Interferons and their receptors in human papillomavirus lesions of the uterine cervix

M. Cintorino[1], *M.D.*; **S. A. Tripodi**[1], *M.D., Ph.D.*; **R. Romagnoli**[2], *B.Sci.*; **F. Ietta**[2], *B.Sci.*; **M. G. Ricci**[3], *M.D.*; **L. Paulesu**[2], *M.D.*

[1]*Institute of *Pathological Anatomy and Histology;* [2]*General Physiology and* [3]*Obstetrics and Gynecology of the University of Siena, Siena (Italy)*

Summary

Purpose of investigation: In this study we analyzed the immunohistochemical expression of specific types of interferon (IFN) in human papillomavirus (HPV) associated cervical lesions.

Methods: Reactivity to anti-IFN-α,-ß and -γ and to anti-IFN-α/ß- and γ-receptors was tested in 33 cervical punch biopsies from 24 HPV-infected women and nine healthy controls. The HPV-infected cases were subdivided into low-risk and high-risk groups, according to the known "oncogenic" potential of the HPV-types detected by PCR.

Results: Cervical epithelium and stroma in HPV-negative as well as low-risk HPV-positive samples were diffusely stained by anti IFN-α, ß and γ antibodies. In contrast, a significantly lower percentage of high-risk HPV-infected tissues was immunoreactive to IFN-ß in the stroma and IFN-γ in the epithelium. There were no relevant differences between control and HPV cases in the expression of IFN-receptors.

Conclusion: We show that a decreased production of some specific classes of IFN is associated with high-risk-type HPV lesions suggesting an important role of IFN distribution patterns in the pathogenesis of HPV lesions.

Key words: IFN; HPV; Uterine Cervix.

Introduction

Many studies have demonstrated that HPV infection plays an important role in cervical carcinogenesis [1-3]. Furthermore, an association between specific types of human papillomavirus (HPV) and risk of progression towards cervical intraepithelial neoplasia (CIN) has been established [4]. Some of the genital HPVs identified so far (e.g., 16, 18, 31 and 33) are considered high-risk (HR) types on account of their frequent association with genital lesions which rapidly progress through increasing grades of CIN into invasive squamous cell carcinoma (SCC). Other genital HPV types (e.g., 6 and 11) appear to play a less significant role in this process since they are usually (but not exclusively) associated with benign lesions and they are considered low-risk (LR) types [5]. The reason for the differences in the oncogenic potential of each HPV subtype in cervical carcinogenesis remains unknown. Oncogenes E6 and E7 of HPV have been suggested to play an important role in the differences in oncogenic potential of each HPV subtype in cervical carcinogenesis [6]. In addition to the oncogenic potential of each HPV type, the highly variable biological and clinical behavior of genital HPV infections appears to be influenced by a number of other factors. In particular, the role of systemic or local immunity in the pathogenesis of HPV lesions has been suggested by many studies, both in vivo and in vitro. Conditions associated with systemic immunosuppression, such as pregnancy [7] or corticosteroid treatment [8] or HIV infection [9, 10], increase the susceptibility of cervical cells to HPV infections, which in turn may result in a higher risk of neoplastic transformation [11]. Similarly, localized immune dysfunction has been associated with HPV-induced lesions [12].

One of the key functional parameters of local immune response is the production of cytokines, including Interferon (IFN), which is also well-known as a potent antiviral substance [13]. IFN-α, IFN-ß and IFN-γ are the three major groups of the IFN family and are classified as type-I (α, ß) and type-II (γ). Type-I IFN has sufficient structural homology to act via the same surface receptors (IFN-α/ß-R) whereas type-II IFN has a separate binding protein (IFN-γ-R) [14]. Previous studies have shown this family of cytokines to have a role in the pathogenesis of HPV infection [15], but the relationship between the presence and distribution of IFN and HPV strains in cervical HPV-associated lesions has not yet been investigated. As such, we analyzed the immunohistochemical expression of the three major classes of IFNs in uterine cervix biopsies in relation to HPV type. Moreover, since IFNs are known to exert their biological activities by binding to specific cell surface receptors, the distribution of immunoreactive receptors binding IFN-α/ß and IFN-γ, respectively, was also analyzed.

