythrin [PE]-conjugated anti–IL-2 [MQ1–17H12], -IL-4PE [MP4–25D2], and -IL-13PE [JES10–5A2]). All MAbs were purchased from BD Pharmingen (San Diego, California). The anti-CD4 MAb and the anti-CD8 MAb were allophycocyanin and peridinin chlorophyll labeled, respectively (Becton Dickinson, Mountain View, California). Four-color staining was performed, and at least 5000 cells were analyzed on a FACSCalibur (Becton Dickinson) equipped with a two-laser system (488- and 630-nm wavelength, respectively). All cytokines were combined with anti–IFN-γ and were stained in conjunction with CD4 and CD8. The data were analyzed with CELLQuest software (Becton Dickinson), and the results are expressed as the percentage of cytokine-producing cells. In terms of the generation of BPH-derived T lymphocyte cell lines, small cell clusters containing highly viable cells were separated from cellular debris by 5 minutes of simple segmentation in a 50-ml tube. The remaining clumps were extensively washed and cultured in RPMI 1640 plus 10% FCS (Gibco) in six-well plates (Falcon, Becton Dickinson) until the clumps became adherent (48 to 72 hours). Thereafter the culture medium was changed, and cells were further cultured in RPMI 1640 + 10% FCS and 100 U/ml of recombinant IL-2 (British Biotechnology Products, Ltd., Abingdon, United Kingdom) and 1% phytohematoglutinin (Gibco). Media was changed twice a week, and outgrowing T cells were cultured in suspension cultures for three passages before testing.

Lymphokine Secretion Assay

After 24 hours T cell harvest cells were re-suspended in a medium containing no recombinant IL-2. On the day of harvest, cells were washed once and adjusted to a number of 1.5×10^6 BPH T cells/ml. BPH T cells were cultured either unstimulated (RPMI-1640+10% FCS) or stimulated with PMA, or the combination of PMA + anti-CD3 (OKT 3, Ortho, 1 μg/ml). After 24 hours supernatants were collected and frozen at $-70°$ C.

Cytometric Bead Array

Fifty μl of supernatant were incubated with 10 μl of anti–IL-2, -4, -5, and -10, TNF-α, and IFN-γ-conjugated cytokine capture beads (human Th1/Th2 cytokine CBA, Becton Dickinson, Mountain View, California) and with PE-conjugated detection antibodies for 3 hours at room temperature in the dark. The amount of cytokines was calibrated using recombinant standards. Following acquisition of sample data using FACSort (Becton Dickinson), the results were generated in graphical and tabular format using BD CBA analysis software.

Total RNA Preparation

RNA preparation of tissues was performed by a modified guanidium thiocyanate/phenol/chloroform extraction technique using TRIzoL Reagent (Gibco, Grand Island, New York). Reverse transcription was performed using 0.1 μg total RNA (analyzed by 260 nm) and GeneAmp RNA PCR kit (Perkin Elmer, Foster City, California). The mixture was incubated for 1 hour at 42° C, and excess of enzyme was inactivated at 95° C for 5 minutes. All cDNAs were normalized to similarly integrated β-actin control signals. Amplified products were analyzed on 1.5% agarose gel (Bio-Rad, Hercules, California) and stained with ethidium bromide.

Reverse Transcriptase PCR

Amplification of cytokine/receptor-coding cDNA was performed with DNA polymerase (AmpliTaq Gold, Perkin Elmer) using 30 cycles of PCR of similar amounts of cDNA, with specific primers. Resulting PCR fragments were separated by electrophoresis on 1.5% agarose gels (Bio-Rad Laboratories) in combination with a 100-bp size marker (Advanced Biotechnologies, Surrey, United Kingdom) and visualized and photographed after ethidium bromide staining under UV transillumination. cDNA of prostatic cells (normal prostatic SC, BPH-SC, BPH-EC, BPH-T, and CaP cells) and of positive control cells, ie, peripheral blood lymphocytes either unstimulated or stimulated with PHA M (Gibco BRL), was tested for lymphokine receptor mRNA expression using primer specific for the following: IFN-γR α-chain (Yano et al, 1996); IL-2R γ-chain (Meazza et al, 1998); IL-2R β-chain (Meazza et al, 1998); IL-2R α-chain (Clontech, Palo Alto California); IL-4R α-chain (Clontech); IL-13R α-chain (Ogata et al, 1998); and β-actin (Clontech).

Real-Time PCR

TaqMan Real-Time PCR was used to quantify IFN-γ, IL-2, IL-4, IL-13, and TGF-β gene amplification using dual-labeled fluorogenic primers and probes specific for IFN-γ, IL-2, IL-4, IL-13, and TGF-β and β-actin (PE Biosystems, Foster City, California). Reaction was performed in a Gene Amp 5700 Sequence Detection System (PE Biosystems). The amount of amplified lymphokine mRNA was correlated to β-actin mRNA content.

The impact of infiltration on lymphokine mRNA expression was analyzed by comparing infiltrated with noninfiltrated tissue samples. The amount of infiltration was graded 0 through 4 (0 for no infiltration, 1 for scattered infiltration without clusters, 2 for single foci of intense infiltration, and 3 for multi-focal intense infiltration). Tissue-derived cDNA was grouped as follows: normal prostate ($n = 5$), BPH tissue samples exhibiting normal histology but that were infiltrated (Grades 1 and 2, $n = 5$), BPH tissue samples exhibiting BPH histology that were noninfiltrated (Grade 0, $n = 10$), and BPH tissue samples exhibiting BPH histology that were infiltrated (Grades 2 and 3, $n = 10$). In addition the central area of macroscopically identified BPH nodules ($n = 10$) was separated and used for cDNA preparation. The differences between the amounts of cytokine mRNA expressed in the different experimental groups were compared statistically (Table 4).

Triple Staining Confocal Laser Scanning Microscopy

IL-4R α-chain was stained using rabbit anti–IL-4R α-chain antiserum (Santa Cruz, St. Cruz, California) followed by species-specific biotinylated goat anti-rabbit (Biosource) and streptavidine conjugated red 670 (Gibco, Inchinnan, Scotland). Both PEC-specific mAbs were visualized using species-specific FITC-conjugated goat anti-mouse antiserum (Biosource, Camarillo, California) and either cytokeratin (ck)-13–specific mAb (anti-cyt 8.12, clone K8.12, Sigma, St. Louis, Missouri) for basal or anti-ck18 mAb (Boehringer Mannheim, Mannheim, Germany) for secretory PEC. The free anti-mouse binding sites were blocked using 1:10 diluted mouse serum (Dako, Glastrup, Denmark) at room temperature followed by direct conjugated rat monoclonal anti–IL-13 PE (Pharmingen, Becton Dickinson, San Jose, California) antibody. Reactivity was analyzed simultaneously using confocal laser microscope with 3 photomultiplier (cLSM; Zeiss, Jena, Germany). Sensitivity of each of the three channels was adjusted using appropriate double reactive controls in which always one of the three antibodies was replaced by equal amounts of control sera or mouse IgG_1. Single anti–IL-4R α-chain reactivity is indicated in red, anti-IL13 reactivity in blue, and anti-ck18 and -ck13 reactivity in green.

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