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Qualitative and Quantitative Analysis of Cytosol Retinoid Binding Proteins in Human Skin*

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The distribution of the cytosol retinol and retinoic acid binding proteins are known to vary greatly within the different layers of the eye, a retinoid target organ. We have analyzed the cytosol retinoid binding from adult human skin, another retinoid target organ, and examined the relative contribution of the epidermis and dermis to the total retinoid binding. The mean specific activity of [^{3}H]retinol (0.52 ± 0.06 pmol/mg protein) and [^{3}H]retinoic acid (3.20 ± 0.45 pmol/mg protein) binding to cytosol preparations from different specimens of adult human skin was determined. On the average these skins bound 7-fold more retinoic acid than retinol. When skin was treated with EDTA and separated into epidermal and dermal fractions, [^{3}H]retinol and [^{3}H]retinoic acid binding was found in the cytosol derived from epidermis (0.36 ± 0.03 pmol/mg protein, 3.69 ± 0.13 pmol/mg protein, respectively) but not from dermis. To confirm that the absence of dermal binding was not due to loss during the EDTA separation, we assayed skin keratomed at 0.1, 0.2, and 0.3 mm. The skin obtained at 0.1 mm was upper epidermis and exhibited binding for both retinol and retinoid acid. The 0.2 mm skin, which added lower epidermis but little dermal contamination, had higher specific activities for both retinol and retinoic acid binding. The 0.3 mm skin which added primarily dermis, had lower specific activities for binding both retinoids. This is consistent with the concept that the epidermis is responsible for the majority of retinoid binding in adult human skin obtained from the lower limb.

Retinoids are necessary for the normal growth and differentiation of many tissues. Clinical administration of both the naturally occurring and synthetic retinoids has profound effects on the skin. Different retinoids exert variable effects on the epidermis (scaling, peeling) vs dermal structures such as sebaceous glands (atrophy) and hair follicles (alopecia). Except for their role in vision, the exact molecular mechanisms through which the retinoids act are unclear.

Two distinct cytosol binding proteins, one specific for vitamin A alcohol (retinol), the other specific for vitamin A acid (retinoic acid), have been identified in many normal and abnormal tissues [1–3]. The cytosol retinoic acid binding protein (CRABP) and the cytosol retinol binding protein (CRBP) have been identified in skin samples from the chick embryo, rat, and human [4–8].

Although the cellular role of these binding proteins is as yet unclear, they may mediate some retinoid effects in a manner similar to the mechanism proposed for steorid hormone effects on nuclear transcription. An intracellular protein is postulated to bind a hormone in the cytoplasm and facilitate translocation to the nucleus as seen in liver cells where CRBP facilitates the interaction of retinol with the nucleus [9,10]. Once inside the nucleus, another receptor protein may further mediate hormone action. There is evidence in cultured Y-79 retinoblastoma cells suggesting translocation of the retinoic acid–CRABP complex into the nucleus [11]. All-*trans*-retinoic acid, 13-*cis*-retinoic acid, and etretinate are retinoids with profound effects on skin. These three retinoids bind to CRABP as evidenced by their ability to compete with [^{3}H]all-*trans*-retinoic acid binding. The clinical effects of these retinoids may, therefore, be mediated through these binding proteins.

Qualitative and quantitative analyses of these binding proteins in various components of the eye have been performed and striking differences in the pattern of binding have been observed between the different layers [12–14]. In the present study, we have examined and quantified the retinol and retinoic acid binding to cytosol fractions from human skin. In addition, cytosol obtained from EDTA-separated epidermis and dermis and also from skin keratomed at various thicknesses were analyzed to characterize the epidermal and dermal contribution to the total retinoid binding found in skin.

MATERIALS AND METHODS

Tissues

Adult human skin was obtained from lower limb amputation specimens using a Castroviejo keratome set at 0.1, 0.2, or 0.3 mm. These specimens were placed in phosphate-buffered saline (PBS) solution and transported to the laboratory on ice. Skin was stored frozen at −70°C until assayed. For determination of mean retinoid binding, 0.3 mm keratomed skin was obtained from several different specimens; cytosol preparations from each sample were prepared and assayed for binding to each radiolabeled retinoid. Several samples were assayed in duplicate. In these cases, the average of both values was used.