Materials and Methods

Selection of cervical tissue samples

The material for our study consisted of 33 alcohol-fixed and paraffin-embedded cervical punch biopsies, selected from the files of the Institute of Pathology of the University of Siena. All samples came from women aged between 25 and 38 years. Twenty-four biopsies were diagnosed as CIN HPV-associated

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and nine as histologically normal, HPV-negative cervical biopsies. CIN was classified as either low-grade (CIN1) or high grade (CIN2/3), according to standard criteria. Consecutive section of 4-5 μm were employed for polymerase chain reaction (PCR) and immunohistochemistry.

Polymerase chain reaction

HPV typing was performed using the PCR method as previously described [16], with slight modifications. Briefly, DNA was extracted from sections of the alcohol-fixed, paraffin-embedded material according to Wan *et al.* [17]; 5-μm sections were prepared from blocks from which the excess paraffin had been trimmed. The sections were handled with clean tweezers and placed (one per tube) into 1.5-ml microfuge tubes. The sections were dried under vacuum. Fifty μl of digestion buffer (50 μM Tris-pH 8.5, 1mM EDTA) containing 200 μg/ml of proteinase K (Boehringer, Mannheim, Germany) were added to the extracted, dried samples. After incubation (3 h at 55°C), protease activity was stopped by heating the sample at 95°C for 10 min. Any residual tissue was removed by centrifugation. The supernatant template DNA was utilized for the amplification. PCR was then carried out in a M. J. Research Mini-cycler using general primers amplifying different mucosotropic HPV types by polymerase chain reaction (PCR). The sequences of the primers were TATGGCTATTCTGAAGTGGAA (sense) and TTGATATACCTGTTCTAAACCA (antisense); the expected size of the amplified fragment was 526-595 bp long, depending on the HPV size [16]. The reaction mix contained 5 μl of the template DNA, PCR buffer (Polymed-Biotechnology Division, Florence, Italy), 1.5 mM of $MgCl_2$, 0.5 U of Taq Polymerase (Polymed-Biotechnology), 25 pM of each primer and H_2O up to 50 μl. Each reaction mix was overlaid with a small drop of mineral oil.

The PCR cycle was carried out according to the hot-start technique: denaturation (95°C for 30 min), annealing (40°C for 40 sec) and extension (71°C for 50 sec) for 38 cycles. Ten μl of the product of amplification, when positive, was digested (2 h at 37°C) with 6 U of restriction endonuclease enzyme ALU I (Boehringer) after addition of 7.5 μl of H_2O and 2 μl of PCR I buffer 10x (Polymed-Biotechnology). Digestion patterns were analyzed on 1.2% agarose gel and visualized with ethidium bromide stain. With this method, we determined the correct typing of the HPV DNA by examining the patterns of different restriction maps: HPV 6 and 11 produced the same pattern (a large fragment of 555 bp), while the amplification products from HPV types 16, 18, 31 and 33 produced a unique pattern for each type: HPV 16 (343, 240 bp), HPV 18 (267, 238, 90 bp), HPV 31 (231, 157, 102, 36 bp) and HPV 33 (287, 141, 116, 21 bp). For each series of reactions, positive and negative samples were added.

Immunohistochemistry

Immunohistochemistry was performed using the alkaline phosphatase anti-alkaline phosphatase (APAAP) method MASON. The characteristics of the monoclonal antibodies (mAbs) [18-21] used in this study and the conditions for their use are listed in Table 1.

Table 1 — *Monoclonal antibodies used in this study.*

Monoclonal antibodies	Specificity	Source	Concentration
LI-1	Hu IFN-α	F. Hoffmann-La Roche Basel, CH	2.5 μg/ml
F14F11	Hu IFN-β	F. Hoffmann-La Roche	5.0 μg/ml
γ69	Hu IFN-γ	F. Hoffmann-La Roche	40 μg/ml
IFN-αR3	Hu IFN-α/β-R	O. Colamonaci Memphis, USA	10 μg/ml
R38	Hu IFN-γ-R	F. Hoffmann-La Roche	5.0 μg/ml

The specificity of the mAbs against human IFN-α, ß, γ and γ-R was confirmed by previous incubation of the mAbs (30 min at 37°C) with human IFN-α, ß, γ (NIAID, Bethesda, MD) and soluble IFN-γ-R (Hoffmann-La Roche, Basel, Switzerland), respectively, at a molar ratio of 1:1 before they were used for tissue staining. The specificity of the anti-IFN-α/ß-R mAbs was confirmed by absorption of the antibody (1 h at 0°C) into Daudi cells (Burkitt lymphoma cell line).

Under light microscopy, the reactive epithelial or stromal inflammatory cells (when present) were revealed by a diffuse cytoplasmic Magenta red staining.

