Expression of Simple Epithelial Keratins 8 and 18 in Epidermal Neoplasia

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A systematic study of keratin expression in epidermal lesions (six actinic keratoses, 10 Bowen's disease, seven squamous cell carcinomas) has been undertaken by using a large panel of monoclonal antibodies to individual keratins. Expression of differentiation-specific keratins was frequently delayed or lost from dysplastic regions. Novel expression of the embryonic, or simple epithelial, keratins 8 and 18 was widely observed in intradermal areas of poorly differentiated squamous cell carcinomas. In addition, the most proliferative of in situ malignancies (Bowen's disease) also contained small numbers of cells expressing simple epithelial keratins. These observations suggest that the expression of simple epithelial keratins may be of functional importance in malignancy of keratinocytes and could be related to tumor invasion and/or to changes in epithelial-mesenchymal interactions. *J Invest Dermatol* 97:763–770, 1991

Keratins, the major structural proteins of epithelial cells, including keratinocytes, display the greatest heterogeneity of all the intermediate filament proteins [1]. They form a complex family of at least 30 polypeptides divided about equally between two gene families (type I and type II intermediate filaments). They are distinguishable from one another on the basis of isolectric point and molecular weight and are numerically classified as such [2]. As with other intermediate filaments, keratins are usually conserved during malignant transformation when all other identifying criteria of the cell of origin may have been lost [3], so that their identification in poorly differentiated tumors can help to establish the epithelial origin of the malignancy [4]. A close correlation has been established between the histologic category of an epithelium and its keratin profile, with primary keratins 8 and 18 characterizing simple epithelium and primary keratins 5 and 14 characterizing stratifying epithelia [5]. This facilitates further classification of undifferentiated epithelial tumors into adenocarcinoma (simple epithelium derived) or squamous cell carcinoma (stratified epithelium derived) [2]. In addition to the diagnostic importance of keratin detection, it is clear that keratin expression is closely linked to epithelial differentiation; as well as the primary keratins, a number of pairs of secondary keratins are expressed by the terminally differentiating cells of stratified epithelia, with each pair characteristic of a particular histologic type of epithelium [5].

The keratin profile of a stratified epithelium such as skin is complex, but the pattern of expression follows basic rules [5], central to which is the concept of expression of keratins in pairs, whereby keratin filaments are heteropolymers of equimolar amounts of one acidic (type I) and one basic (type II) keratin [6,7]. Beyond this, the keratin profile of an individual cell is dependent not only on the epithelial cell type (simple or stratified), but also on the differentiation program (cornifying or non-cornifying) and the rate at which the tissue cells are turning over [5]. In normal human skin, a sequence of keratin changes occurs as the keratinocyte moves from the basal layer through the spinous and granular layers to the stratum corneum [8]. Basal keratinocytes contain the primary keratins 5 and 14 [9] and on leaving the basal layer secondary keratins 1 and 10 additionally appear [8], this keratin pair being characteristic of cornifying, or skin-type, differentiation [5]. Non-cornifying stratified epithelia such as esophagus express secondary keratins 4 and 13, and corneal epithelium expresses keratins 3 and 12 upon commitment to terminal differentiation [10]. In situations of faster cell turnover, suprabasal expression of keratins 6 and 16 occurs [11], overlapping with the tissue-specific secondary pair [12]. Keratins 8 and 18 are expressed by simple epithelial cells that in the skin are represented by cells of the sweat gland (secretory cells) [13], but, with the exception of Merkel cells [14], are not present in the epidermis.

Keratins may be detected biochemically or immunohistochemically, each technique having advantages and disadvantages. The main shortcoming of biochemical techniques is their inability to differentiate in tissues between a large number of cells producing a low level of keratin, and a few cells producing high levels. Even two-dimensional gel electrophoresis will not detect subtle differences in keratin expression by small populations of cells. Immunocytological methods allow in situ visualization of heterogeneous populations, and the recent generation of monoclonal antibodies has substantially increased the range of available markers. A number of highly specific antibodies are now in existence, such that most of the keratin expression can now be identified unequivocally in situ, in frozen tissue sections [10,15,16,17]. Using a comprehensive panel of monoclonal antibodies to human keratins we have examined the
development of epidermal squamous malignancy in normal skin, pre-malignant (actinic keratosis), in situ malignant (Bowen’s disease), and invasive malignant (squamous cell carcinoma) lesions of the epidermis. Such studies may help in the understanding of the biologic mechanisms of cutaneous carcinogenesis, and may have important diagnostic and prognostic implications.

