Endogenous Skin Fluorescence is a Good Marker for Objective Evaluation of Comedolysis

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Objective evaluation of comedone lesions, especially *in vivo,* remains a challenge. We have used the rhino mouse model in combination with topical application of all-*trans* retinoic acid as a comedolytic agent, to investigate the potential of fluorescence spectroscopy as a noninvasive technique in the assessment of noninflammatory acne. The results indicate that there is a strong correlation between the fluorescence excitation spectral features assessed *in vivo,* and the histologic changes identified, particularly the size of the utriculi as well as the dermal and epidermal thickness. We conclude that fluorescence excitation spectroscopy represents a promising novel and useful tool in the quantitative evaluation of the pseudocomedones and could also be used for the rapid and noninvasive assessment of comedolysis induced by the application of pharmacologic agents such as retinoids. ***Key words: acne/comedones/fluorescence spectroscopy/retinoids/rhino mouse. J Invest Dermatol 115:100–105, 2000***

The rhino mouse ($hr^{rh}hr^{rh}$) is an allele of the hairless mouse ($hr^{hr}hr^{hr}$) and is an experimental animal model for noninflammatory acne. The skin of the rhino mouse is characterized by pilosebaceous apparati with abnormal differentiation and maturation, deep dermal cysts, and a large number of horn-filled structures (remnants of hair shafts) called utriculi, which resemble typical retentional acneic lesions or comedones. It has been shown that retinoids cause the utriculi to empty and collapse (Mann, 1971; Kligman and Kligman, 1979; Bernerd *et al*, 1991; Fort-Lacoste *et al*, 1999). Retinoids are also known to moderate growth and differentiation processes in both developing organisms and adult tissues, including the skin (Elias *et al*, 1981; Kubilus, 1983; González *et al*, 1997). They are also beneficial as therapeutic agents for cutaneous diseases in which alterations of epidermal proliferation and differentiation play a key part, such as acne vulgaris and psoriasis (Kligman *et al*, 1969; Van Scott, 1972; Peck, 1981; Ehmann and Voorhees, 1982). Retinoids such as all-*trans* retinoic acid (tRA), also cause a cutaneous inflammatory response in these animals, which depends on the particular compound, the concentration, and the frequency of application. The rhino mouse model has been widely used in the screening of various topical comedolytic agents, mainly retinoids (Kligman and Kligman, 1979; Mezick *et al*, 1984; Bernerd *et al*, 1991; Sundberg, 1994; Fort-Lacoste *et al*, 1999).

An important problem in the screening of topical comedolytic agents is the detailed measurement and evaluation of their comedolytic action. Using the rhino mouse model, this is usually done by histology. Routine histology, however, is an invasive method, which entails the removal, fixation, sectioning, staining, and visual examination of a tissue sample under the microscope. Furthermore, it requires a multistage sample preparation process, which is time consuming, labor intensive, and can introduce artifacts. Current noninvasive tools for assessing the effects of retinoids on skin do not allow the evaluation of their comedolytic activity, such as transepidermal water loss (Marks and Edwards, 1992; Fullerton and Serup, 1997) and silicone rubber replicas (Marks and Edwards, 1992) or if they do, they do not provide comedolytic effect over time on the same treated skin site, such as the cyanocrylate skin surface stripping (Mills and Kligman, 1982; Piérard *et al*, 1995; Piérard-Franchimont *et al*, 1999). A noninvasive method that could provide a rapid and efficient assessment over time of the same skin site of the acne-like condition *in vivo* would be of great value in both animal models and in humans.

Fluorescence spectroscopy is such a noninvasive technique for investigating skin. The endogenous fluorescence of normal skin has been the subject of a significant number of investigations (Utz *et al*, 1993; Zeng *et al*, 1995; Lucchina *et al*, 1996; Kollias *et al*, 1998) and there is evidence that specific fluorescence spectral features can be related to particular histologic and morphologic features, or structural and functional characteristics of the skin. For example, certain fluorophores have been identified in the skin of the hairless mouse, which are related to the aging and photoaging processes (Kollias *et al*, 1998).