Epidermal-Dermal Separation

For each experiment, 0.3 mm keratomed skin was obtained from one specimen and assayed either as the full-thickness, 0.3 mm skin or as EDTA-separated epidermis or dermis. The 0.3 mm keratomed (full thickness) skin was floated dermal side down on a solution of 0.02% EDTA for 2–4 h at 37°C. Epidermal sheets were then peeled off the dermis, and each was washed in PBS. Skin was not frozen prior to EDTA separation, but after separation tissues were stored at −70°C until assayed.

Cytosol Preparation, Incubation, and Sucrose Density Gradient Analysis

After overnight lyophilization, tissues were placed in a motor-driven, glass-on-glass homogenizer with approximately 1 cc of Tris buffer for each 1 cm^2 of tissue and homogenized until a uniform consistency was obtained. The buffer was 10 mM Tris buffer with 10 mM KCl, 1 mM dithiothreitol, and 1 mM EDTA adjusted to pH 7.6. The homogenate was centrifuged at 27,000 *g* for 10 min at 4°C. The supernatant was recentrifuged at 110,000 *g* for 60 min to obtain the final supernatant

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Abbreviations:
CRABP: cytosol retinoic acid binding protein
CRBP: cytosol retinol binding protein

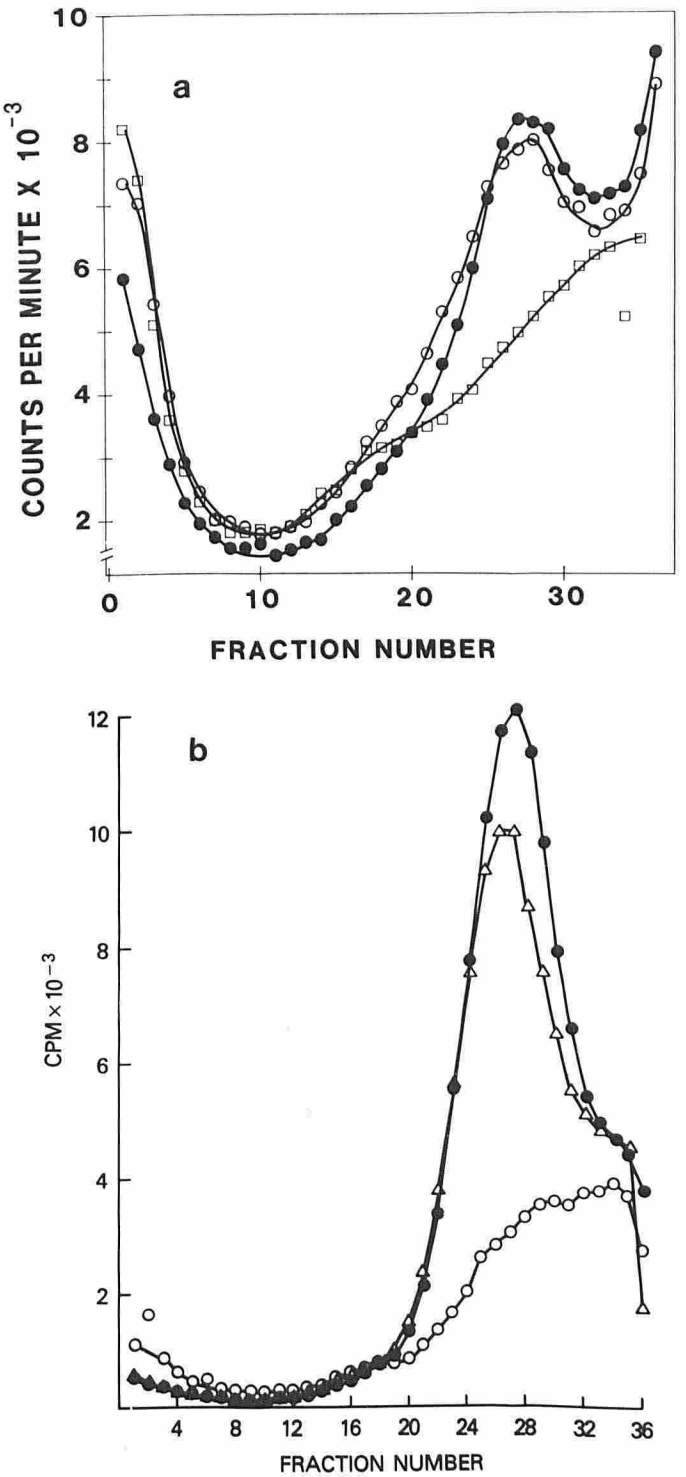

FIG 1. *a*, Sucrose density gradient analysis of [^{3}H]-retinol binding to human skin cytosol. The peak of radioactivity seen after incubation of skin cytosol (500 μl) in 5.9 × 10^{-8} M [^{3}H]retinol (●——●) at 37°C for 15 min corresponds to a sedimentation coefficient of 2S. The peak was eliminated with the addition of a 100-fold excess of nonradiolabeled retinol (□——□) during the incubation period, demonstrating that this binding was saturable. The peak was unaffected by the addition of a 100-fold excess of nonradiolabeled retinoic acid (○——○), demonstrating binding specificity for retinol (alcohol function). Other conditions are as previously given [14]. *b*, Sucrose density gradient analysis of [^{3}H]retinoic acid binding to human skin cytosol. After incubation of skin cytosol (500 μl) in 3.7 × 10^{-8} M in [^{3}H]retinoic acid, at 37°C for 15 min, binding was again seen as a peak of radioactivity (●——●) in the 2S area of the gradient. Saturability and specificity were demonstrated as elimination of the peak with the addition of a 500-fold excess of nonradiolabeled retinoic acid (○——○) but not a 500-fold excess of retinol (△——△). Other conditions are as previously given [15].

(cytosol) fraction. Protein determinations were performed on these final cytosol fractions by the method of Lowry et al [15].