Statistical Analysis

Statistical analysis was performed by the chi-square and Fisher methods using a statistical software package (SYSTAT-7). Statistical significance was established at $p < 0.05$.

Results

All CIN HPV-associated biopsies, both CIN 1 (16 cases) and CIN2/CIN3 (8 cases), showed the presence of HPV DNA (HPV-positive) on the basis of PCR analysis. Biopsies including HPV 6 and 11 were classified as LR (n=6) while those including HPV 16, 18, 31 and 33 (n=18) were classified as HR according to the oncogenic capacity of the HPV type [4]. All cases with no evidence of condylomatous lesions (n=9) were negative for HPV DNA and were used as controls (Table 2).

Table 2 — *Histopathology and HPV typing.*

No. of cases	CIN1	CIN2/CIN3	HPV Type
6	6	—	6.11*
9	4	5	16**
3	3	—	18**
3	2	1	31**
2	1	1	33**
1	—	1	18.33**

* Low-risk (LR) HPV type
**High-risk (HR) HPV type

Immunohistochemistry

Anti-IFN-α, ß and γ antibodies stained epithelium and stroma in HPV-negative as well as HPV-positive cervical lesions. Cervical keratinocytes showed both cytoplasmic and membrane staining. Focally, basal keratinocytes showed nuclear staining (Figure 1). In some cases, intense cytoplasmic staining was present in the reticular elements located in the intermediate layers and compatible with Langerhan's cells (Figure 2). In the stroma, mesenchymal cells and the endothelium of small vessels appeared intensely stained by anti-IFN-α, ß and γ antibodies (Figure 3). The immunostaining of the endocervical epithelium at the squamocolumnar junction was low or absent for IFN-α, ß and γ in normal cases as well as all HPV-associated cases (Figure 4).

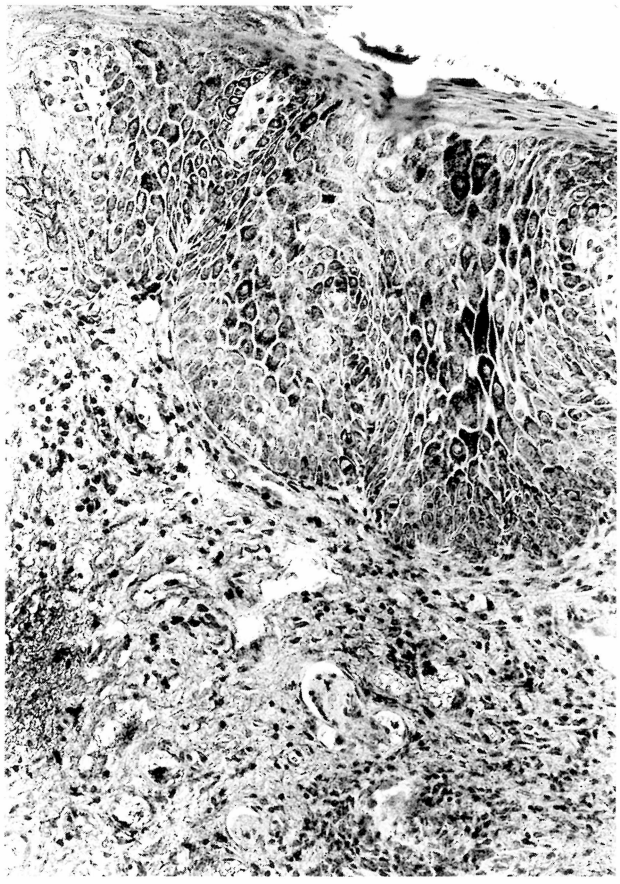

Figure 1. — Immunohistochemical localization of IFN-ß in condyloma (HPV 6 positive) of the exocervix. Strong cytoplasmic immunoreactivity is evident in all the epithelial layers. Some IFN-ß-positive cells are also present in the underlying stroma.