The major fluorophores in the ultraviolet and in the violet-blue end of the visible spectrum are tryptophan, collagen cross-links (pepsin digestible and collagenase digestible), NADH, and to a lesser extent keratin. The fluorescence of these fluorophores was found to change in a predictable manner with photoaging induced by ultraviolet light (Kollias *et al*, 1998). It has also been demonstrated that in acne there are fluorophores of the porphyrin family associated with *Propionibacterium acnes* (Lucchina *et al*, 1996), and fluorophores associated with ''horn'', which is contained in the comedones. Both of these materials produce strong characteristic fluorescence when excited with ultraviolet light and this fact generates great interest for utilizing fluorescence spectroscopy in the noninvasive assessment of the state of closed comedones.

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Abbreviations: FES, fluorescence excitation spectroscopy; tRA, all-*trans* retinoic acid.

In this study, we have investigated the potential of fluorescence excitation spectroscopy (FES) for the quick and noninvasive quantitative assessment of comedolytic agents on acne-like lesions *in vivo*. To perform this task, we have used the rhino mouse model and the effects of the topical application of tRA were assessed by FES as well as by standard histology and fluorescence microscopy. The results of the FES analysis were correlated with the results of histology and specific features were identified in the fluorescence spectra associated with the microscopic features of comedones.

MATERIALS AND METHODS

Chemicals tRNA was obtained from Roche Pharmaceuticals (Nutley, NJ) and was dissolved in acetone for the first phase of the study, and in ethanol/propylene glycol mixture (70:30, vol/vol) during the repeat phase of this study.

Animals Thirty-six female 7 wk old rhino mice ($hr^{h}hr^{h}$) were obtained from Charles River Laboratories (Wilmington, MA). The animals were provided with food and water *ad libidum*, and were divided into six groups of six animals each. Groups I, II, III, and IV received topical application of tRA at concentrations of 0.1, 0.01, 0.001, and 0.0001%, respectively. The animals in group V received vehicle only, and group VI served as the control. tRA was applied once per day in doses of 100 μl on the entire dorsal skin, for five consecutive days each week, for a total period of 2 wk. Animals in all groups were killed 24 h after the last treatment by cervical dislocation and biopsies were obtained immediately following killing. Fluorescence excitation spectra were collected from dorsal skin on a daily basis during treatment. During the measurements, the animals were sedated with inhalation of methoxyflurane (Metofane, Pitman-Moore, Mundelein, IL). In all cases, solutions were prepared daily before use. The study protocol received approval by the Subcommittee on Research Animal Care of the Massachusetts General Hospital.

To test the validity and reproducibility of the results, and to further investigate the histology with fluorescence microscopy, this study was repeated with five new animal groups of six animals each, of the same age, in exactly the same way, and tRA was applied in serial dilutions of 0.025%, 0.0025%, and 0.00025%, to animal groups I, II, and III, whereas group IV received the vehicle, and group V served as the control. Animals were studied for an additional recovery period of 30 d following the end of treatment with tRA.

Fluorescence excitation spectroscopy Fluorescence excitation spectra were obtained *in vivo* with a fluorescence spectrophotometer (SkinSkan, SPEX, Edison, NJ), equipped with a 450 W Xenon lamp, double monochromators on the excitation and emission, a photomultiplier detector (R928P, Hamamatsu, Hamamatsu City, Japan) connected to a single photon counting system and a bifurcated quartz fiber bundle (Model 1950, SPEX Ind., Edison, NJ) for light delivery and collection. The individual fibers were 100 μm in diameter and were randomly arranged to form a bundle 6 mm in diameter. The resolution was 4 nm, the intensity of the excitation radiation was in the range of 1–20 mW per cm^2, and the total delivered radiation dose was below the erythema threshold. Each fluorescence measurement consisted of a set of eight serial excitation spectra collected by positioning the emission monochromator from 340 to 480 nm in increments of 20 nm and scanning the excitation monochromator from 260 nm to within 20 nm of the emission monochromator setting. Light from the excitation monochromator was focused into one leg of the bifurcated fiber bundle. The other leg of the fiber bundle was focused into the input of the emission monochromator. The joined end of the fiber bundle was brought into direct contact with the skin site measured. Care was taken to clean properly the fiber bundle end with an alcohol swab between measurements on different animals and that gentle pressure was applied to the animals skin during measurements. All fluorescence spectra were corrected for instrument response.