MATERIALS AND METHODS

Tissue Samples  Elliptic biopsy of epidermal lesions was performed for diagnostic purposes using 1% lignocaine (no adrenaline) as local anaesthetic. Specimens were bisected, half undergoing routine processing for histopathologic assessment while the other half was placed in liquid-nitrogen-cooled isopentane using OCT cryo-embedding medium (Tissue Tec, Miles, USA) prior to storing in liquid nitrogen at −70°C until required. The diagnosis of squamous cell carcinoma (seven cases), Bowen’s disease (10 cases), and actinic keratoses (six cases) was confirmed by conventional histologic analysis. Patients receiving immunosuppressive therapy or suffering from medical illnesses likely to compromise their immune status were excluded from this study. In addition, normal skin was examined as control material.

Immunohistochemistry  Five-micrometer cryostat sections were cut and stained using a routine three-step peroxidase anti-peroxidase reaction as previously described [18]. Frozen sections were used throughout this study as formalin fixation diminishes the immunocytochemical detectability of keratins with most monoclonal antibodies [19]. Briefly, cryostat sections were air-dried for 20 min prior to fixing in acetone for 5 min, followed by washing in Tris-buffered saline (TBS) for 5 min. Following incubation with 20% swine serum for 10 min, 100 µl of the optimal dilution (see Table 1) of each primary antibody was applied to the section for 60 min at room temperature, followed by 30-min incubations of a rabbit anti-mouse bridging antibody, followed by peroxidase-anti-peroxidase complexes (all second-layer antibodies were obtained from DAKO Ltd., High Wycombe, Bucks, U.K.). Sections were thoroughly washed with TBS between all stages of staining. Finally, the sections were incubated with 3,3′-diaminobenzidine (DAB) for 10 min in the dark, prior to rinsing and counterstaining in Mayer’s hematoxylin and mounting in DPX.

Antibodies Used  Antibodies used, their dilutions, and keratin specificity are listed in Table I. This set was selected from a large bank of over 70 monoclonal antibodies as being the strongest and most reliable antibodies in their tissue reactivity. With the exception of 2.1.D7 (a rat monoclonal) and the rabbit polyclonal antiserum to keratins 5 and 1, all antibodies used were mouse monoclonal antibodies. For each keratin, a number of different antibodies were used to reduce the likelihood of a negative result being due to conformational change or sequestration of the keratin epitope rather than true absence of the keratin protein. Antibodies to the intermediate filament proteins vimentin, desmin, neurofilament, and glial fibrillary acid protein were also used.

Controls  Secretary cells of sweat glands acted as positive internal controls in skin sections for the simple epithelial keratins 7, 8, 18, and 19 [13]. In addition, all simple epithelial keratin antibodies were tested on either lung or intestine as positive controls. Sections of cornea and oral mucosa were used as positive controls for keratins 3 + 12 and 4 + 13, respectively. Perilezial skin acted as a positive internal control for keratins 5 + 14 and keratins 1 + 10 and basal cell markers. Skin from porocarcinoma lesions was used as a control for keratins 6 + 16 [11]. Negative controls included omission of primary antibodies, substitution by irrelevant monoclonal antibodies of similar isotype, omission of second- and third-step reagents, and reaction of sections with DAB alone.

RESULTS

Epithelial Reactivity of Antibodies in Normal Tissue  Antibodies to the same keratin or keratin group produced similar staining patterns.

Keratins 5 and 14:  The antibodies LLOO1 and LLOO2 [20] were raised against a synthetic carboxyterminal peptide of K14 and gave panepithelial staining of epidermal cells up to the granular layer. The monoclonal antibody 2.1.D7 [20] and polyclonal RaK5 [20], both to keratin 5, stained all basal and parabasal cells but did not extend as far towards the epidermal surface as the anti-keratin 14 antibodies.

