Specific Quantification of Mouse and Human Keratin Proteins by Radioimmunoassay

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Mouse epidermal keratin proteins were purified and labelled with ^{125}I by chemical techniques. A radioimmunoassay method was established with a rabbit antibody elicited against the mouse keratin. This assay method was utilized to quantify keratin proteins of mouse and human epidermal extracts, both from intact tissues and cells in culture. As little as 30 ng of mouse keratin proteins (0.5 pmol) was quantifiable.

The principal differentiation product of mammalian stratified squamous epithelia is a structural protein, keratin, the major components of which are a fibrous protein, keratin filaments and a histidine-rich matrix protein (Fraser *et al.*, 1972; Dale *et al.*, 1978). During the last decade, a great deal has been learned about the structure and composition of the keratin filaments. The established procedure for their isolation has been by total dissolution of the tissue with a denaturing solvent that releases the keratin filaments in the form of their constituent polypeptide chain subunits. The subunits constitute a heterogeneous group of α -helix-rich polypeptides of molecular weights within the range of about 47 000–68 000 (Steinert & Idler, 1975; Lee *et al.*, 1976; Sun & Green, 1978; Steinert *et al.*, 1979). A remarkable property of these subunits is their ability to re-form filaments *in vitro* under appropriate conditions (Steinert *et al.*, 1976). Bovine (Steinert, 1978*a*) and probably all epidermal keratin filaments and related structures (Steinert, 1978*b*) are composed of a basic three-chain structural building block. Amino acid analysis of the polypeptides from a variety of species indicates striking similarities in composition (Steinert & Idler, 1975; Fuchs & Green, 1978). Likewise, purified polypeptides from a single species display complete immunological cross-reactivity, and furthermore display cross-reactivity with the polypeptides of other mammalian species (Lee *et al.*, 1976; Fuchs & Green, 1978; Steinert *et al.*, 1979). Recent findings have demonstrated that epidermal keratin-like proteins may exist in tissues other than the epidermis or stratified squamous epithelia. By the use of both biochemical (Steinert *et al.*, 1978) and immunological (Sun *et al.*, 1979; Frank *et al.*, 1979) techniques, keratin-like molecules have been local-

ized to the 10 nm (or 'intermediate') filaments associated with the cytoskeleton of cultured cells and tissues *in situ*. Thus keratin-like proteins may be ubiquitous components of the cytoskeleton of many eukaryotic cell types (Steinert *et al.*, 1980).

We have previously described a mouse epidermal culture system in which normal keratin proteins are synthesized (Steinert & Yuspa, 1978). These polypeptides have been isolated and characterized and used for the production of rabbit anti-(mouse keratin) sera (Steinert *et al.*, 1979). The antisera cross-reacted with epidermis from human tissues when assayed by qualitative techniques. This present paper describes the development of a quantitative radioimmunoassay method for epidermal keratin proteins and its application to problems of quantification of specific cellular proteins. This assay method provides a powerful tool for the analysis of keratin proteins and for the identification of keratin-like proteins in cells other than keratinocytes.

Materials and Methods

Cells and tissues

Newborn-mouse epidermis, dermis and stratum corneum were obtained by a previously described trypsin flotation procedure (Yuspa & Harris, 1974). The same tissues from human foreskin were obtained in a similar manner through the courtesy of Ms. Pamela Nelson, Microbiological Associates, (Bethesda, MD, U.S.A.). Epidermal cells and dermal fibroblasts were grown as described previously (Yuspa *et al.*, 1976). Low-sulphur proteins of mouse hair, which constitute the subunits of the hair keratin filaments, were isolated as described by Kemp & Rogers (1970).

Preparation of cell extracts

Monolayer cultures or intact tissues (minced) were washed three times with phosphate-buffered saline (Yuspa *et al.*, 1976) and extracted with 8M-urea/buffer (Steinert *et al.*, 1979) at 37°C for 2h. Insoluble material in extracts was centrifuged at 2000g for 10min and the supernatants were frozen in portions. One portion was used for the assay of total protein by the procedure of Bramhall *et al.* (1969). Other portions were used for radioimmunoassay. Purified mouse or human keratin filaments were prepared from tissue extracts by assembly *in vitro* as described previously (Steinert *et al.*, 1979) and redissolved in 8M-urea/buffer for radioimmunoassay.