Histology Skin samples approximately 5 mm in diameter were obtained from the skin of each animal following killing, from the mid-line of the anterior portion of the dorsum skin. All specimens were fixed in 10% buffered formaldehyde for 24 h, and later embedded in paraffin. Tissue sections 5 μm thick were cut perpendicularly to the epidermal surface and stained with hematoxylin and eosin for light microscopic examination. For each animal, the epidermal and dermal thickness, utriculi diameter, and number of capillaries containing more than five erythrocytes were assessed with a micrometer eyepiece adapted to a microscope (Leitz SM-LUX, Ernst Leitz, Wetzlar, Germany) using a 40× objective for the measurement of epidermal and dermal thicknesses, and utriculi diameter, and a 20× objective for the count of the dilated capillaries.

Fluorescence microscopic analysis was performed during the repeat phase of the study on 5 μm thick unstained frozen tissue sections. Microscopic images of frozen sections were taken using a fluorescence microscope (Axiophot, C. Zeiss, Oberkochen, Germany) equipped with a thermo-electrically cooled CCD camera having 12 bit resolution (Photometrics, Tucson, AZ). Excitation light at 365 nm was provided by an appropriate bandpass filter, and emission was set at wavelengths longer than 420 nm with the use of a long-pass filter.

RESULTS

Fluorescence excitation spectroscopy Significant changes were observed in the fluorescence excitation spectra as a result of the application of the comedolytic agent, which were confirmed during the repeat phase of this study. **Figure 1** presents a representative picture of these changes as observed during the first phase. **Figure 1**(***a***) shows the average fluorescence spectra measured before the application of tRA. Note the two characteristic peaks, the first located approximately at 295 nm, which is related to epidermal tryptophan and the second at 340–370 nm, which is attributed to the collagen cross-links (Kollias *et al*, 1998). The second peak is characterized by greater intensity, as compared with the first peak, before tRA treatment. **Figure 1**(***b–e***) shows the average fluorescence spectra at the end of the 2 wk treatment period with tRA in concentrations 0.0001%, 0.001%, 0.01%, and 0.1%, respectively. The intensity of the second peak exhibited a dramatic decrease, even after the application of tRA at the lowest concentration (0.0001%, **Fig 1*b***). The intensity of the second peak remained approximately constant as a function of tRA concentration (**Fig 1*b–e***), whereas a significant increase in the intensity of the first peak was observed. In addition, the fluorescence intensity at excitation wavelengths greater than 400 nm showed a definite decrease with application of increasing concentration of tRA. Finally, finer changes were also observed in the line shape of the fluorescence spectra, i.e., a small ''shoulder'' developed at about 275 nm, especially at high concentrations of tRA.

Figure 2 shows the intensity of the two main peaks of the fluorescence spectra, as a function of time (i.e., increasing number of consecutive applications of tRA) during the course of this study, for the various concentrations of tRA. Note the gradual increase of the 295 nm peak intensity (**Fig 2*a***) and the corresponding decrease in the 370 nm peak (**Fig 2*b***). The changes were stronger at higher tRA concentrations.

Histology Microscopic analysis at 2 wk (**Fig 3**) showed a hyperplastic interutricular epidermis as well as marked decrease in the utricular diameter in all retinoid-treated animals. The utriculi appeared in the collapsed state for the treated skin sites at high tRA concentration (**Fig 3*c***).

