

# Localization of Low-sulfur Keratin Proteins in the Wool Follicle Using Monoclonal Antibodies

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**Abstract.** Monoclonal antibodies that recognize components of the low-sulfur keratin proteins extracted from Merino wool have been used to locate these components within the wool follicle. Immunoblotting procedures showed that all of the monoclonal antibodies bound more than one of the eight low-sulfur protein components, indicating that these proteins have antigenic determinants in common. Immunofluorescence studies showed that those antibodies specific

for the component 7 family of the low-sulfur proteins bound to the developing wool fiber, whereas those antibodies recognizing the component 8 family bound to areas throughout the wool follicle, particularly the inner and outer root sheaths, but also to the fiber, the cuticle, and the epidermis. One of the monoclonal antibodies also bound to intermediate filament networks of cultured human epithelial cells.

THE structure of the wool fiber has been the subject of intensive investigation over the past two decades (8, 13). A combination of chemical and structural studies has demonstrated that the fiber is a composite structure of keratinized cells, each containing longitudinally arranged microfibrils embedded in an amorphous matrix. It has also been shown that the microfibrils are composed of a distinct set of proteins, the low-sulfur proteins, and that the matrix is composed of a mixture of proteins designated the high-sulfur and high-tyrosine proteins (13). Cross-linking by disulfide bridges stabilizes the keratin structures and renders the proteins insoluble (14).

Although much of the earlier biochemical, chemical, and physical properties of keratin proteins have been defined in keratins extracted from wool fibers (13), almost all of the immunological studies have used either keratin extracted from epidermis (1, 15, 22, 29, 30) or cytokeratin (3, 4, 12, 15, 21). Therefore, there is a need to add an immunological approach to the earlier biochemical studies on the structure of components of wool keratins, and to further define the relationships between keratins from the diverse sources.

Two serious difficulties limited earlier immunological studies of wool keratins (9–11, 19). The first was cross-reactivity between the different wool keratins (9), a consequence of the high degree of amino acid sequence homology between the components (6). The second was the presence of S-carboxymethyl groups introduced into solubilized keratin extracted from wool to prevent re-formation of disulfide bridges (11, 19). S-carboxymethyl groups are powerful haptens and antisera prepared using such proteins exhibited cross-reactivity with all S-carboxymethylated proteins. The development of monoclonal antibodies has allowed the preparation of antibodies specific for wool keratin proteins without the problem of cross-reactivity with carboxymethyl groups (16). Of the

three major groups of wool proteins, the low-sulfur group alone has homologies with intermediate filament proteins from other epithelial tissues (6). The low-sulfur group has been subdivided into two families of which one, designated the component 7 family, has similar properties to type II intermediate filament protein and the other, designated the component 8 family, is similar to type I intermediate filament protein (13). This paper describes the use of monoclonal antibodies in the study of low-sulfur protein synthesis and deposition in wool follicles.

## Materials and Methods

### Low-sulfur Wool Proteins

Low-sulfur wool proteins were extracted by reduction and alkylation of Merino wool as previously described (5).

### Preparation of Anti-low-sulfur Keratin Monoclonal Antibodies

Immunizations and cell fusions were carried out as previously described (16). Screening of hybridoma supernatants was by alkaline phosphatase enzyme-linked immunosorbent assay. 96-well polyvinyl chloride EIA plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 200 ng/well of either low-sulfur keratin proteins or of S-carboxymethylated bovine serum albumin (BSA) in 0.1 M carbonate-bicarbonate buffer, pH 9.6. Hybridoma supernatants were diluted 1:1 in phosphate-buffered saline (PBS) containing 0.1% Tween-20 and incubated at room temperature for 1 h. After washing, rabbit anti-mouse IgG conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) diluted 1:1,000 was added to each well and incubated as above. The substrate used was *p*-nitrophenyl phosphate, 1 mg/ml in 10% diethanolamine buffer, pH 9.6. After color development, the absorbances were read in a Dynatech Mini-reader II at 410 nm. Hybridomas secreting antibodies to low-sulfur proteins but not to S-carboxymethylated BSA were cloned by limit dilution and propagated in culture.

