Comparison of the Uptake and Metabolism of Retinol Delivered to Primary Mouse Keratinocytes Either Free or Bound to Rat Serum Retinol-binding Protein

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Serum retinol-binding protein (RBP) is believed to be responsible for the transport of retinol from its storage site in the liver to vitamin A requiring target cells such as keratinocytes. We have used primary mouse keratinocytes as a model system to compare the uptake and metabolism of [^{3}H] retinol delivered to them either free in solution or bound to RBP. RBP was purified from rat serum, loaded with [^{3}H]retinol, and the [^{3}H]retinol-RBP complex purified by affinity chromatography on human transthyretin-Sepharose. Keratinocytes incubated with either free [^{3}H]retinol or [^{3}H]retinol-RBP complex accumulated [^{3}H]retinol in a time and temperature dependent manner. However, cells incubated with free [^{3}H]retinol acquired 15- to 20-fold more ligand than if the retinol was delivered via RBP. The uptake of free [^{3}H]retinol or [^{3}H]retinol from RBP was not inhibited by excess unlabeled free retinol. The uptake of [^{3}H]retinol from RBP was inhibited by high concentrations of holo-RBP, with half maximal inhibition occurring at 3 μM holo-RBP. However, no specific binding of ^{125}I-labeled RBP to monolayers of keratinocytes or membranes prepared from them was found indicating the absence of a high affinity RBP receptor on keratinocytes. Surprisingly, 50% of the [^{3}H]retinol delivered to the keratinocytes during a 30-min uptake period was released from them within 30-min irrespective of whether or not it was initially delivered to them as free [^{3}H]retinol or bound to RBP. The remaining 50% was lost at a much slower rate, but only 20% remained 24-h after delivery. Studies on retinol metabolism demonstrated that 7%–12% of the total cell-associated [^{3}H]retinol delivered during a 90-min uptake period was esterified (mostly as retinyl palmitate) whether or not it was given free in solution or bound to RBP. Additionally, [^{3}H]retinol taken up by the keratinocytes during the initial 90-min incubation was not chased into a stable retinyl ester pool in a subsequent 9.5-h incubation, but instead, retinyl ester was lost from the cells with kinetics similar to those of total cell-associated radioactivity. These results suggest that a function of RBP is to protect cells from a rapid accumulation of the vitamin which occurs when it is delivered free in solution. However, the cellular fate and metabolism of retinol appears to be the same whether the vitamin is delivered free in solution or bound to RBP. *J. Invest Dermatol 92:283–289, 1989*

In vivo studies have demonstrated that vitamin A is required for the proper growth and differentiation of epithelia including the epidermis [1]. Numerous investigations have also shown that the regulation by vitamin A of growth and differentiation can be studied in vitro using cultured keratinocytes, which undergo several morphologic and biochemical changes in response to retinoids [2–6]. In general, retinoids enhance features of a secretory epithelium, whereas a deficiency of the vitamin accentuates the markers of terminal epidermoid differentiation. For example, retinoic acid inhibits calcium and phorbol ester induced terminal differentiation [5,6] and cornified envelope formation in keratinocytes [7,9,10], as well as phorbol ester induced ornithine decarboxylase activity [11,12]. Furthermore, the addition of retinoids to cultured keratinocytes induces tissue transglutaminase [7,8], regulates the expression of various keratins [13–15], increases the number of cell surface EGF receptors [16,17], and stimulates the incorporation of labeled sugars into glycoconjugates [18–20]. Although these studies have clearly shown that retinoids can have a profound influence on several cellular parameters related to growth and differentiation, in these studies the vitamin was introduced to the cells in a non-physiologic way, by adding it directly to the tissue culture media dispersed in solvent. However, in vivo, cells do not come in contact with the vitamin in the free form because retinol circulates in complex with a specific binding protein, the serum retinol-binding protein (RBP). It is not known whether the same responses would occur if retinol was delivered to the cells bound to RBP.

RBP is a low molecular weight (21 kDa) protein which is responsible for the transport of retinol from its storage site in the liver to target tissues such as the skin [21]. After synthesis in the liver, RBP binds a single molecule of retinol, and is secreted into the circulation

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Abbreviations:

BSA: bovine serum albumin
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC: high-performance liquid chromatography
MEM: Eagle's minimal essential medium without $CaCl_2$
PBS: calcium- and magnesium-free Dulbecco's phosphate buffered saline
RBP: (holo- or apo-) plasma retinol-binding protein (with or without retinol)
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TTR: transthyretin (prealbumin)

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where it forms a strong non-covalent interaction with transthyretin (TTR). The retinol-RBP-TTR complex is thought to deliver bound retinol to target cells via a transient interaction with a specific cell surface receptor which recognizes RBP. In fact, several reports present evidence in favor of an RBP receptor on several cell types including keratinocytes [22–29], although purification and characterization of an RBP receptor has not been achieved.