Figure 4 shows the results obtained with fluorescence microscopy during the second phase of this study for the animal group treated with 0.025% tRA. **Figure 4**(***a***) presents an image of an unstained frozen section of the untreated skin of a control animal, and **Fig 4**(***b***) shows a fluorescence image of the same section. Characteristic bright fluorescence is clearly seen, originating from the material inside the utriculi as opposed to the weaker fluorescence from the surrounding collagen. **Figure 4**(***c***, ***d***) shows the corresponding images taken on a frozen section immediately after the end of the 2 wk period of tRA treatment. The utriculi appear to be collapsed in the image taken with white light (**Fig 4*c***), and the fluorescence signal associated with the material inside the utriculi is absent in **Fig 4**(***d***). At the end of the 30 d recovery period, some utriculi-like structures can be observed under white light illumination (**Fig 4*e***), whereas the corresponding fluorescence image (**Fig 4*f***) does not show any characteristic fluorescence associated with the utriculi.

Table I summarizes the quantitative results of the basic histologic findings from the second phase of this study, which consist of the

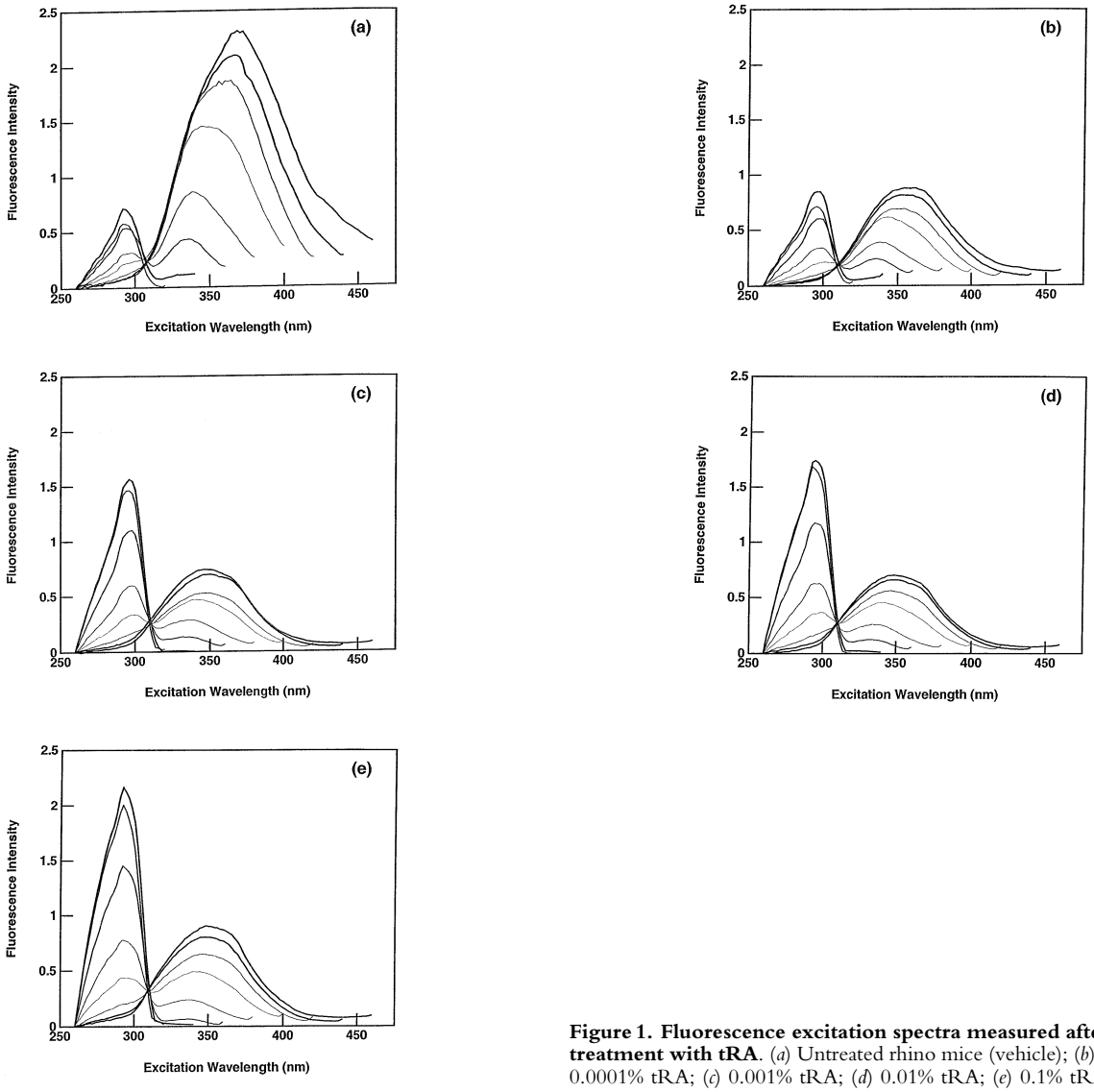

Figure 1. Fluorescence excitation spectra measured after the end of treatment with tRA. (*a*) Untreated rhino mice (vehicle); (*b*) Treated with 0.0001% tRA; (*c*) 0.001% tRA; (*d*) 0.01% tRA; (*e*) 0.1% tRA.

following parameters: dermal thickness, epidermal thickness, size of the utriculi, and density of capillaries containing more than five erythrocytes. Note the definite increase in the epidermal thickness after the application of tRA, and the corresponding increase in the dermal thickness. The size of the utriculi exhibited the most dramatic correlation with the concentration of tRA and no utriculi could be identified after the end of treatment with 0.025% tRA. Finally, the surface density of dilated capillary loops, as a measure of the inflammation, revealed good correlation with tRA concentration.

DISCUSSION