Basal Markers:  LH6, LH8 [20], and PAb421 [21,22] recognize epitopes specific to basal cells. These epitopes appear to be conformation-dependent, as they are not identifiable by immunoblotting, although the observed affinity of these antibodies for keratin filaments suggests the epitopes may involve K5 and K14. The restriction of staining of these antibodies to the basal layer only presumably relates to a conformational masking [21] of the epitope due to the addition of suprabasal keratins.

Keratins 1 and 10:  All suprabasal keratinocytes stained strongly with these antibodies (LH1, LH2, RKSE60, K8.6.0, RaK1) [13,21].

Keratins 6 and 16:  Monoclonal antibody LMM3 to keratin 16 [23] gave no staining in normal interfollicular epithelium. Keratins 6 and 16 are expressed suprabasally in glabrous skin and in situations of epidermal hyperproliferation and wound healing [24], as well as in normal suprabasal oрогenital mucosa [12].

Keratins 4 and 13:  AE6 and 6B10 gave no staining in normal skin; KS13.1 showed widespread cross-reaction with tissue sections and could not therefore be interpreted. Keratins 4 and 13 are normally present suprabasally in stratified, non-cornifying epithelium [12,25].

Keratins 3 and 12:  AE5 produced variable cross-reactivity with normal epidermis, making interpretation difficult, although it gave strong staining in cornea [10] control section.

Keratins 8 and 18:  Staining in skin with antibodies to these keratins (Table I) was restricted to secretory cells in the sweat glands, and Merkel cells in the epidermis [14,26].

Keratin 7:  LP1K and LP5K gave staining of the inner hair root sheath cells down to the hair bulb as previously documented [13]. Sebaceous glands and secretory sweat glands were stained by all antibodies to keratin 7 (RCK105, CK7, LP1K, LP5K). Thus, sebaceous gland epithelial cells are unique in expressing keratin 7 in the absence of other “simple epithelial keratins,” K8, K18, and K19.

Keratin 19:  As with K8/18/7, sweat gland secretory cells were positive, but the keratin 19 antibodies (LP2K, K19.1, KM4.62, BA16, K19.2) also stained the sweat gland duct right up into the epidermis [15]. A population of cells of the outer hair root sheath immediately below the insertion of the sebaceous glands reacted with these markers as previously described [13,15,27]. K19 has also been described in basal cells around nipple ducts [15].

Keratins in Partial-Thickness Epidermal Dysplasia  A total of six actinic keratoses removed from the face or arms all showed similar changes from normal epidermis. Occasional lesional keratinocytes failed to stain with either of the K14-specific antibodies, LLOO1 and LLOO2. Serial sections revealed these cells to be still expressing K5. As the basal conformational markers stained many cell layers beyond the basal zone (Fig 1), including the LLOO1/ LLOO2 negative cells, we cannot rule out that such negative cells are the result of partial proteolysis of the terminal region of K14 to give loss of this C-terminal epitope, rather than reflecting complete absence of the polypeptide. The differentiation keratins 1 and 10 were delayed in appearance, staining only the upper zones of dysplastic areas (Fig 2). This delay mirrored the expanded staining zone of the basal markers, suggesting that the presence of keratins 1 and 10 expression is directly responsible for the restriction of the basal conformationally determined epitopes. K16 was present suprabas-
**Table I. Monoclonal Antibodies to Cytokeratins**

<table>
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<tr>
<th>Ab</th>
<th>Reference/Source</th>
<th>Dilution*</th>
<th>Target</th>
<th>Ab</th>
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<td>Vimentin</td>
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*All primary antibodies used as undiluted tissue culture supernatant unless otherwise stated.

sally in keeping with the hyperproliferative profile of the lesion. All other epidermal keratin staining was negative.

**Keratin Markers in In Situ Malignancy** Of the 10 cases of Bowen's disease studied, all showed full-thickness atypia histologically, but only two cases were markedly proliferative. In seven of the 10 cases, widespread loss of suprabasal staining with LLOO1 and LLOO2 for keratin 14 was seen, although a single layer of cells on the basal lamina always remained positive. Like actinic keratoses, serial sections showed that the LLOO1-negative cells still expressed K5 (Fig 3). In five of 10 cases, basal-specific conformation-dependent markers were negative in much of the basal layer, suggesting that K14 and its association with K5 is abnormal down to and including the basal layer. The individual keratin profile of cells confirmed the histologic impression of full-thickness atypia in Bowen's disease. In the two most proliferative cases, however, widespread staining of cells with LH8 and LH6 (not PAb421) was noted, together with associated widespread loss of keratins 5 and 10. In the non-proliferative cases, keratins 1 and 10 showed delayed expression similar to the pattern seen in actinic keratoses. Widespread suprabasal expression of keratin 16 was observed.