### Isotype Enzyme-linked Immunosorbent Assay

The isotype of each monoclonal antibody was determined by an enzyme-linked immunosorbent assay. 96-well PVC EIA plates were coated with low-sulfur

keratin proteins and hybridoma supernatants were added as described above. Affinity-purified rabbit antisera to each murine immunoglobulin subclass (IgG<sub>1</sub>, IgG<sub>2a</sub>, IgG<sub>2b</sub>, IgG<sub>3</sub>, IgM, IgA) (Mallinckrodt Inc., Australia) were diluted 1:500 and added to the plate for 1 h at room temperature. Goat anti-rabbit Ig conjugated to horseradish peroxidase (Bio-Rad Laboratories, Richmond, CA) was diluted 1:200 and added to each well. The substrate used was *o*-phenylenediamine, 0.34 mg/ml in 0.1 M citrate buffer, pH 6, to which 0.075% (vol/vol) H<sub>2</sub>O<sub>2</sub> was added. The absorbances were read on a Dynatech Minireader II at 490 nm.

### Western Transfer and Immunoblotting

Low-sulfur keratin proteins were electrophoresed on 10% polyacrylamide SDS gels according to the method of Laemmli (17). The proteins were transferred to nitrocellulose membranes (0.45 μm pore size; Schleicher & Schull, Inc., West Germany) in a transfer buffer consisting of 0.025 M Tris-HCl, 0.192 M glycine, 20% (vol/vol) methanol, pH 7.4, for 16 h at room temperature according to the method of Towbin et al. (28). After blocking the nitrocellulose membranes with a skim milk solution designated "BLOTTO" (18), immunoblotting was performed using monoclonal antibodies in the form of undiluted hybridoma culture supernatants. After a 1-h incubation at room temperature, the strips were washed with Tris-buffered saline and goat anti-mouse Ig conjugated to horseradish peroxidase (Bio-Rad Laboratories) diluted 1:2,000 in BLOTTO was added to the nitrocellulose strips. After incubation and washing, the binding of individual monoclonal antibodies to the low-sulfur keratin proteins was visualized using a 0.3% (wt/vol) solution of 4-chloro-1-naphthol in 20 ml methanol, to which 0.06% (vol/vol) H<sub>2</sub>O<sub>2</sub> in 100 ml Tris-buffered saline, pH 7.4, had been added.

### Immunofluorescence

6-μm-thick frozen sections were cut from biopsies of Merino sheep skin using a cryotome. The sections, both longitudinal and transverse, were placed onto gelatin-coated glass microscope slides and air dried at 4°C for 1–2 h. Undiluted hybridoma culture supernatants were applied to the sections for 45 min at room temperature. After washing in two changes of PBS, sheep anti-mouse Ig conjugated to fluorescein isothiocyanate (Silenus Laboratories, Melbourne) was diluted 1:20 in PBS containing 0.1% Tween-20 and added to the sections. After incubation and washing as above, the sections were mounted in 90% (vol/vol) glycerol containing 1 mg/ml *p*-phenylenediamine in PBS, pH 8.0, and examined under incident light excitation with illumination at 450 nm.

### Immunoelectron Microscopy

Biopsies of sheep skin were fixed in 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at room temperature. The specimens were then

transferred to 0.5 M NH<sub>4</sub>Cl in 0.1 M phosphate buffer, pH 7.4, for 2 h at room temperature and rinsed in 0.1 M phosphate buffer. The specimens were dehydrated through a graded series of ethanols and embedded in an acrylic resin, LR White, overnight. The resin was polymerized at 55°C in the absence of oxygen and ultra-thin sections were cut and placed onto Formvar-coated copper grids.