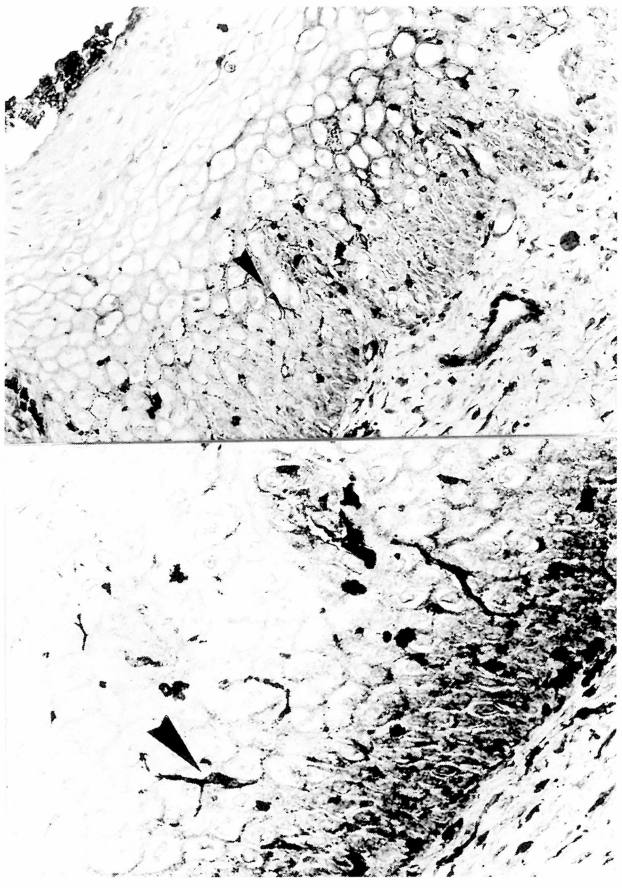

Figure 2. — In this HPV18-positive case, the epithelial cells are not reactive to anti-IFN-ß mAbs. However, immunostaining with the same mAbs is evident in the reticular elements located in the lower epithelial layers and compatible with Langerhan's cells.

Semiquantitative evaluation of immunohistochemical results indicated that both epithelium and stroma were diffusely stained in 100% of HPV-negative and LR HPV-positive samples. In contrast, a lower percentage of HR cases was immunoreactive to IFN-ß and IFN-γ in the epithelium, and to IFN-α and IFN-γ in the stroma. These results are expressed as histograms of frequency (Figure 5). Such differences were statistically significant ($p < 0.05$) for IFN-ß in the stroma and IFN-γ in the epithelium, but not for IFN-α.

All of LR HPV-positive as well as HPV-negative samples were immunostained by anti-IFN-α/ß-R and -IFN-γ-R antibodies. The staining was present in both epithelial and stromal cells. A lower percentage of HR cases was stained by anti-IFN-α/ß-R and -IFN-γ-R antibodies but differences were minimal and not statistically significant (Figure 6). There was neither relevant difference between the distribution patterns nor staining intensity for the two receptors.

Discussion

Several studies have indicated that IFN-α, IFN-ß and IFN-γ, the three major groups of the IFN family, play an antiviral role in HPV infections. This antiviral effect is mediated by several mechanisms such as decreased expression of histocompatibility-related antigen (HLA) Class I molecules by infected keratinocytes [22] or an upregulation of ICAM-I [23]. The present study analyzed for the first time the distribution of this family of cytokines in normal and HPV-infected cervical tissues. Our data show that all three major types of IFN- are expressed by cervical epithelium and stroma in HPV-negative as well as in HPV-positive cervical biopsies infected with HPV strains at low risk of malignant transformation. In contrast, a lower number of high-risk HPV-positive cases reveals staining for IFN-ß and IFN-γ. IFNs, particularly IFN-γ, are considered, type-I response cytokines together with Interleukin-2 (IL-2) and IL-12. It has been demonstrated that type-I cytokines activate cell-mediated immunity, which has a pivotal role in protection against viruses and tumors. The decrease of IFNs in HR HPV lesions seems to be in agreement with several data suggesting a TH1/TH2 shift in the immune response in HPV-induced genital lesions. Firstly, a depressed production of IL-2 and IFN-γ by peripheral blood mononuclear cells has been observed in patients with HPV condylomatous lesions [24]; secondly, a decreased interleukin-2 and

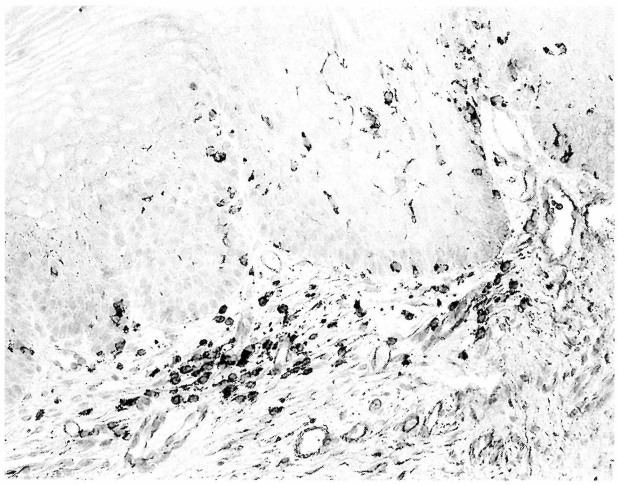

Figure 3. — Strong immunoreactivity is evident in stromal and endothelial cells of this HPV18-positive condylomatous lesion stained with anti-IFN-γ-R mAbs.