Although an understanding of the mechanism and biochemical steps involved in the delivery of retinol from RBP to target cells would appear to be crucial to determining the molecular mechanism of action of the vitamin, surprisingly little is currently understood about this delivery process. As a first step toward determining whether the way retinol is delivered to, and interacts with, target cells is a critical determinant in the cellular response elicited by the vitamin, we compared the uptake, metabolism, and cellular fate of [^{3}H]retinol delivered to primary mouse keratinocytes either bound to purified rat RBP or free in solution. In this report we demonstrate that keratinocytes accumulate 15- to 20-fold more [^{3}H]retinol when delivered free in solution than when bound to RBP. However, 50% of the delivered retinol is rapidly (within 30 min) released from the cells and the fraction of the total cell-associated retinol that is metabolized to retinyl ester is independent of the mode of delivery.

MATERIALS AND METHODS

Materials [11, 12(n)-^{3}H]All-trans-retinol (60.0 Ci/mmol) was purchased from New England Nuclear. 13-cis-Retinoic acid, all-trans-retinoic acid, and all-trans-retinyl acetate were from Eastman Kodak Company, and retinyl palmitate from Hoffmann-La Roche. Phenyl-Sepharose CL-4B, Sephadex G-50 (fine), and the Mono Q HR 5/5 column were from Pharmacia Fine Chemicals. Human TTR was obtained from Calbiochem and BSA from Miles Laboratories. SDS-PAGE analysis showed that both the TTR and BSA preparations used in these studies were apparently free of RBP contamination.

Cell Culture Cultures of primary newborn BALB/c mouse epidermal cells were prepared from trypsin-separated epidermal sheets [30]. Cultures were plated (1.5×10^6 cells per 35 mm culture dish) and maintained in MEM (MA Bioproducts) containing 8% Chelex-treated (Bio-Rad Chelex) fetal calf serum, 1% Antibiotic-Antimycotic (Gibco), and supplemented with $CaCl_2$ to 60–70 μM. Culture medium was changed every other day and experiments were conducted 7–10 days after plating.

Purification of Retinol-Binding Protein RBP was purified from rat serum (Pel Freeze) by the following modification of the procedure described by Berni et al [31] for the purification of RBP from human serum. RBP from the Sephadex G-50 gel filtration step of the procedure by Berni et al [31] was diluted 1 : 1 with 0.1 M Tris containing 6.0 M urea, pH 7.0, and chromatographed on a column (2.6 × 80 cm) of Sephadex G-50 fine equilibrated in 50 mM Tris containing 3 M urea, pH 7.0 [32]. Purified RBP was stored at 0.5–1.0 mg/ml in PBS at −80°C.

Formation and Isolation of [^{3}H]Retinol-RBP Complex RBP (100 μg) in 1.0 ml of PBS was added to [^{3}H]retinol (100 μCi in 50 μl of ethanol) in a small brown vial and rotated overnight at room temperature. [^{3}H]Retinol-RBP complex was isolated free of unbound retinol by affinity chromatography on a column of human TTR-Sepharose [33]. The specific activity of the [^{3}H]retinol-RBP complex ranged from 250–700 cpm/ng RBP. The 330 to 280 nm ratio of the affinity purified [^{3}H]retinol-RBP complex averaged 0.90, indicating the preparation contained 90% holo-RBP.

Iodination of Retinol-Binding Protein RBP was iodinated using Iodo-Beads (Pierce Chemical Co.) according to the manufacturer's recommendations. Briefly, RBP (100 μg) in 200 μl of PBS, containing 1.0 mCi of Na^{125}I (New England Nuclear) and a single Iodo-Bead, was allowed to react at room temperature for 10 min. PBS (800 μl) was then added to the reaction, and the labeled RBP was separated from free iodine by gel filtration on a prepacked disposable PD-10 column (Pharmacia) equilibrated in PBS. The specific activity of the labeled RBP ranged from 1 to 5 μCi/μg. Autoradiography of the iodinated RBP, following electrophoresis on a 10% SDS polyacrylamide slab gel, showed a single band with an apparent molecular weight of 21,000 daltons, the expected molecular weight of RBP.