Immunolocalization studies were performed using an adaptation of the immunogold technique (25). Monoclonal antibodies were purified from hybridoma supernatants by precipitation with 50% saturated ammonium sulfate at 4°C and diluted to 50 μg/ml in PBS containing 1% (vol/vol) Tween-20, and 3% (wt/vol) BSA. Grids holding ultra-thin sections were inverted onto a drop of antibody for 10 min at room temperature. After washing in PBS/Tween, the

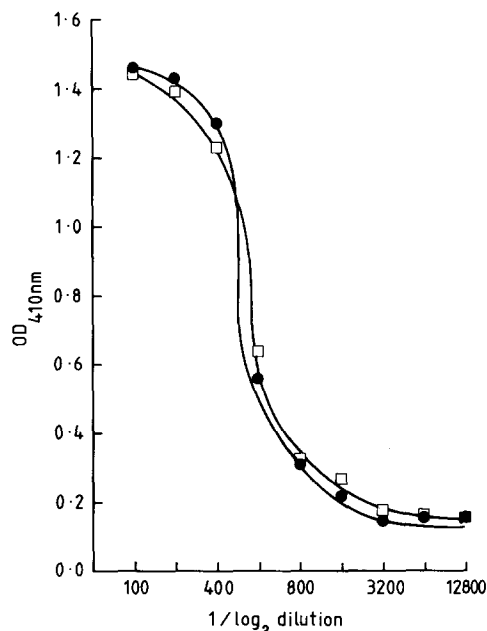


Figure 1. Titration curves of antiserum from a BALB/c mouse immunized with low-sulfur wool keratin proteins against low-sulfur protein (●) and an S-carboxymethylated preparation of BSA (□) in order to indicate the antigenicity of the S-carboxymethyl group.

Table I. Immunoglobulin Subclass, Protein Specificity, and Localization in the Wool Follicle of the Monoclonal Antibodies Raised to the Low-Sulfur Family of S-carboxymethylated Wool Proteins

Monoclonal antibody	Subclass	Low-sulfur protein components bound	Wool follicle localization
αK7	IgG <sub>1</sub>	7a, 7b, 7c, 5	fiber, base
αK8	IgG <sub>1</sub>	7a, 7b, 7c, 5	fiber
αK4	IgG <sub>1</sub>	7a, 7b, 5	fiber, base
αK6	IgG <sub>1</sub>	7a, 7b, 7c, 5	fiber, base
K7C22	IgG <sub>1</sub>	7a, 7b, 7c, 5	fiber, base
K2F2	IgM	5, 7a, 7c	irs*, trichohyalin, fiber
K2B9	IgG <sub>1</sub>	8c-1, 8c-2, 8b	irs, cuticle, base
KF24	IgM	5, 8c-1, 8c-2, 8b	fiber, irs, ors <sup>†</sup>
KF29	IgG <sub>1</sub>	5, 8c-1, 8a	fiber, base
αK1	IgG <sub>1</sub>	5, 8c-1, 8c-2, 8a	fiber, base
αK2	IgG <sub>1</sub>	8c-1, 8c-2, 8b, 8a	ors, epidermis
KF25	IgM	5, 8c-1, 8c-2, 8b, 8a	irs, ors, epidermis
F23	IgG <sub>1</sub>	7a, 7b, 7c, 5, 8c	fiber, base
αK3	IgG <sub>2b</sub>	7b, 8c-1, 8c-2, 8b, 8a	fiber
αK5	IgG <sub>1</sub>	7a, 7b, 5, 8c-1, 8c-2, 8b	irs, cuticle, base, epidermis
KF27	IgM	7a, 7b, 7c, 8c-1, 8b, 8a	irs, epidermis, fiber
KF28	IgG <sub>1</sub>	7a, 7b, 7c, 5, 8c-2, 8b, 8a	fiber, base
K42	IgM	7a, 7b, 7c, 5, 8c-1, 8c-2, 8b, 8a	ors, epidermis
KF26	IgG <sub>1</sub>	7a, 7b, 7c, 5, 8c-1, 8b	ors, cuticle

\* irs, inner root sheath.