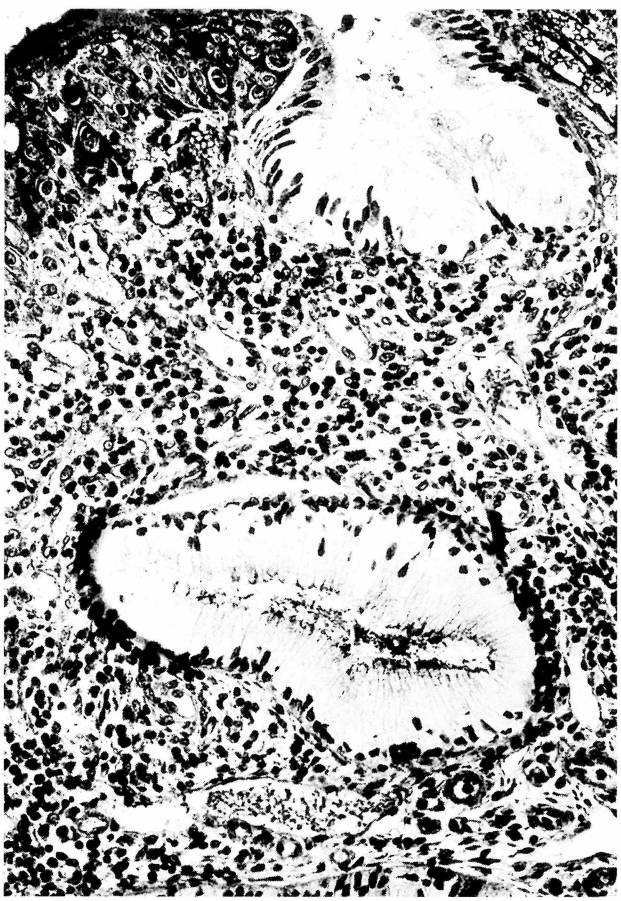

Figure 4. — The immunostaining of the endocervical epithelium at the squamocolumnar junction was low or absent for INFγ in this HPV11-associated lesion. In contrast, strong immunoreactivity is evident in epithelial keratinocytes and stromal cells.

interferon-γ production together with a depressed natural killer activity have been shown in patients with condyloma acuminatum lesions [25]. Finally, the impairment of

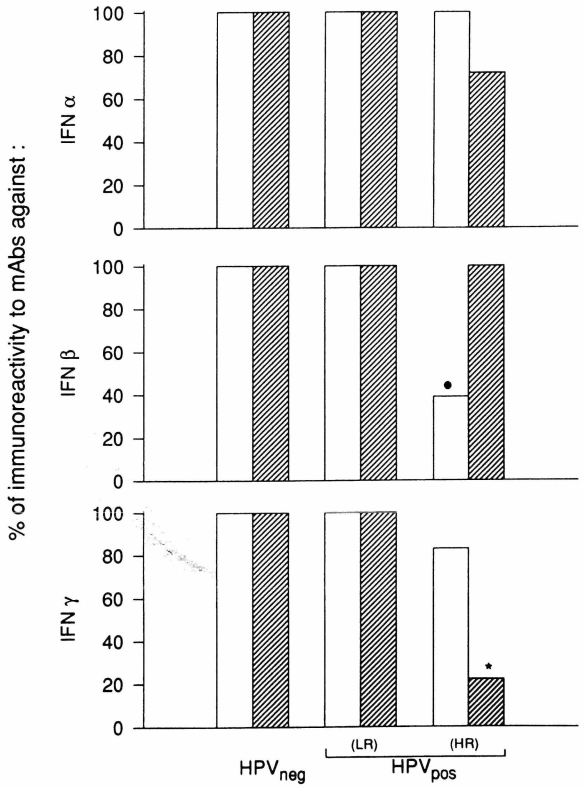

Figure 5. — Percentages of cervical biopsy samples with immunoreactivity to anti-IFN-α, ß and γ according to the presence and type of HPV.
HPV-negative = negative for HPV DNA; HPV-positive = positive for HPV DNA; □ = epithelial cells; ▨ = stromal cells; • = HR HPV-positive vs HPV-negative $p < 0.05$; * = HPV-positive vs HPV-negative $p < 0.025$.

the IFN response in HPV-16 infected cells has recently been demonstrated [26] as well as the inactivation of the interferon regulatory factor-1 tumor suppressor protein by HPV E7 oncoprotein as a mechanism for immune evasion in cervical carcinogenesis [27].