In this study, we present evidence that comedolysis on rhino mouse skin can be evaluated quantitatively *in vivo* using FES. We have observed a direct correlation between the size of the utriculi and specific features of the fluorescence spectra, following successive applications of tRA in the rhino mouse model. We have also established that both the fluorescence and the histology of the rhino mouse skin following treatment with tRA, present a picture resembling that of the hairless mouse (Kollias *et al*, 1998). This indicates that the material filling the utriculi has characteristic fluorescence, which can be used to measure the size and density of the utriculi in the skin in a noninvasive way using FES. We obtained further confirmation for this observation by examining the fluorescence of frozen skin sections using microscopy techniques, and identifying a characteristic fluorescence signature related to the utriculi. Previous studies of the fluorescence characteristics of the hairless mouse skin (Kollias *et al*, 1998) have attributed the 295 nm fluorescence band to epidermal tryptophan, and the 340 and 370 nm bands to pepsin and collagenase digestible collagen cross-links, respectively. The results of this study suggest that the fluorescence from the content of the utriculi contributes significantly to the 370 nm band as a correlation was observed between the fluorescence intensity of that band and the diameter of the utriculi as assessed by histology. In addition, fluorescence microscopy showed that utriculi exhibit a strong and characteristic fluorescence with excitation at 365 nm and emission in the visible range.

The increase in the 295 nm band was due to proliferation and inflammation induced by the retinoids, which is a well established and studied reaction of the skin caused by this family of compounds (Ashton *et al*, 1984; González *et al*, 1997). In a previous study (González *et al*, 1997), we have studied the proliferation induced by tRA in the rhino mouse skin and confirmed the significant increase in both the basal and suprabasal proliferative indices. The increase in epidermal thickness after treatment with tRA, also confirms the increase of the 295 nm band intensity.

As can be seen in **Fig 1**(***d***) and **Fig 1**(***e***) an additional fluorescence band at approximately 275 nm appears after treatment with tRA,