† ors, outer root sheath.

grid was placed onto a drop of rabbit anti-mouse Ig diluted to 50  $\mu\text{g}/\text{ml}$  for 10 min at room temperature. After washing, the grid was transferred to a drop of protein A-colloidal gold (14-nm particles) (a gift of Dr. Stuart Craig, Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, Canberra), diluted 1:20. The sections were washed, stained in 2% aqueous uranyl acetate, followed by lead citrate, and examined in a JEOL transmission electron microscope.

## Results

### Monoclonal Antibodies to Low-sulfur Wool Keratins

Table I summarizes the reactivities of the 19 anti-low-sulfur wool keratin monoclonal antibodies. Most of the monoclonal antibodies were of the IgG<sub>1</sub> subclass (65%), with the remainder being either IgG<sub>2b</sub> or IgM. There was no correlation between the subclass of the antibody and the components recognized. The previously published subclass assignments for  $\alpha\text{K}8$  (IgG<sub>2b</sub>) and  $\alpha\text{K}2$  (IgM) (16) were found to be incorrect. The isotyping system used in this paper has been found to be more sensitive than that used previously.

The S-carboxymethyl groups introduced into extracted keratin proteins are major antigenic determinants (10, 19). This

is demonstrated by the experiment shown in Fig. 1 using polyclonal antiserum from a BALB/c mouse immunized with S-carboxymethyl low-sulfur wool keratins. The antiserum was titrated in an enzyme-linked immunosorbent assay against a preparation of these keratins and against S-carboxymethyl BSA. The titration curves are very similar, indicating a high proportion of S-carboxymethyl cysteine-reactive antibodies in the antiserum. This result highlights the advantage of using hybridoma technology to select for antibodies binding to epitopes on keratin proteins rather than to the immunodominant S-carboxymethyl group.

### Determination of the Specifications of the Low-sulfur Wool Keratin Monoclonal Antibodies by Western Transfer Immunoblots

SDS PAGE of low-sulfur keratin proteins reveals eight major protein components (Fig. 2). These can be divided on the basis of amino acid composition into two families, the components 7 (7a, 7b, 7c) and components 8 (8a, 8b, 8c-1, 8c-2), plus component 5 which has similarities to both families (8, 13). All of the 19 monoclonal antibodies raised to the low-sulfur keratin proteins bind to more than one of these eight components, confirming the observations of an earlier study (16). Seven are specific for the components 7, six are specific for the components 8, and six bind to components of both families. Fig. 2 shows representative immunoblots of each of these patterns. In addition, one of the monoclonal antibodies binds primarily to component 5 and as such is the most nearly monospecific of the monoclonal antibodies.