An unexpected finding in the two proliferative cases was the presence, at the deep dermal invaginations, of occasional cells that were positive for one or more of keratins 7, 8, 18, and 19 (Fig 4). Such cells were rare, but were unequivocally positive with multiple antibodies.

**Keratin Markers in Invasive Malignancy** Within the seven cases of squamous cell carcinoma (SCC) studied, considerable heterogeneity of staining was observed. Well differentiated areas within tumors were quite distinct from poorly differentiated zones. In well differentiated areas staining for keratins 5 and 14 was well preserved, more so than in Bowen's disease. However, poorly differentiated areas showed widespread loss of keratin 14 staining, which (unlike that in Bowen's disease and actinic keratosis) was now accompanied by similar loss of keratin 5 staining. The border between well differentiated (K14+ve) and poorly differentiated (K14−ve) areas was often abrupt. In keratin 5− and 14−negative areas, basal markers were also negative. By contrast, in keratins 5− and 14−positive areas, PAb421, LH8, and LH6 stain full-thickness epidermis, reflecting the extended accessibility of the basal cell epitope in the absence of keratins 1 and 10. Whether well or poorly differentiated areas were examined, staining for keratins 1 and 10 was absent (Fig 5), suggesting that cells were not following ordered skin-type differentiation pathways. No alternative pathways of terminal differentiation such as oral (K4/13) or corneal (K3/12) keratin expression could be demonstrated. Keratin 1− and keratin 10−negative cells were stained with LMM3, demonstrating the expression of keratin 16 in these cells, which is usually associated with rapid proliferation.

The most profound abnormality demonstrated in this study was the widespread expression by all SCC of keratins normally restricted to simple epithelial cells, keratins 7 (3 of 7 cases), K8 (4 of 7), K18 (6 of 7), and K19 (5 of 7) were demonstrated by multiple antibodies of the relevant specificity (Fig 6). Although well differentiated areas did express small amounts of simple epithelial keratins, most of this staining was seen in keratin 14-negative, poorly
Figure 1. Expression of reactivity of LH8 from a single basal layer found in normal skin to many cell layers beyond on entering an area of actinic keratosis (magnification \( \times 125 \)).

differentiated areas (Fig 7). Serial sections demonstrated coexpression of multiple simple keratins.

The cells in SCC stained with LP34, which detects both simple and stratified epithelial keratins, and no expression of other intermediate filaments of vimentin, desmin, neurofilaments, or glial fibrillary acid protein was seen.

DISCUSSION

A number of previous studies have looked at the expression of keratins in epidermal neoplasias, but the overall results have not been consistent. This may be due to the insensitivity of gross biochemical analyses, to tumor heterogeneity, and to differences in minority cell numbers, or to substantial differences in the specificity and sensitivity of antibodies used in different laboratories. To reduce these problems, we have used peroxidase anti-peroxidase techniques for in situ resolution of cellular heterogeneity; we have deployed a selected group of monoclonal antibodies that are all consistently strong and not cross-reactive, and have used multiple antibodies nonspecific for each keratin wherever possible to avoid ambiguities caused by conformational artefacts. In addition, we have avoided including squamous carcinomas arising in immunosuppressed patients (such as renal transplant patients) because squamous-proliferative lesions arising in such patients may constitute a specific subgroup of epidermal tumors (in particular, related to papilloma virus).

The results of this study have indicated that simple epithelial keratin expression by lesional keratinocytes occurs in squamous cell carcinomas of the skin, that in epidermal neoplasia, the expression pattern of the keratinocyte keratins 5/14 is often disturbed, and that expression of differentiation-specific keratins 1/10 is reduced.