Iodination of keratin proteins

Isolated mouse keratins (K_1 , mol.wt. 68000, or K_2 , mol.wt. 60000) (Steinert & Yuspa, 1978), dissolved in 8M-urea/buffer at a concentration of 3–10 $\mu\text{g}/25\mu\text{l}$, were added to 50 μl of 5mM-sodium phosphate buffer, pH 7.5, and allowed to react with 1.5mCi of carrier-free Na^{125}I (New England Nuclear, Boston, MA, U.S.A.) in the presence of 25 μg of chloramine-T (Eastman Organic Chemicals, Rochester, NY, U.S.A.) (10 μl) in water for 30s (Greenwood *et al.*, 1963). The reaction was stopped by the addition of 62.5 μg of sodium metabisulphite, and the reaction mixture was immediately applied to a Sephadex/polyacrylamide P-60 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) gel column previously equilibrated with 50mM-sodium phosphate buffer, pH 7.5, and coated with a 2% solution of bovine serum albumin. The column was eluted with 50mM-sodium phosphate buffer and fractions of organic ^{125}I -labelled keratin were collected into tubes containing 100 μl of 8mM-Tris/HCl buffer, pH 7.5, containing 1% sodium dodecyl sulphate and 1 mg of dithiothreitol. After overnight incubation at 22°C and brief heating to 95°C, the mixture was applied to polyacrylamide gels (Steinert *et al.*, 1979) and electrophoresed. The ^{125}I -labelled keratin bands were excised, eluted and stored in assay dilution buffer at -70°C .

Radioimmunoassay

The preparation of antiserum has been described (Steinert *et al.*, 1979). All dilutions of the assay were in 10mM-Tris/HCl buffer, pH 7.5, containing 1% bovine serum albumin, 0.01% sodium dodecyl sulphate and 0.01% Thiomersal. In a final volume of 1.0 ml were added: 0.1 ml of rabbit anti-(keratin K_1) or anti-(keratin K_2) serum (1:600 initial dilution), 0.1 ml of ^{125}I -labelled keratin (80–140 $\mu\text{Ci}/\mu\text{g}$, about 10000 c.p.m.) and 0.1 ml of standard keratin (keratin K_1 isolated from mouse epidermal cells) or 0.01–0.05 ml of unknown sample (both in 8M-urea/buffer)

and adjusted to volume with the dilution buffer. This mixture was incubated for 2h at 37°C followed by 15h at 4°C. Then 0.1 ml of goat anti-(rabbit IgG) antibody (0.2mg) was added and the mixture was incubated for 6h at 4°C. Next 3 ml of 10mM-Tris/HCl buffer, pH 7.5, was added to each tube and the immunoprecipitate was isolated by centrifugation at 1200g for 25 min at 4°C. The supernatant was decanted and the radioactivity of the pellets counted in a Searle model 1185 Gamma Counter.

Fig. 1. Standard curve for the radioimmunoassay of keratin

Keratin (K_1) was iodinated as described in the Materials and Methods section. Approx. 10000 c.p.m. of ^{125}I -labelled keratin K_1 was added to the assay medium containing rabbit anti-(mouse keratin) serum at a dilution of 1:6000. Increasing amounts of authentic keratin K_1 , isolated from newborn-mouse epidermis, were added as non-radioactive competitor, and the decrease in the radioactivity in the immunoprecipitate was expressed as a percentage of the non-competed (no keratin K_1) control. Each point represents the mean of six different standard curve determinations, which varied by less than $\pm 10\%$. Pre-immune rabbit serum, at final dilutions of 1:100, failed to precipitate ^{125}I -labelled keratin K_1 above precipitable radioactivity observed in control assay media lacking serum.

Results

Optimum conditions for binding of ¹²⁵I-labelled keratin (K₁) to immune serum occurred with ¹²⁵I-labelled keratin K₁ specific radioactivities between 80 and 140 μCi/μg and final antisera dilutions between 1:3000 and 1:6000. When increasing quantities of unlabelled keratin K₁ were competed against ¹²⁵I-labelled keratin (K₁), a linear competition curve was obtained that saturated near 90% inhibition (Fig. 1). In repeated assays with the same iodinated keratin K₁, this standard curve varied by less than ±10% and at 50% inhibition detected 125 ng of keratin K₁ (approx. 2 pmol).

When increasing amounts of urea extracts of mouse epidermal cells were assayed by the radioimmunoassay method, a competition profile similar to the standard curve was obtained (Fig. 2). In this assay, quantification of keratin was possible with less than 1 μg of total extracted protein added, and keratin could be detected at 30 ng (0.5 pmol) or less. Urea extracts of epidermal cells and stratum corneum from human tissues also reacted with anti-(mouse keratin) sera (Steinert *et al.*, 1979). When these extracts were assayed by the radioimmunoassay method, the keratin content of human extracts was lower (Table 1). However, competition assays comparing purified keratin filament proteins of human and mouse origin revealed that solutions of human keratin proteins (competed 10-fold less effectively for anti-(mouse keratin) serum. In contrast, the assay detected virtually 100% of the mouse protein prepared by filament assembly *in vitro*.