Component 5 appears to have antigenic similarities to both

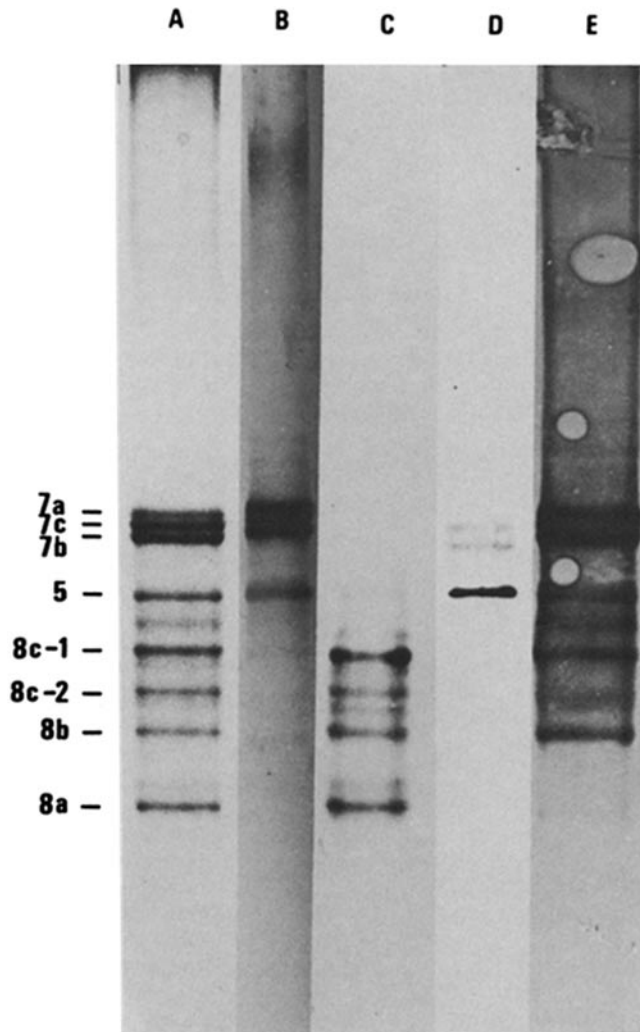


Figure 2. Western transfer immunoblots of extracted wool low-sulfur keratin proteins probed with monoclonal antibodies of varying specificity. (A) Coomassie Blue-stained 10% polyacrylamide gel of low-sulfur protein. (B-E) Immunoblots of the same preparation probed with antibodies  $\alpha\text{K}7$  (B),  $\alpha\text{K}2$  (C), K2F2 (D), and  $\alpha\text{K}5$  (E).