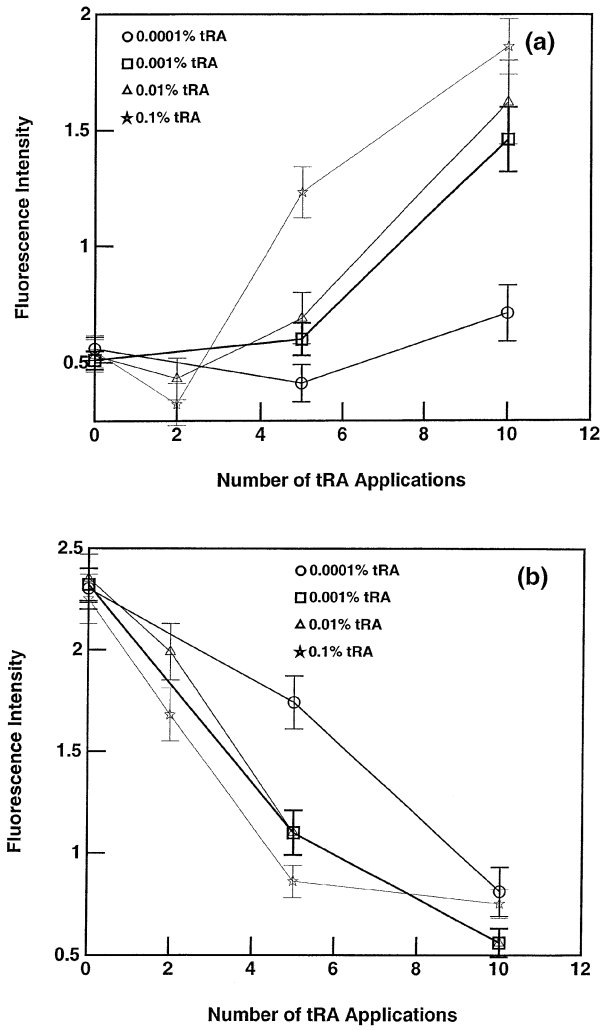

Figure 2. Fluorescence intensity as a function of consecutive applications of tRA. (*a*) 295 nm excitation, 340 nm emission; (*b*) 370 nm excitation, 480 nm emission. Errors shown are mean ± SD.