Uptake of Free [^{3}H]Retinol or [^{3}H]Retinol from RBP by Primary Mouse Keratinocytes Cultures of primary mouse keratinocytes (in 35 mm culture dishes) were washed twice with 2 ml of ice-cold MEM containing 10 mM HEPES and 1 mg/ml BSA (MEM-HEPES-BSA). MEM-HEPES-BSA (1.0 ml) containing either free [^{3}H]retinol (added to the media in ethanol, final concentration 0.05%–0.10%) or [^{3}H]retinol-RBP complex was added to the cells in the amounts indicated. The cells were incubated at 37°C for the times indicated, placed at 4°C, and washed twice with 2 ml of ice-cold PBS containing 1 mg/ml BSA and then twice with 2 ml of ice-cold PBS. PBS or water (1.0 ml) was then added, and the cells were frozen, thawed, and scraped. Radioactivity was determined in an aliquot (0.7–0.8 ml) of the lysate in 10 ml of Aquasol (New England Nuclear).

HPLC Analysis of [^{3}H]Retinol Metabolites Formed by Mouse Keratinocytes Mouse keratinocytes fed either free [^{3}H]retinol or [^{3}H]retinol-RBP in calcium-free CMRL-1066 media supplemented with BSA (5 mg/ml), hydrocortisone (5 μg/ml), glutamine (2 mM), insulin (5 μg/ml), and $CaCl_2$ (50 μM) (subsequently referred to as supplemented CMRL) were washed and extracted with methanol as indicated in the Figure legends. Retinoid standards (50–200 ng) were added and the methanol evaporated under a stream of N_2. The extract was solubilized in 40 μl of methanol and analyzed by HPLC on a Partisil-10 ODS-2 column (Whatman) eluted with a methanolic gradient [34]. Radioactivity in the eluate was monitored either by a Flo-One Model HS radioactivity flow detector (Radiomatic Instrument and Chemical Co.) or by collecting fractions and determining radioactivity in a liquid scintillation counter.

RESULTS

Preparation and Characterization of [^{3}H]Retinol-RBP Complex The purified rat RBP used in these studies exhibited a single band on an SDS-PAGE slab gel (not shown) and chromatographed as a single peak on a Mono Q HR 5/5 column (Fig 1). In order to compare the delivery and uptake, by primary cultures of mouse keratinocytes, of [^{3}H]retinol bound to RBP to that of free [^{3}H]retinol it was necessary to prepare and purify [^{3}H]retinol-RBP complex free of any unbound [^{3}H]retinol. To accomplish this we incubated RBP with [^{3}H]retinol and purified the [^{3}H]retinol-RBP complex by affinity chromatography on human TTR-Sepharose

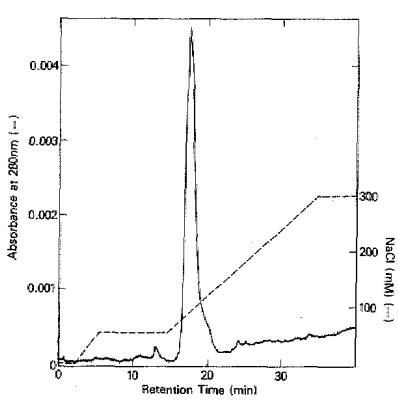

Figure 1. HPLC of purified rat RBP on a Mono Q HR 5/5 anion exchange column. Purified rat RBP (18 μg) was chromatographed on a Mono Q column eluted with a gradient of NaCl in 5 mM Tris, pH 8.0 [35]. Absorbance at 280 nm was monitored.

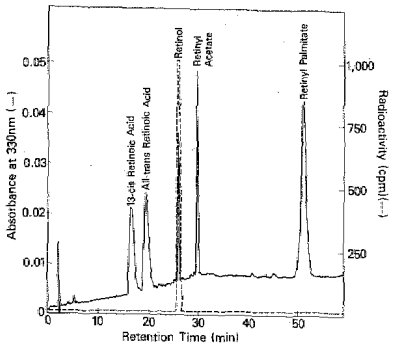

Figure 2. Radioactivity extracted from [^{3}H]retinol-RBP complex co-chromatographs with standard retinol on a Partisil-10 ODS-2 column. [^{3}H]Retinol-RBP (1,800 cpm) was extracted with methanol, and the extract was co-chromatographed with standard retinoids (50–200 ng) on a Partisil-10 ODS-2 column eluted with an increasing gradient of methanol in water [34]. Radioactivity *(dashed line)* and absorbance at 330 nm *(solid line)* were monitored.