Disturbed Expression of Keratins 5 and 14 In all stages of keratinocyte transformation studied, from premalignancy through carcinoma in situ to invasive malignancies, reductions from the normal staining pattern of antibodies LLOO1 and LLOO2 to keratin 14 were seen. Keratin 5-reactive antibodies and antibodies recognizing conformationally determined basal cell epitopes mostly showed normal staining of the LLOO1/LLOO2-negative cells. Some of the negative results may be due to antigenic alteration rather than true loss. Other antibodies to the keratin 14 carboxy-terminal peptide similar to LLOO1 and LLOO2 also do not stain the stratum corneum in normal epidermis, where programmed proteolysis of keratins takes place as part of terminal differentiation [20,28]. However, in poorly differentiated areas of SCC all markers associated with keratin 5 and keratin 14 were negative, possibly representing a real downregulation or loss of these keratins rather than loss or sequestration of specific epitopes. Reduction in K14

Figure 2. Delayed expression of keratin 10 in actinic keratosis using RKS660 antibody compared to the normal expression in epibasal cells (adjacent section of Fig 1). (Magnification \( \times 125 \).)

Figure 3. Many keratinocytes in Bowen’s disease fail to stain with keratin 14 antibodies but continue to stain normally for the paired keratin 5, here using RaK5 (magnification \( \times 125 \)).

Figure 4. Isolated keratinocytes at the tips of proliferative Bowen’s disease reactive with numerous simple epithelial keratin antibodies. Shown here, keratin 18 staining with CK2 (magnification \( \times 330 \)).
expression in some squamous and basal cell carcinomas has been noted previously [29], and SV40-transformed cultured keratinocytes also show reduced expression of keratins 5 and 14 [30], suggesting that loss or downregulation of these primary stratified epithelial keratins frequently occurs as part of the process of transformation.

Delay or Downregulation of Expression of Keratins 1 and 10

The normal progression of differentiation of keratinocytes from the basal layer up towards the stratum granulosum was markedly disordered in all the lesions studied, in agreement with earlier observations [2,30,31]. Both actinic keratoses and Bowen's disease showed a delay in expression of differentiation keratins 1 and 10 in that these first appeared at a higher-than-normal position in the epidermis. Partial- and full-thickness dysplasia of the epidermis is therefore still associated with stratifying differentiation, but an expanded compartment of poorly differentiated basal-like cells remains, possibly with delayed commitment to their final differentiation pathway.

Invasive squamous cell carcinoma differed as keratins 1 and 10 were absent. These keratins were absent from well differentiated keratin 14-positive areas as well as poorly differentiated keratin 14-negative areas. It is interesting that keratin 16 continues to be expressed suprabasally in the absence of keratins 1 and 10, supporting the concept that these two suprabasal keratin pairs are regulated independently. Overlapping expression of these two keratin patterns has also been seen in oral epithelia [12]. Recent data suggest that K6/K16 expression is not directly coupled to the proliferating state [32].

Appearance of Expression of Simple Epithelial Keratins

This occurs in poorly differentiated invasive areas of squamous cell carcinomas, and rarely in individual cells in highly proliferative in situ malignancies (Bowen's disease). Together with the above loss of keratins in poorly differentiated SCC, there was widespread expression of keratins 18, 8, 7, and 19 by the transformed keratinocytes. In adjacent islands of well differentiated and poorly differentiated SCC, heterogeneous populations of cells were seen, some expressing keratins 5 and 14, others keratins 8 and 18. The expression of keratin 19 may not represent a great divergence from normal differentiation patterns because keratin 19 can be normally expressed in both simple and stratified epithelia [33] and is very closely related to the keratinocyte-specific keratins [15]. Simple epithelial keratins 7, closely related 8, and 18 have not previously been reported in interfollicular epidermis (other than in the non-keratinocyte Merkel cells), although recent reports suggest a low level of expression in the basal cells of some mucosal epithelia [34] and parts of scalp hair follicles [27].

Keratins 8 and 18 are the characteristic (primary) keratins of simple epithelia. They are the earliest keratins to be expressed in the preimplantation embryo [35] when they are present in all embryonic cells, but are subsequently lost from most differentiating tis-
sues. As the epidermis begins to differentiate, expression of keratins 8 and 18 in skin is lost from presumptive keratinocytes and restricted to the periderm [13,36] and then later lost from the epidermis altogether as the periderm is sloughed off from the underlying stratified layers. Thus, the expression of keratins 8 and 18 in keratinocytes of squamous cell carcinomas derived from epidermis represents a major change in differentiation from the phenotype of their non-transformed counterparts.