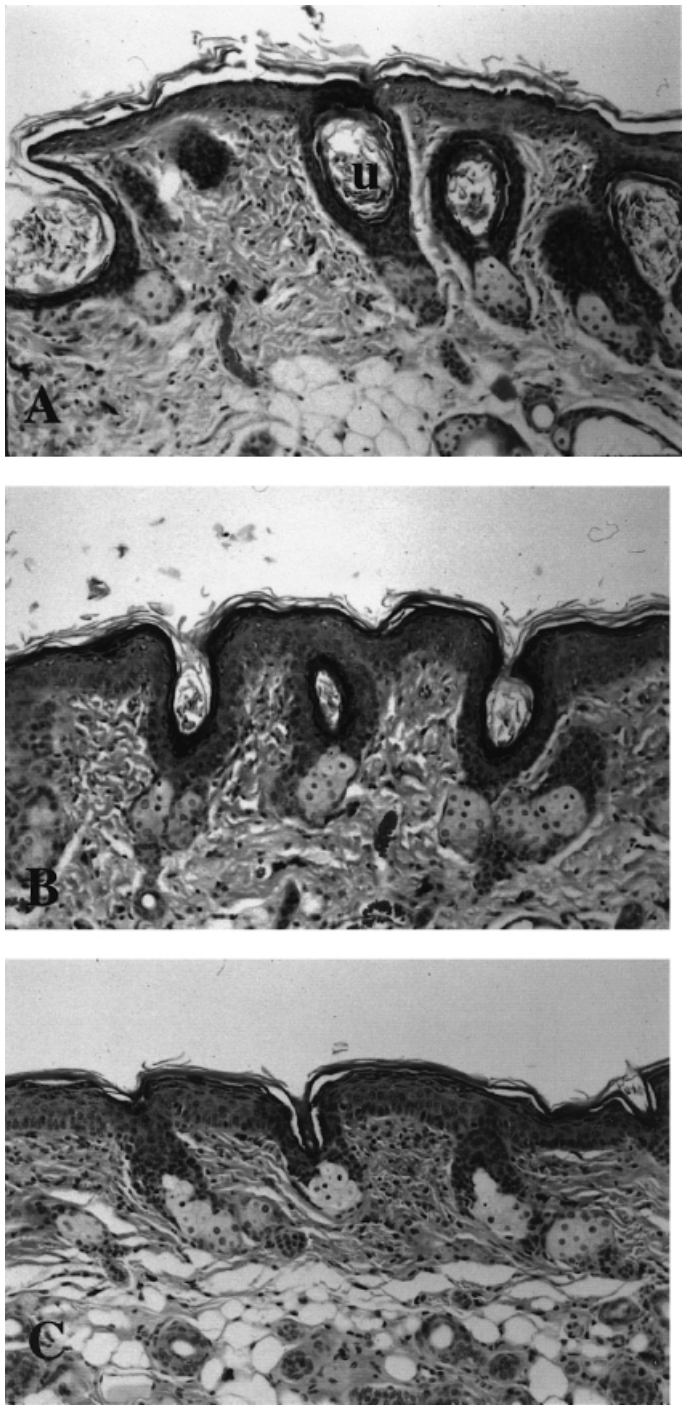

Figure 3. Hematoxylin and eosin sections of the animal skin after the end of treatment with tRA. (*a*) Control; (*b*) 0.0001% tRA (lowest concentration); (*c*) 0.1% tRA (highest concentration). The utriculi (oval-shaped structures marked "u") size and density decrease progressively as a function of tRA concentration.

especially at high tRA concentrations. This band is probably due to neutrophils associated with inflammation as has already been reported (Monici *et al*, 1995). Proliferation and production of new collagen in the dermis is also partially responsible for the drop in the 340–370 nm band intensity. This may be due to the fact that new collagen is characterized by a smaller number of cross-links, and therefore fluorescence of smaller intensity (Kollias *et al*, 1998).

In a previous study we investigated the fluorescence excitation spectra of the hairless mouse (Kollias *et al*, 1998). The main histologic difference between the hairless mouse and the rhino mouse is the presence of the utriculi (Mann, 1971). In this study, we have established that the fluorescence of the rhino mouse differs from that of the hairless mouse, and the fluorescence of the rhino mouse becomes similar to that of the hairless mouse after treatment with tRA. This indicates that the initial difference in the fluorescence of the two animal types is connected to the presence of the utriculi (decrease of the 370 nm band intensity), and the thickness of the epidermis (increase of the 295 nm band).

Finally, during the 30 d follow-up period in the second phase of this study, we observed a partial reconstitution of the utriculi using routine histology. This change was not correlated with any significant changes in the fluorescence signal, which is an indicator that the new utriculi are not characteristic of the acne condition.

In summary, we have studied the fluorescence excitation spectra of the rhino mouse skin, and showed that the histologic changes induced by comedolytic agents such as tRA can be monitored noninvasively. In particular, the identification of a characteristic fluorescence signature associated with the utriculi in the rhino mouse opens the way for the development of a new specific and powerful technique for the noninvasive assessment and monitoring of pseudocomedones. Although we have evaluated closed comedones, this technique could also be important in monitoring open comedones as well. Additional studies are warranted to apply the method presented here on human skin and to establish the exact relationship between the changes in the fluorescence and the changes induced in skin by the retinoids.

Table I. Histologic parameters[a]

Animal group	Epidermal thickness (μm)	Dermal thickness (μm)	Utriculi diameter (μm)	Dilated capillary loops
I (0.00025% tRA)	42.5 ± 8.6*	298 ± 66	43 ± 29**	20.1 ± 3.4**
II (0.0025% tRA)	45 ± 10*	341 ± 125	21 ± 4**	18.2 ± 5.5**
III (0.025% tRA)	78 ± 28**	399 ± 91*	–	39.8 ± 8.4**
IV (vehicle)	16.4 ± 2	264 ± 12	114 ± 38	2.8 ± 2.1
V (control)	20 ± 5	225 ± 25	110 ± 31	3.1 ± 2.2

[a]Epidermal and dermal thickness and utriculi diameter were assessed in each mouse, in three fields per mouse. The number of dilated capillaries in the papillary dermis (each containing five or more erythrocytes in their lumen) were counted per field for each mouse. Errors shown are mean ± SD: *$p < 0.05$; **$p < 0.005$.