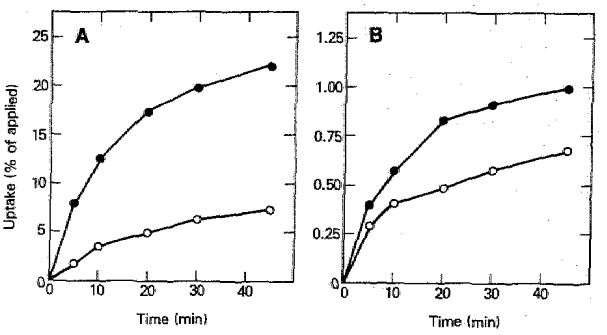

Figure 3. Time and temperature dependent uptake of [^{3}H]retinol delivered to mouse keratinocytes either free in solution or bound to RBP. Mouse keratinocytes were incubated at either 4°C *(open circle)* or 37°C *(closed circle)* with free [^{3}H]retinol (445,000 cpm, 7 nM) *(A)* or [^{3}H]retinol-RBP complex (445,000 cpm, 50 nM) *(B)* for the times indicated and the cell-associated radioactivity determined.

[33]. The [^{3}H]retinol-RBP complex co-chromatographed with the unlabeled RBP on a Mono Q HR 5/5 column (not shown) and the radiolabeled retinoid moiety chromatographed as all-trans-retinol (Fig 2).

Comparison of the Uptake by Primary Mouse Keratinocytes of Free [^{3}H]Retinol to [^{3}H]Retinol Delivered Via RBP Primary mouse keratinocytes were cultured in low calcium MEM (60 μM). Under these conditions the cells assume morphologic and biologic characteristics typical of basal cells [30]. Serum-free MEM containing free [^{3}H]retinol (added directly to the media in ethanol) or [^{3}H]retinol-RBP complex was added to the keratinocytes which were then incubated at either 4° or 37°C. At various times cell-associated radioactivity was determined. A time as well as temperature-dependent uptake of [^{3}H]retinol was found, independent of whether or not it was delivered free in solution or bound to RBP (Fig 3). However, cells incubated with free [^{3}H]retinol accumulated 15- to 20-fold more ligand than those in which the [^{3}H]retinol was delivered via RBP (Fig 3). In fact, about 20% of the added retinol became cell-associated in only 30-min when delivered free in solution, with only 1% delivered to the cells when given bound to RBP. The uptake of free [^{3}H]retinol or [^{3}H]retinol from RBP was not inhibited by a several-fold molar excess of unlabeled retinol (Table I). In contrast, the uptake of [^{3}H]retinol from RBP was inhibited by high concentrations of holo-RBP (Fig 4*A*), with 50% inhibition of [^{3}H]retinol uptake requiring 70 μg/ml RBP (3 μM) (Fig 4*B*). However, any binding of RBP to a putative cell surface RBP receptor must be of a low affinity because no specific binding of ^{125}I-labeled RBP to monolayers of keratinocytes, or membranes prepared from them, was found (not shown). Uptake of [^{3}H]retinol from RBP by keratinocytes does not appear to involve TTR because the addition of a 100-fold molar excess of TTR over [^{3}H]retinol-RBP had no effect on retinol uptake (Table II). A 500-fold molar excess of TTR over free [^{3}H]retinol inhibited [^{3}H]retinol uptake by 23%, possibly due to the TTR forming a complex with the free [^{3}H]retinol and making it less available to the cells (Table II). Consistent with previous results with other cell types [22,24], our results indicate that the transfer of [^{3}H]retinol from RBP to keratinocytes does not involve internalization of RBP. We found no evidence for adsorptive pinocytosis of ^{125}I-labeled RBP and also found that the uptake of free [^{3}H]retinol or [^{3}H]retinol from RBP was not influenced by chloroquine (1 mM) or ammonium chloride (10 mM), two classic inhibitors of receptor-mediated endocytosis [36] (not shown).

Cellular Fate and Metabolism of [^{3}H]Retinol Delivered to Keratinocytes Either Bound to RBP or Free in Solution Mouse keratinocytes were incubated at 37°C for 30 min in media containing either free [^{3}H]retinol or [^{3}H]retinol-RBP complex. The medium was then removed, the cells were washed, fresh media was added, the cells were returned to 37°C, and at various times the cell-associated [^{3}H]retinol was determined. Surprisingly, 50% of the [^{3}H]retinol delivered to the cells during the initial uptake period was released from the cells within 30 min, irrespective of whether or not it was initially delivered to them as free [^{3}H]retinol or bound to RBP (Fig 5). The remaining 50% of the cell-associated [^{3}H]retinol was lost at a much slower rate. About 35% remained after 9.5 h (Fig 6) and by 24 h only 20% remained (not shown), again, independent of whether it was originally delivered to the cells as free [^{3}H]retinol or via RBP. The [^{3}H]retinol lost from the cells was always quantitatively recovered in the medium.