Aliquots (500 μl) of the cytosol were incubated with [^{3}H]retinoid under dim red light at either 4°C for 2 h or 37°C for 15 min. Assay techniques have been previously described [13,14]. Preliminary studies with skin (data not shown) have demonstrated that both incubation conditions yield similar amounts of binding. Within each experiment, only one incubation condition was used such that comparisons were done using the same conditions. A 250-μl aliquot of each reaction mixture was then layered on 5–20% sucrose gradients (4.6 ml) and centrifuged at 243,000 *g* for 16 h. Each gradient was then fractionated into approximately 36 fractions and radioactivity determined in each fraction. The amount of binding was calculated as the amount of specific binding by integration of the 2S peak. Error values are given as standard errors of the mean.

[^{3}H]all-*trans*-retinol (sp act = 5.0 Ci/mmol), purchased from New England Nuclear, Boston, Massachusetts, was used in 4 of the 14 retinol binding analyses comprising the mean values. All other retinol binding determinations were performed using [11,12^{3}H]all-*trans*-retinol (sp act ranging from 43–55 Ci/mmol) purchased from Amersham Corporation, Arlington Heights, Illinois. [11,12^{3}H]all-*trans*-retinoic acid (sp act = 28.7 or 32 Ci/mmol) was a kind gift from Dr. W.E. Scott of Hoffman-La Roche, Inc., Nutley, New Jersey.

RESULTS

Sucrose Density Gradient Analysis of [^{3}H]Retinol and [^{3}H] Retinoic Acid Binding to Human Skin

Analysis for CRBP: Fig 1*a* depicts a representative analysis of retinol binding to 0.3 mm keratomed adult human skin cytosol. After incubation of cytosol with [^{3}H]retinol, a peak of radioactivity was observed at approximately fraction 27. In this system, this area of the gradient corresponds to a sedimentation coefficient of 2S. CRBP has been extensively studied in the chick embryo, particularly in the chick embryo retina [16]. When the cytosol from chick embryo retina was incubated with [^{3}H]retinol, the peak of radioactivity appeared in the same area of the gradient (fraction 27, data not shown).

Interaction of a ligand with a receptor or binding protein is normally saturable, i.e., there are a limited number of available binding sites. Thus, nonradiolabeled retinoids would be expected to compete with labeled retinoid for the limited number of binding sites, such that, in the presence of an excess amount of nonradiolabeled retinol, the binding of [^{3}H]retinol would be extensively reduced. After incubation of cytosol from the same sample with [^{3}H]retinol in the presence of a 100-fold excess of nonradiolabeled retinol the peak was virtually eliminated. Receptor binding is also usually specific for one or a small number of structurally similar compounds. Incubation of this cytosol with [^{3}H]retinol in the presence of a 100× excess of nonradiolabeled retinoic acid (a structurally similar retinoid except that the alcohol group is replaced by an acid function) did not interfere with the binding peak.