Besides the differences in IFN distribution pattern among the different HPV types, our study underlines the role of each cervical mucosa cell type in determining IFN production. In addition to keratinocytes, which have already been demonstrated to produce IFN [28-30] we showed that the epidermal dendritic cells, primarily represented by Langerhan's cells, are another important source of IFNs in the cervical epithelium. The capacity of cultured epithelial Langerhan's cells to synthesize and secrete IFN has already been reported [31, 32] and, recently, a dendritic cell precursor has been identified as the natural interferon-producing cell [33]. Viral activation represents a powerful stimulus on dendritic cells to release large amounts of IFN, which in turn triggers a cascade of responses to inhibit viral replication. It is noteworthy that relevant modification of dendritic cell number has been reported in HPV-associated cervical lesions [34-38].

The immunoreactivity of stromal cells, such as fibro-

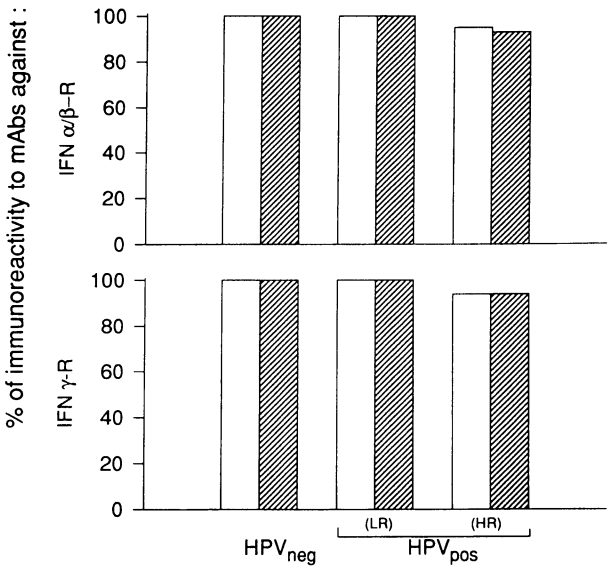

Figure 6. — Percentages of cervical biopsy samples with immunoreactivity to anti-IFN-α/ß- and γ-R according to the presence and type of HPV.
HPV-negative = negative for HPV DNA; HPV-positive = positive for HPV DNA; ▨ = epithelial cells; □ = stromal cells.

blasts, lymphomonocytes and endothelial cells, indicates that the stroma is another important source of IFN in cervical tissues. In particular, the decreased immunostaining of stromal cells with IFN-γ and IFN-α antibodies suggests that cervical fibroblasts and lymphomonocytes actively participate in the immune response against HPV. The importance of fibroblasts in the immune network and their role as sentinel cells against viral infections has been recently recognized [39].

Finally, we observed a low or absent IFN expression in endocervical glands of histologically normal as well as HPV-infected biopsies. The scarcity of such immunostimulating cytokines in the transformation zone, the region of the cervix most sensitive to lesion development, seems to be relevant considering that high expression of immunosuppressive cytokines (IL10, TGF-ß) has been demonstrated in the same location [12].

The effects of IFN are mediated by interactions with cell surface receptors; type-I IFN shares the same receptor (IFN-α/ß-R), while IFN-γ binds to a different one (IFN-γ-R) [20, 21]. Our data indicate no statistically significant differences between healthy and HPV-infected subjects, thereby suggesting that the expression of IFN-R is not relevant with respect to the actions and effects of IFN.

On the whole, our results indicate that the type and distribution of IFN may be of importance in the defense mechanisms against HPV and show that a complex network of cells, such as epithelial and Langerhan's cells in the epithelium, fibroblasts and lymphomonocytes in the stroma, actively participate by secreting IFNs during the immune response against HPV-assosciated cervical infections.

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Address reprint requests to:
S. TRIPODI M.D.
Institute of Pathological Anatomy and Histology
Via delle Scotte
53100 Siena (Italy)