We also studied the metabolism of [^{3}H]retinol by mouse keratinocytes. As shown in Fig 7, the major metabolite found, after incubation of the cells for 90-min with free [^{3}H]retinol, co-chromatographed with standard retinyl palmitate. A second peak, most likely

Table I. Excess Unlabeled Retinol Does Not Inhibit the Uptake by Mouse Keratinocytes of Free [^{3}H]Retinol or [^{3}H]Retinol from RBP[a]

Retinol Concentration (μM)	Ratio (M/M) Retinol / [^{3}H] Retinol-RBP	Ratio (M/M) Retinol / [^{3}H] Retinol	Uptake (cpm/plate) [^{3}H] Retinol-RBP	Uptake (cpm/plate) [^{3}H] Retinol
0	0	0	5,583 ± 420	73,946 ± 1545
0.1	2.5	10	6,303 ± 266	72,562 ± 2182
1.0	25	100	6,268 ± 747	76,527 ± 1687
10.0	250	1000	7,183 ± 966	77,087 ± 2543

[a] Mouse keratinocytes were incubated at 37°C for 20 min with [^{3}H]retinol-RBP (500,000 cpm, 40 nM) or [^{3}H]retinol (505,000 cpm, 10 nM) in the presence of the indicated concentration of unlabeled retinol and the cell-associated radioactivity determined. Mean ± SD, N = 3.

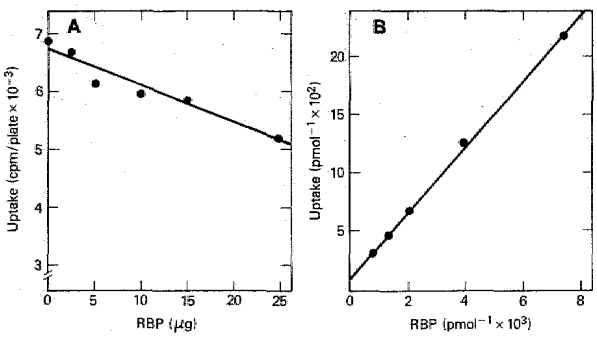

Figure 4. Inhibition of the uptake of [^{3}H]retinol from [^{3}H]retinol-RBP complex by excess unlabeled holo-RBP. Mouse epidermal cells were incubated at 37°C for 20 min with [^{3}H]retinol-RBP (196,000 cpm, 0.3 μg) in the presence of increasing concentrations of unlabeled holo-RBP and the cell-associated radioactivity determined *(A)*. *Panel B* shows a double reciprocal plot of the data shown in *panel A*.

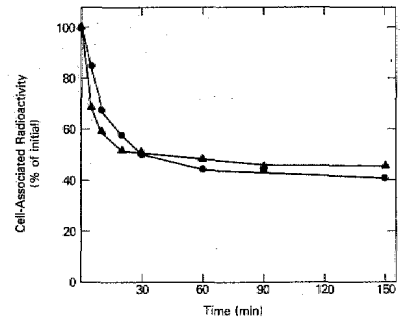

Figure 5. Time course of the loss of cell-associated radioactivity following delivery of [^{3}H]retinol to mouse keratinocytes either free in solution or bound to RBP. Mouse keratinocytes were incubated at 37°C for 30 min with either [^{3}H]retinol-RBP complex (1,500,000 cpm, 112 nM) *(closed triangle)* or free [^{3}H]retinol (1,500,000 cpm, 25 nM) *(closed circle)* in 1.0 ml of supplemented CMRL medium. The medium was then removed, the cells were washed twice with 2.0 ml of supplemented CMRL, 1.0 ml of fresh supplemented CMRL was added, and the cells were placed at 37°C. At the times indicated the cells were washed twice with 2.0 ml of PBS containing BSA (1 mg/ml) and then 1.0 ml of methanol was added directly to the culture dish. The cells were then scraped into the methanol and the plate washed with an additional 0.4 ml of methanol. The extracts were combined, and radioactivity in an aliquot was determined.

another retinyl ester, eluted 6–7 min prior to retinyl palmitate (Fig 7). However, most (80%) of the radioactivity eluted as [^{3}H]retinol, while the two esters accounted for about 13% of the recovered radioactivity. Significant radioactivity was not observed co-eluting with either 13-cis or all-trans-retinoic acid standards, suggesting that retinoic acid is not a major metabolite from retinol in keratinocytes. We did not find any significant difference in the individual metabolites formed, or the extent of esterification (the percentage of the total cell-associated radioactivity which was esterified), whether the [^{3}H]retinol was delivered to the keratinocytes free in solution or bound to RBP.