Analysis for CRABP: After incubation of 0.3 mm adult human skin cytosol with [^{3}H]retinoic acid, a peak of radioactivity was again observed at approximately fraction 27 (Fig 1*b*), the area of the gradient indicating a sedimentation coefficient of 2S. In contrast to the situation with [3H]retinol, little if any excess, nonspecifically bound or free radiolabeled retinoic acid is observed in these gradients. The reason for this is not known although it is likely that excess ligand is removed by binding to nonspecific sites on centrifuge tubes, glass vessels, etc. Saturability and specificity are indicated by the elimination of the 2S peak after incubation in the presence of a 500-fold excess of nonradiolabeled retinoic acid but not a 500-fold excess of nonradiolabeled retinol. The small decrease in [^{3}H]retinoic acid binding seen after the addition of a large excess of nonra-

diolabeled retinol is most likely due to oxidation of some of the nonradiolabeled retinol to the acid form and subsequent competition of the nonradiolabeled acid form with the [^{3}H]retinoic acid.

Retinoid Binding in 0.3 mm Keratomed Adult Human Skin Cytosol: Mean Values

Keratomed adult human lower limb skin (0.3 mm) was obtained from several different specimens and the results of the analysis for CRBP and CRABP are shown in Fig 2 using the techniques delineated in Fig 1. The mean specific activity for [^{3}H]retinol binding from these specimens was 0.52 ± 0.06 pmol/mg protein (range 0.22–0.85, N = 14). The mean [^{3}H]retinoic acid binding (3.20 ± 0.45 pmol/mg protein (range 1.62–5.81, N = 12)) was significantly higher ($p < 0.01$) than retinol binding.

In all 9 cases where the analysis for retinol and retinoic acid binding was performed on the same cytosol, the specific activity of the retinoic acid binding was higher than for retinol. The mean ratio of the specific activities for retinoic acid binding compared to retinal binding was 7.2 (range 2.2–15.6).

A typical histologic section of the skin obtained after keratoming at 0.3 mm is shown in Fig 3, top.

Retinoid Binding to EDTA-Separated Epidermis and Dermis

Keratomed skin samples (0.3 mm) were assayed as obtained (full thickness) or after EDTA separation into epidermis and dermis. Fig 3 shows the histology of samples of the tissues used in this study. The keratomed skin is of uniform thickness. The epidermal-dermal separation appears to be complete with no visible epidermal cell loss.

The quantitative distribution of [^{3}H]retinoid binding in the full-thickness keratomed skin vs its component epidermis and dermis after EDTA separation is shown in Fig 4. The capacity for retinol binding found in the epidermis alone (0.36 ± 0.03 pmol/mg protein) was the same as found in the full-thickness skin (0.36 ± 0.09 pmol/mg protein). There was no retinol binding detected in the dermal cytosol.

The specific activity of retinoic acid binding found in the epidermis (3.69 ± 0.13 pmol/mg protein) was slightly higher than that found in the 4 samples of 0.3 mm full-thickness skin (2.11 ± 0.38 pmol/mg protein) obtained from the same (paired) samples. This probably represents a similar amount of binding. The discrepancy is most likely due to the small number of samples (N = 4). This is supported by the observation that the average value for retinoic acid binding to 0.3 mm keratomed skin obtained from the larger numbers of hetrogenous samples shown in Fig 2 (3.43 pmol/mg protein) is much closer to the value obtained for epidermis and not significantly different from either the epidermal or untreated values. Another possible