Extracts from primary cultures of mouse and human dermal fibroblasts did not contain significant

Fig. 2. Radioimmunoassay profile of extracts of mouse epidermal cells
Mouse epidermal cells were grown in culture for 72 h as described in the Materials and Methods section. Monolayers were extracted with 8 M-urea/buffer, and samples were radioimmunoassayed as described in the legend to Fig. 1. Symbols (●, ■) represent results from independent assays.

Table 1. Quantification of keratin proteins by radioimmunoassay

Cultured cells or tissues were extracted with 8 M-urea/buffer as described in the Materials and Methods section, and samples of extracts were radioimmunoassayed against ¹²⁵I-labelled keratin K₁. The keratin/extracted protein ratio was determined from a standard curve competing purified keratin K₁ against the same ¹²⁵I-labelled keratin K₁. Protein added was determined in the 8 M-urea extracts by the procedure of Bramhall *et al.* (1969).

Cell or tissue	Keratin/total extracted protein (μg/mg)	Protein added to assay medium (μg)
Cultured cells		
Mouse keratinocytes	37-135*	4
Mouse dermal fibroblasts	Not detectable	65
Human keratinocytes before plating	31 (301†)	3
Human keratinocytes	6 (60†)	25
Human dermal fibroblasts	Not detectable	22
Tissues		
Mouse stratum corneum	479	0.7
Human stratum corneum	69 (690†)	5
Mouse hair (low-sulphur)	Not detectable	12
Bovine serum albumin	Not detectable	300

* Varies depending on time in culture.
† Value corrected for 10% cross-reactivity of human keratin proteins and anti-(mouse keratin) serum.

amounts of keratin-reactive material even when up to 100 μg of total protein was added to the assay medium. Bovine serum albumin did not inhibit the assay with the addition of up to 300 μg of protein, suggesting that the values obtained from cells and tissues listed in Table 1 represent specific inhibition by keratin proteins. In addition, mouse hair keratin, an embryologically and structurally related fibrous protein previously shown to be antigenically distinct from epidermal keratin (Kemp & Rogers, 1970), did not compete in this assay.

Discussion

The present work is the first report of a quantitative radioimmunoassay method specific for mammalian epidermal keratin proteins. Previously a radioimmunoassay method was reported for amphibian keratin (Reeves, 1975; 1977), but this assay method did not recognize keratin of mammalian origin. Antibodies to mammalian hair or epidermal keratin also do not recognize amphibian (Lee *et al.*, 1976) or avian (Kemp & Rogers, 1970) keratin. The assay method for mammalian epidermal keratin reported here is able to detect nanograms of keratin from cell or tissue extracts with facility and high reproducibility. Other non-specific proteins (bovine serum albumin, undefined protein in non-epidermal cell extracts) or hair keratins do not inhibit in the assay even when relatively large quantities (300 μg) are added. As the epidermal keratin proteins from two species (mouse and human) are easily quantified, this assay method is useful for a variety of experimental and clinical studies.

Previous results obtained by the use of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis have indicated that keratin proteins from mouse epidermal cells at most times in culture comprise approx. 20% of the total urea-extractable proteins and 70–80% of the total protein from the stratum corneum (Steinert & Yuspa, 1978). In the radioimmunoassay analysis, however, a maximum of 14% of the extractable protein from mouse epidermal cells and about 50% of the proteins extracted from mouse stratum corneum were recognized as keratin. Likewise only about 70% of the expected 85% keratin proteins (Sun & Green, 1978) from human stratum corneum was detected. This slightly lower relative concentration detected by the radioimmunoassay method in protein-rich tissue extracts is very probably due to aggregation of the keratin proteins (filament formation) during the radioimmunoassay procedure, wherein denaturing conditions cannot be maintained to minimize aggregation. Thus several molecules of antigen, as a filament aggregate, may compete for a single antibody binding site. When dilute solutions of purified keratin proteins are assayed, aggregation does not seem to be a problem.

The results obtained by use of the sensitive technique described in the present paper illustrate the utility of this powerful analytical method for the quantification of specific keratin-like proteins in epidermal cells. Since molecules with similar properties to keratin proteins are reportedly a component of the cytoskeleton of many eukaryotic cell types (Steinert *et al.*, 1978; Sun *et al.*, 1979; Frank *et al.*, 1979), such questions as the cellular specificity, chemical and structural similarity and biosynthesis and modulation of these proteins during cellular growth can now be approached in a quantitative manner.

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