Figure 6 follows the retinyl ester formed during an initial 90-min incubation with either free [^{3}H]retinol or [^{3}H]retinol-RBP complex during a subsequent 9.5-h incubation in the absence of radiolabeled retinol. Two points should be emphasized. First, [^{3}H]retinol, which was taken up by the cells during the initial 90-min incubation, did not chase into a stable retinyl ester pool. In fact, total retinyl ester was lost from the cells with kinetics paralleling those of total cell-associated radioactivity. However, unlike retinol, intact retinyl ester was not released from the cells into the media but must have been hydrolyzed prior to release (not shown). Second, whether or not [^{3}H]retinol was given to the cells free in the media or bound to RBP, the amount of delivered radioactivity esterified during the initial 90-min incubation (7% – 12%), as well as the kinetics of the loss of retinyl ester during the subsequent 9.5-h incubation period, were similar (Fig 6).

DISCUSSION

In this study we have used primary mouse keratinocytes as a model system to compare the delivery and metabolism of [^{3}H]retinol provided to them either free in solution or bound to RBP. We thought that keratinocytes would be particularly well suited for these studies because it had been shown that terminal differentiation in these cells is controlled by retinoids [4–10,13–15]. Additionally, immunofluorescence studies using anti-RBP antibodies indicated that RBP displayed a sustained interaction with keratinocytes in the epidermis [37]. Also, RBP has been shown to occur around epidermal cells at a concentration of about 30% – 40% of the plasma concentration, demonstrating that keratinocytes have access to RBP in vivo [38]. The results of the study presented here demonstrate the following major points. First, keratinocytes accumulate 15 – 20 times more retinol when delivered free in solution rather than bound to RBP. Second, cultured mouse keratinocytes do not express a high affinity cell surface RBP receptor. Third, about 50% of the retinol delivered to keratinocytes either free in solution or bound to RBP is rapidly released from the cells into the media. Fourth, the extent and rate of metabolism of retinol to retinyl ester by keratinocytes is not influenced by the mode of delivery. Finally, [^{3}H]retinol delivered to keratinocytes in an initial incubation as either free [^{3}H]retinol or bound to RBP does not chase into a stable retinyl ester pool in a subsequent incubation in the absence of labeled retinol.

We found that mouse keratinocytes accumulated 15- to 20-fold more retinol when delivered free in solution rather than bound to RBP. These results suggest that an important function of RBP in vivo may be to actually protect cells from a rapid uptake of the vitamin by biologic membranes which takes place if retinol is not bound to RBP. A similar conclusion was reached in in vivo studies of hypervitaminosis A in humans [39] and rats [40]. Those studies

Table II. Uptake of [^{3}H]Retinol from RBP or Free [^{3}H]Retinol by Mouse Keratinocytes in the Presence of Excess TTR[a]

TTR Concentration (μM)	Ratio (M/M) TTR / [^{3}H] Retinol-RBP	Ratio (M/M) TTR / [^{3}H] Retinol	Uptake (cpm/plate) [^{3}H] Retinol-RBP	Uptake (cpm/plate) [^{3}H] Retinol
0	0	0	5,853 ± 359	74,410 ± 2169
0.5	10	50	5,929 ± 106	70,136 ± 3683
1.0	20	100	5,723 ± 254	68,469 ± 399
2.0	40	200	4,957 ± 207	65,112 ± 978
5.0	100	500	5,573 ± 123	57,062 ± 3330

[a] Mouse keratinocytes were incubated at 37°C for 20-min with [^{3}H]retinol-RBP (470,000 cpm, 50 nM) or [^{3}H]retinol (470,000, 10 nM) in the presence of the indicated concentration of TTR and the cell-associated radioactivity determined. Mean ± SD, N = 3.