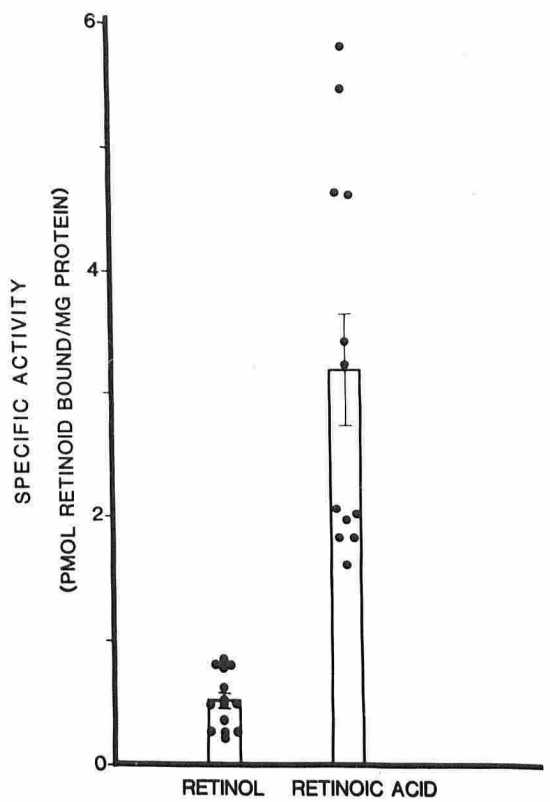

FIG 2. Mean specific activities for [^{3}H]retinol and [^{3}H]retinoic acid binding in cytosols prepared from 0.3 mm keratomed human skin obtained from several different specimens. Binding was analyzed using the sucrose gradient technique delineated in Fig 1. These adult human skin samples had a 7-fold greater capacity for binding retinoic acid compared to retinol. *Bars* = SEM.

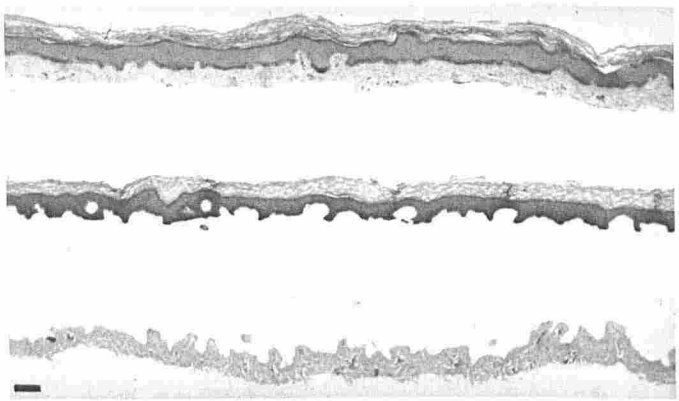

FIG 3. Histology of representative samples of the 0.3 mm keratomed skin before and after EDTA-separation. *Top*, The 0.3 mm keratomed skin was of uniform thickness. *Middle*, The EDTA-separated epidermis consisted of stratum corneum and epidermis with no visible loss of epidermal cells. The preservation of the basal layer can be seen as retention of pigmented cells. *Bottom*, The dermis retained normal histology and was free of epidermal cell contamination. The *bar* represents 0.1 mm.

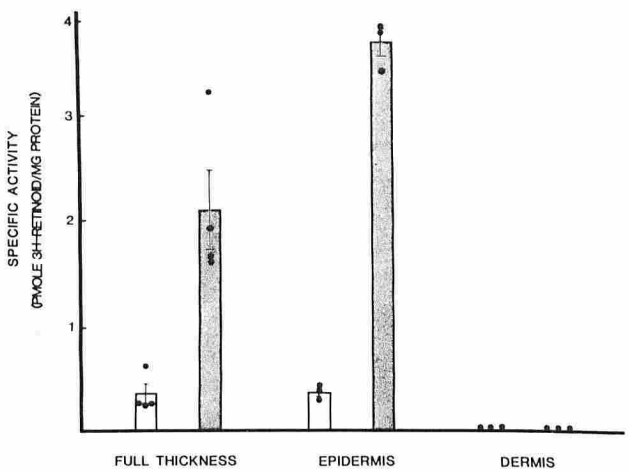

FIG 4. The distribution of [^{3}H]retinoid binding with and without EDTA separation. Binding was determined as given in Fig 1. After EDTA separation of skin, the [^{3}H]retinol (□) and [^{3}H]retinoid acid (□) binding was confined to the epidermis. The specific activities for [^{3}H]retinoic acid binding was significantly greater than for [^{3}H]retinol binding in the cytosol derived from both full-thickness skin ($p < 0.05$) and epidermis ($p < 0.01$). *Bars* = SEM.

explanation for a lower specific activity of binding seen in the untreated skin vs epidermis may be that the full-thickness keratomed skin adds dermis, which does not bind retinoic acid but does add protein, thereby diluting out the specific activity.