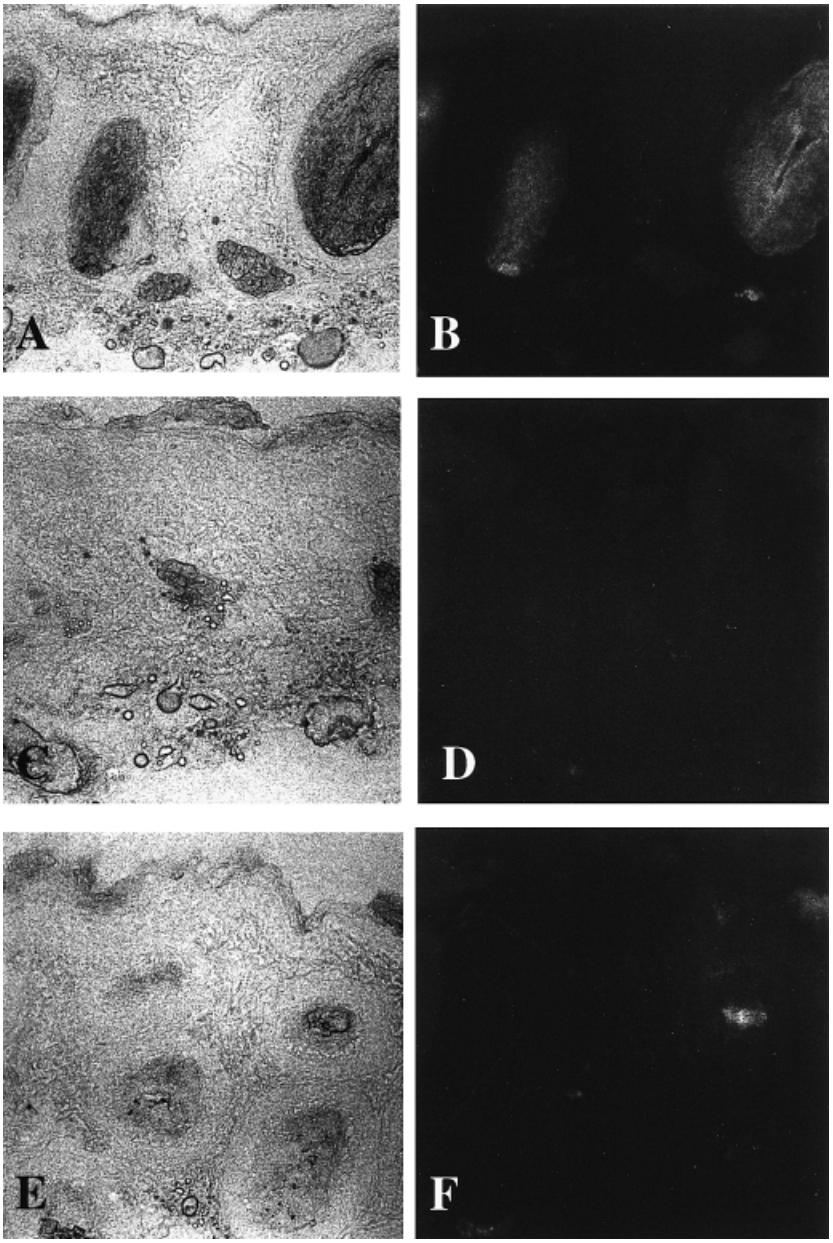

Figure 4. Microscopic examination of unstained frozen tissue sections. (*a*) Before tRA application (*b*) corresponding fluorescence microscopy section. Note the characteristic fluorescence associated with the utriculi (oval-shaped structures). (*c*) After the end of treatment with tRA (shows the collapse of the utriculi). (*d*) Corresponding fluorescence image (note the absence of the characteristic utriculi fluorescence). (*e*) After the end of the 30 d follow-up period (note the partial reconstitution and reappearance of the utriculi). (*f*) Corresponding fluorescence image (no fluorescence is observed from the utriculi).

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