One explanation for the significance of simple epithelial keratins in small populations of skin keratinocytes in Bowen’s disease may be that this is related to potential invasion of the dermis. A previous report of occasional cells positive for PKK1 in Bowen’s disease [37] is comparable to our findings of simple epithelial keratin expression by occasional cells, as the antibody PKK1, though not monospecific, does react with simple epithelial keratins 8, 18, and 19 and not with interfollicular keratinocytes.

Previous studies of squamous cell carcinoma in skin have not suggested that de novo expression of simple epithelial keratins 8 and 18 is a characteristic feature of invasive squamous cell carcinomas [2,29,31,38–41]. Although the association we observe is consistent, the staining is focal and the protein levels may be too low to detect biochemically. Our results probably reflect the greater sensitivity of immunohistochemical methods now being used, particularly with good affinity monospecific antibodies. The presence of large quantities of other major keratins could also mask small amounts of simple epithelial keratins in biochemical studies if small samples are analyzed. In contrast, when squamous cell carcinomas from other non-cornifying stratifying epithelial sites, such as esophagus and ectocervix, have been examined immunohistochemically, keratins 8 and 18 have frequently been observed [42–48]; and similar results have been obtained from bladder [49]. All these studies have used antibodies known to be particularly strong. In the bladder and cervix studies, it was suggested that these simple epithelial keratins are elevated with increasing degree of dysplasia [47,49]. It is also known that epidermal basement membranes are lost during tumor invasion and the exposed dermal extracellular matrix may have different inductive effects on keratin expression from the basement membrane proteins.

Expression of simple epithelial keratins has been described in transformed epidermal keratinocytes in tissue culture [26,30,50,51], including non-tumorigenic lines and normal epidermal keratinocytes. Interpretation of these data is complicated by possible culture artefacts such as high retinoid levels in the tissue culture medium, which are known to be able to induce mainly keratins 13 and 19 [52]. Over the last 10 years there have been several suggestions that simple epithelial keratin expression in stratifying keratinocytes might be associated with malignant transformation [26,37,46–49]. Our present findings lend weight to these early hypotheses. Such changes are most marked in poorly differentiated areas of tumor in areas of aggressive stromal invasion by transformed keratinocytes.

The in situ studies described in this paper demonstrate the heterogeneity of the malignant cell population in invasive squamous cell carcinoma, together with inappropriate expression of simple epithelial keratins that are normally restricted to very early fetal development or to simple epithelia. It is of particular interest that such changes occur in individual cells within markedly proliferative in situ malignancy that is otherwise not yet frankly invasive. According to the multistep theory of malignancy, the transformation of a normal keratinocyte to a malignant cell would require several steps [53], thought to be random genetic mutations initially occurring in one or two cells [54]. Traditional histologic examination based on morphologic criteria makes it difficult to identify precise boundaries between atypia and carcinoma in situ. “Full-thickness atypia” determined histologically may not represent true biologic carcinoma in situ unless some of the cells have become fully transformed, acquiring the ability to invade and metastasize; many cases of full-thickness atypia exist in the skin for many years as areas of Bowen’s disease and demonstrate a relatively benign growth pattern. We would like to suggest the possibility that aberrant expression of simple epithelial keratins by keratinocytes may be an early marker for the genetic and epigenetic events that render keratinocytes capable of invasion. The substitution of keratinocyte-type cytoskeleton filaments (K5/K14) for a keratin cytoskeleton that is characteristic of simple epithelia (keratins 8/18) may result in a cellular cytoskeleton that is easier to remodel [55], or a cell of greater cytoplasmic plasticity that may consequently be more mobile. Further studies are needed to establish the relationship between the expression of basement membrane proteins and extracellular matrix components with the possible importance of these keratins, preferably in the in vivo context of longitudinal studies of metastatic potential.

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**COURSE ANNOUNCEMENT**

The tenth international course on “Advances on the Basic Sciences applied to Dermatology” will be held October 29 – 30, 1992 in Rome. For further information contact F. Serri and D. Cerimele, Department of Dermatology, Catholic University, Policlinico A. Gemelli, Largo A. Gemelli 8, 00168, Rome, Italy.