Retinoid Binding to Skin Keratomed at Increasing Thickness

To insure that the lack of retinoid binding by EDTA-separated dermis was not due to destruction of the binding protein during the chemical separation, we chose another technique to indirectly assess the contribution of the dermis to the retinoid binding found in full-thickness skin. Skin from one specimen was keratomed at 0.1, 0.2, and 0.3 mm. The histology of these specimens is shown in Fig 5. Compared to the 0.1 mm skin, the 0.2 mm skin, shown in the middle section, added almost all of the lower epidermal cells except for the lowest epidermal ridges, but included very little dermal contamination. The 0.3 mm skin shown in the bottom section added very few additional epidermal cells but did add considerable dermal contamination.

The cytosol retinoid binding from the skins keratomed at increasing thicknesses are shown in Fig 6. The increase in thickness from 0.1 to 0.2 mm, which added the lower part of the epidermis, increased the specific activity of both retinol (from 0.25 to 0.74 pmol/mg protein) and retinoic acid (from 3.36 to 4.95 pmol/mg protein) binding. But the further increase in thickness from 0.2 to 0.3 mm, which added predominantly dermis, yielded a decrease in the specific activity for both retinol (from 0.74 to 0.62 pmol/mg protein) and retinoic acid (from 4.95 to 4.37 pmol/mg protein) binding. This is consistent with the concept that the predominant retinoid binding in adult human lower limb skin is in the epidermis. If there is little or no binding from the dermis, adding more dermis would add inert protein and thereby dilute the specific activity. The binding to 0.3 mm skin measured in this experiment using the high-specific-activity [^{3}H]retinol (0.62 pmol/mg protein) is similar to that measured in determining the mean values using the low-specific-activity [^{3}H]retinol (Fig 2, 0.52 ± 0.06 pmol/mg protein).

Retinoic acid binding to the 0.2 mm skin (4.95 pmol/mg protein) was 47% higher than binding to 0.1 mm skin (3.36 pmol/mg protein). In contrast, retinol binding to the 0.2 mm skin (0.74 pmol/mg protein) which adds the lower epidermal cells was 196% higher compared to the binding observed in the 0.1 mm skin (0.25 pmol/mg protein). The addition of these lower epidermal cells yielded a greater increase in retinol binding compared to retinoic acid binding. These experiments suggest that the lower epidermis had a greater capacity for binding retinol compared to the upper epidermis.

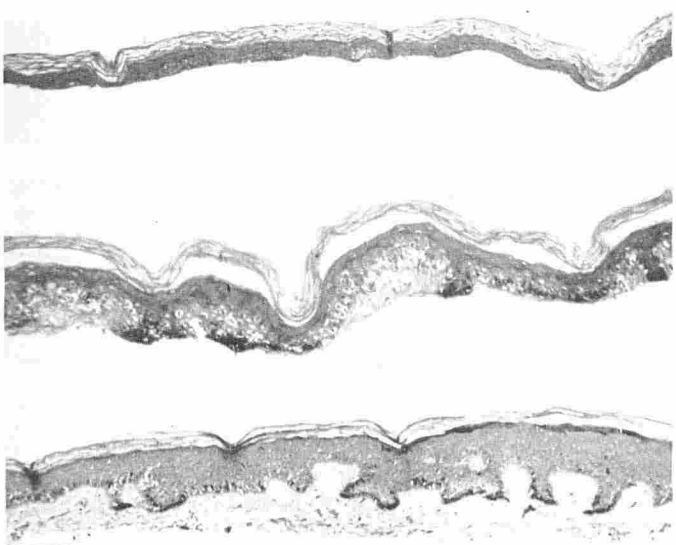

FIG 5. Representative histology of the human skin samples keratomed at increasing thickness. *Top*, The 0.1 mm keratomed skin consisted of upper epidermis. *Middle*, The 0.2 mm keratomed skin added almost all of the lower epidermis. *Bottom*, The 0.3 mm keratomed skin added predominantly dermis. The *bar* represents 0.1 mm.