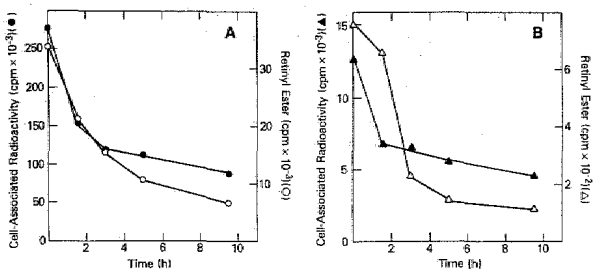

Figure 6. Total cell-associated radioactivity and [^{3}H]retinyl ester content of mouse keratinocytes following delivery of [^{3}H]retinol either free in solution or bound to RBP. Mouse keratinocytes were incubated at 37°C for 90-min with either free [^{3}H]retinol (1,572,000 cpm, 27 nM) *(A)* or [^{3}H]retinol-RBP complex (1,510,000 cpm, 119 nM) *(B)* in 1.0 ml of supplemented CMRL medium. The medium was then removed, the cells were washed with supplemented CMRL, fresh supplemented CMRL was added, and the cells were placed at 37°C as described in Fig 5. At the times indicated the cells were washed twice with 2.0 ml of PBS containing BSA (1 mg/ml) and extracted with 1.4 ml of methanol. Cell-associated radioactivity *(closed circle, closed triangle)* and retinyl ester content *(open circle, open triangle)* (as analyzed by HPLC) were determined.

suggested that vitamin A toxicity occurs when excessive amounts of vitamin A are nonspecifically presented to cell membranes in association with plasma lipoproteins rather than bound to RBP. According to this concept, RBP is acting not only to regulate the supply of retinol to tissues but also to protect cells from the surface-active properties of the vitamin. The interaction of free retinol with the cell surface appears to be via a nonspecific solubilization of the lipophilic vitamin into the lipid bilayer, because the uptake of free [^{3}H]retinol was not competed by unlabeled retinol at concentrations as high as 10^{-5} M, the level at which retinol becomes cytotoxic to keratinocytes. We found that at least 80% of the delivered retinol (either free or bound to RBP) was associated with the pellet following centrifugation of homogenized keratinocytes, indicating predominantly a membrane localization following delivery (unpublished results).

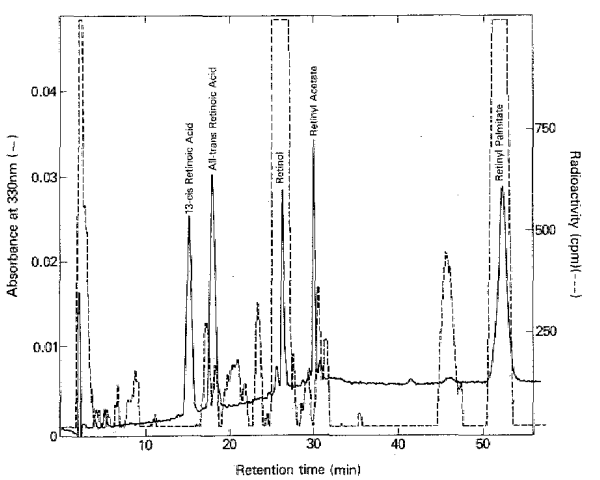

Figure 7. Analysis of retinoid metabolites by HPLC on a Partisil-10 ODS-2 column following incubation of mouse keratinocytes with free [^{3}H]retinol. Mouse keratinocytes were incubated at 37°C for 90 min with free [^{3}H]retinol (1,083,000 cpm, 18 nM) in 1.0 ml of supplemented CMRL medium. The medium was removed, the cells washed twice with 2.0 ml of PBS containing BSA (1 mg/ml), and then extracted with 1.5 ml of methanol. Standard retinoids (50–200 ng) were added and the sample was chromatographed on a Partisil-10 ODS-2 column [34]. Radioactivity *(dashed line)* and absorbance at 330 nm *(solid line)* were monitored. Of the 102,200 cpm applied to the column, 99% was recovered in the eluate with 81,700 cpm eluting as retinol and 11,000 cpm as retinyl palmitate.

A major effort was given toward determining whether the delivery to keratinocytes of retinol from RBP involves a high affinity cell surface RBP receptor. Numerous reports support the existence of such a receptor not only on keratinocytes [29,37] but also on retinal pigment epithelial cells [22,23,27], intestinal mucosal epithelial cells [24,25], membranes isolated from testicular cells [26], corneal epithelial cells [41], interstitial cells of the testis [42], F-9 teratocarcinoma cells [28], hepatocytes and stellate cells [43,44], and placenta [45]. However, to date, the evidence for an RBP receptor is circumstantial and in no case has a protein been purified that exhibits RBP binding activity. Our studies with mouse keratinocytes failed to detect any specific binding of ^{125}I-labeled RBP to monolayers of keratinocytes or to membranes prepared from them, suggesting that a sustained interaction between RBP and an RBP receptor on keratinocytes does not take place. A transient interaction between RBP and a cell surface receptor cannot be completely ruled out. However, experiments using ^{125}I-labeled RBP and heterobifunctional cross-linking reagents, which should have detected even a transient interaction, also proved negative (unpublished results). The finding that high concentrations of excess unlabeled holo-RBP inhibited the uptake of [^{3}H]retinol from RBP is consistent with RBP receptor-mediated uptake of [^{3}H]retinol. However, the high concentrations of RBP required for this inhibition (50% inhibition at 3 μM RBP) would indicate a receptor of very low affinity. Failure to detect specific binding of iodinated RBP has previously been reported for intestinal mucosal cells [24] and corneal epithelial cells [41]. In contrast, binding of ^{125}I-labeled RBP to isolated bovine retinal pigment epithelial cells [22], cultured human retinal pigment epithelial cells [27], testicular membranes [26], and interstitial cells [42] was found. However, the extremely low (<1 nM) concentrations of labeled RBP used in some of the studies make unequivocal interpretation of the results problematic.