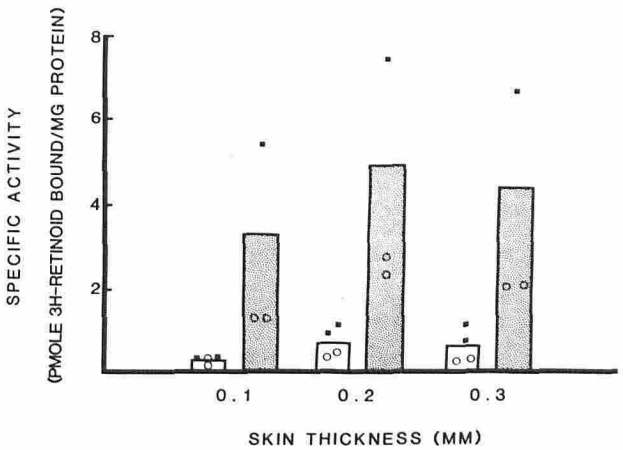

FIG 6. Specific activites of [^{3}H]retinol (□) and [^{3}H]retinoic acid (□) binding from skin keratomed at increasing thickness. Binding was determined as given in Fig 1. The results of 2 separate experiments are shown. The *squares* were all part of one experiment and the *circles* were a completely separate experiment. Duplicates are shown with the same symbol. While the absolute amounts of binding differ between both experiments, the pattern of relative binding within each experiment is the same. Increasing thickness from 0.1 to 0.2 mm (adding the lower part of the epidermis, e.g., basal cell layer) increased the specific activity of both retinol and retinoic acid binding. The further increase in thickness from 0.2 to 0.3 mm, which added predominantly dermis, resulted in a decrease in the specific activity binding to both retinoids.

DISCUSSION

The retinoids are compounds which are essential for normal growth and differentiation and have dramatic effects when administered in pharmacologic doses. The skin is a major retinoid target organ and consequently human skin undergoes dramatic morphologic changes in response to both natural and synthetic retinoids.

Cytosol binding proteins for retinol and retinoic acid have been identified in many tissues. While their role in the mechanism of retinoid action is unclear, they are thought to mediate at least some retinoid effects. To further understand the possible role of these binding proteins in the mechanisms of retinoid action, we have analyzed and quantified the relative retinoid binding in adult human skin.

Few studies are yet available on the presence of the binding proteins in human or nonhuman skin samples. Gates and King [5] have examined the cytosol retinoid binding proteins in untreated chick embryo skin and collagense-separated epidermis and dermis. They found very high levels of CRABP in chick embryo skin with twice as much CRABP in dermis as in epidermis. A lesser amount of CRBP was detected and the levels in epidermis and dermis were similar. We are not surprised by their finding of large amounts of binding to chick embryo dermis in contrast to our inability to identify binding in adult human dermis. These binding proteins are well known to change dramatically during differentiation. In the rat, for example, high levels of CRABP have been reported in fetal tissues with a lesser or nondetectable level found in the corresponding adult tissue [6]. Our inability to measure dermal binding could also have been influenced by other factors. Adult human dermis is a very tough tissue and may have been less completely homogenized than epidermis. In addition, there are fewer cells present in dermis.

Puhvel and Sakamoto have examined epidermal and sebaceous follicle cytosol derived from human facial skin [8]. The levels of [^{3}H]retinoic acid binding which they measured from facial skin were somewhat higher, but of the same order of magnitude as we measured in lower limb skin. Although this difference may be due to technique, the location from which the skin was obtained could account for the difference. Clinically administered synthetic retinoids cause more apparent cutaneous toxicity (scaling, redness) to facial skin than to lower limb skin. A difference in CRABP levels could account for this.

This study analyzes the comparative retinoid binding to human epidermis and dermis. These adult human skin samples bound more retinoic acid (about 7-fold) than retinol. The binding of both retinoids was predominantly if not completely confined to the epidermis. Furthermore, while the retinoic acid binding seemed to be somewhat homogeneously distributed between the upper and lower epidermis, there appeared to be a difference in the relative retinol binding between the upper and lower epidermis, with the lower epidermis binding more retinol than the upper epidermis.

Identifying the pattern of binding and quantifying the amounts of binding in adult human skin will enable us to establish a baseline to compare binding with skin at different stages of differentiation and during pathologic transformation.

Since the submission of this manuscript another article on the same topic has been published in the British Journal of Dermatology 3:647–654, 1984.

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