A surprising result of these studies was that 50% of the [^{3}H]retinol delivered to keratinocytes in an initial incubation either free in solution or bound to RBP was released from the cells within 30 min in a subsequent incubation. The kinetics of release of the remaining 50% was slower. However, only 20% of the initial cell-associated retinol remained after 24 h. The mechanism and biologic significance of this rapid release of delivered retinol remains to be determined. However, it is tempting to speculate that the retinol may be reversibly interacting with a cell surface component of the plasma membrane. A cell surface site of action of retinoids is suggested by experiments in which enucleated cells [46] and cells exposed to immobilized retinoic acid [47] were responsive to the vitamin. Furthermore, the antagonism between the actions of phorbol esters and retinoids [5,48], as well as the inhibition of protein kinase C by retinoids [49], suggests the protein kinase C pathway as a possible site of retinoid action at the cell surface.

Metabolism studies demonstrated that retinyl palmitate was the major metabolite formed from [^{3}H]retinol delivered to mouse keratinocytes either free in solution or bound to RBP. Metabolism to retinoic acid was not detected in these studies. We found no evidence that the rate or extent of esterification was influenced by the mode of retinol delivery. The finding that retinyl esters constituted the major metabolite from [^{3}H]retinol agrees with previous studies showing that in human epidermis about 70% of the vitamin occurs as fatty acyl esters [50], and that mouse epidermal microsomes contain an active acyl-CoA : retinol acyltransferase [51].

Retinyl esters are believed to function as the cellular storage form of the vitamin which can be mobilized in times of cellular need. If this is the case, our results from experiments to determine retinyl ester content of epidermal cells after [^{3}H]retinol delivery would have to be described as unexpected in at least two ways. First, retinol delivered to the keratinocytes during an initial 90-min incubation did not chase into a stable retinyl ester pool in a subsequent incubation, as would be expected if retinyl ester represented a stable storage form of the vitamin in these cells. Second, the loss of total cell-associated retinyl ester formed during an initial incubation with [^{3}H]ret-

inol was unexpectedly rapid, with only 15%–20% of the initial retinyl ester remaining after a 9.5-h chase in the absence of labeled retinol. Again, it would not be expected that a storage form of the vitamin would be utilized so rapidly because the keratinocytes used in these studies should not be in a condition of vitamin A deficiency (they had been cultured in the presence of serum). Although retinyl ester is clearly the storage form of the vitamin in the liver, it may have a different function in the epidermis. Based on in vitro experiments with plasma membranes isolated from pigment epithelial cells, Ottonello et al [52] have proposed a model in which retinol is esterified and deesterified during translocation across the plasma membrane, and retinyl ester occurs only as an intermediate in this process. Their results suggested that the acceptor of the vitamin in the plasma membrane may be a membrane form of the well-characterized cytoplasmic retinol-binding protein. Our results on retinyl ester turnover in keratinocytes are more consistent with retinyl ester performing a similar function (i.e., as an intermediate in membrane translocation), rather than being a storage form of the vitamin. However, we found no evidence of delivered [^{3}H]retinol subsequently being transferred to the cytoplasmic retinol or retinoic acid binding proteins (unpublished data), despite the fact that these binding proteins are expressed at high levels in keratinocytes [53].

A major goal of these studies was to compare the uptake, metabolism, and fate of [^{3}H]retinol delivered to keratinocytes either bound to RBP or free in solution. We have demonstrated that the major difference between these modes of delivery is in the amount of the vitamin which becomes cell-associated. The method of delivery had no measurable effect on either the metabolism of the vitamin to retinyl ester, or the turnover of the delivered retinol. It will now be interesting to determine whether the mode of retinol delivery has any influence on the time course, dose response, and magnitude of cellular responses controlled by retinoids in keratinocytes. In this regard, Moore et al [54] have shown that tissue transglutaminase in mouse macrophages is maximally induced only when retinoic acid is presented to them bound to RBP.

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