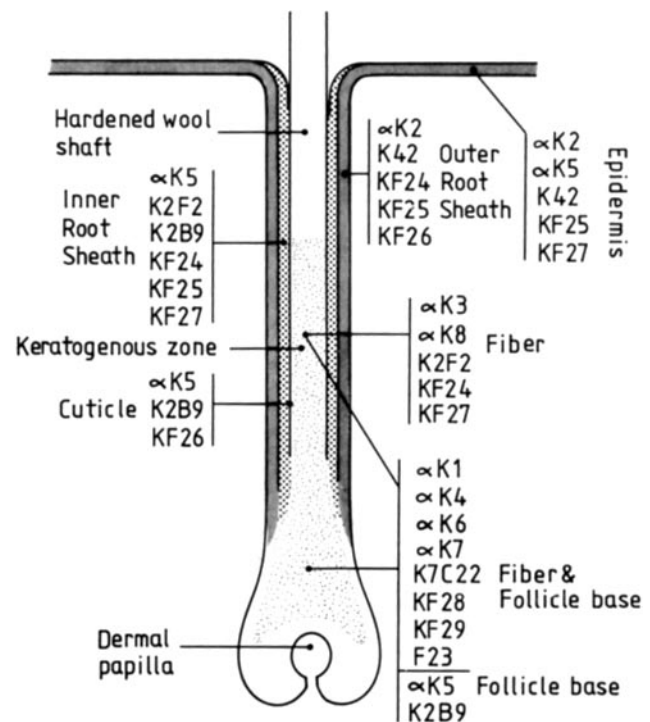
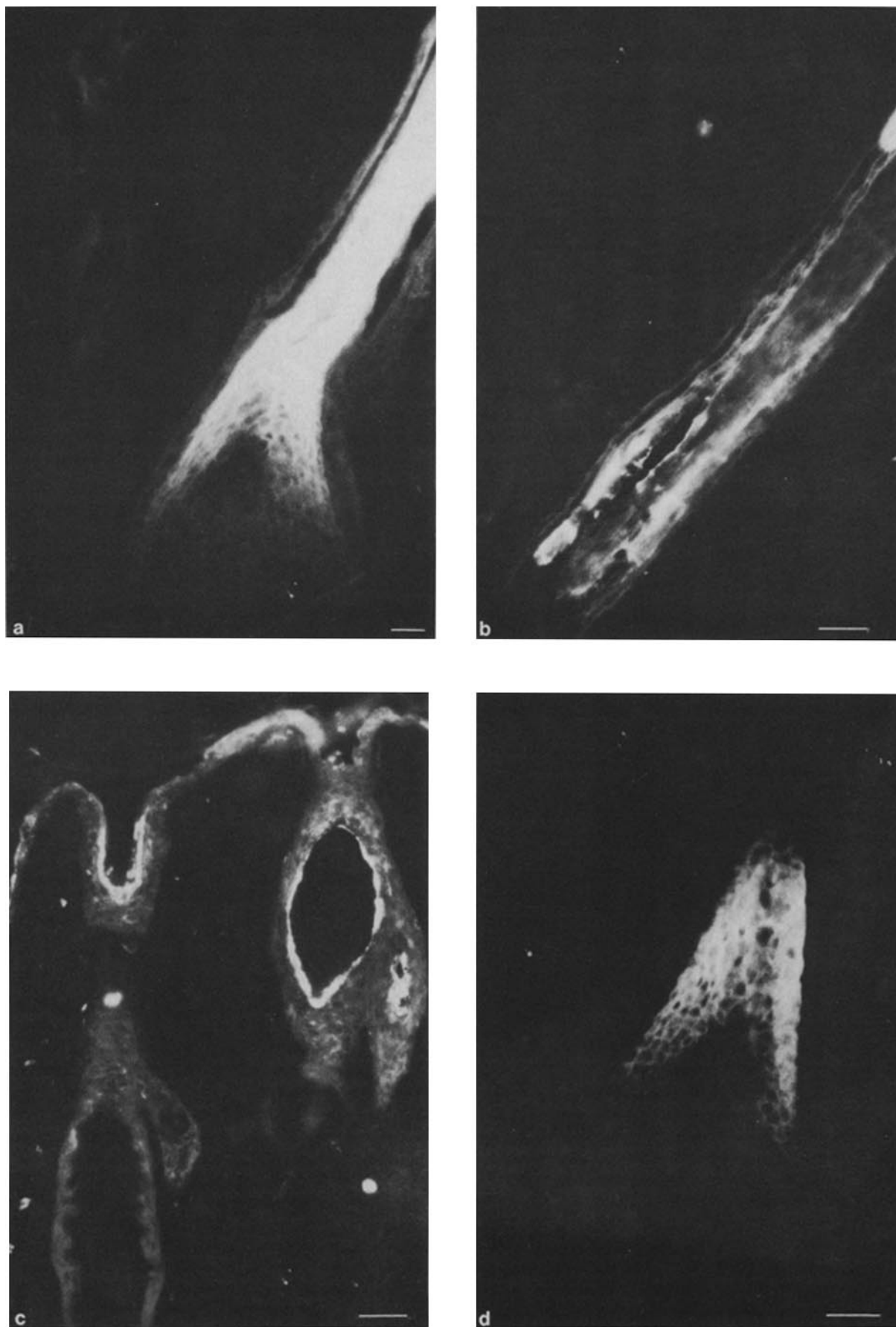


Figure 3. Schematic representation of the binding of low-sulfur keratin monoclonal antibodies to a longitudinal section of a wool follicle. The antibodies are grouped according to the area recognized in indirect immunofluorescence assays using cryotome sections of sheep skin.



**Figure 4.** Indirect immunofluorescence of cryotome sections of sheep skin probed with low-sulfur keratin-specific monoclonal antibodies. (a) Longitudinal section of wool root treated with antibody  $\alpha$ K7. (b) Longitudinal section of developing wool shaft treated with antibody K2B9. (c) Section of sheep epidermis and upper wool follicles treated with antibody  $\alpha$ K2. (d) Oblique section of wool root treated with antibody F29. Bars, 20  $\mu$ m.

families. This similarity is not surprising in view of the similarities in amino acid sequence between components 7 and 5 (7). Thus, four of the antibodies specific for the family 8 components also recognize component 5. It should be noted that bands or smears were frequently seen on gels in the higher molecular weight range, above the components 7, and these were reactive with many of the monoclonal antibodies in immunoblots (see, for example, Fig. 2*b*). These bands or smears were interpreted as being a result of aggregation of two or more of the components, with the apparent retention of the original antigenic sites (16).

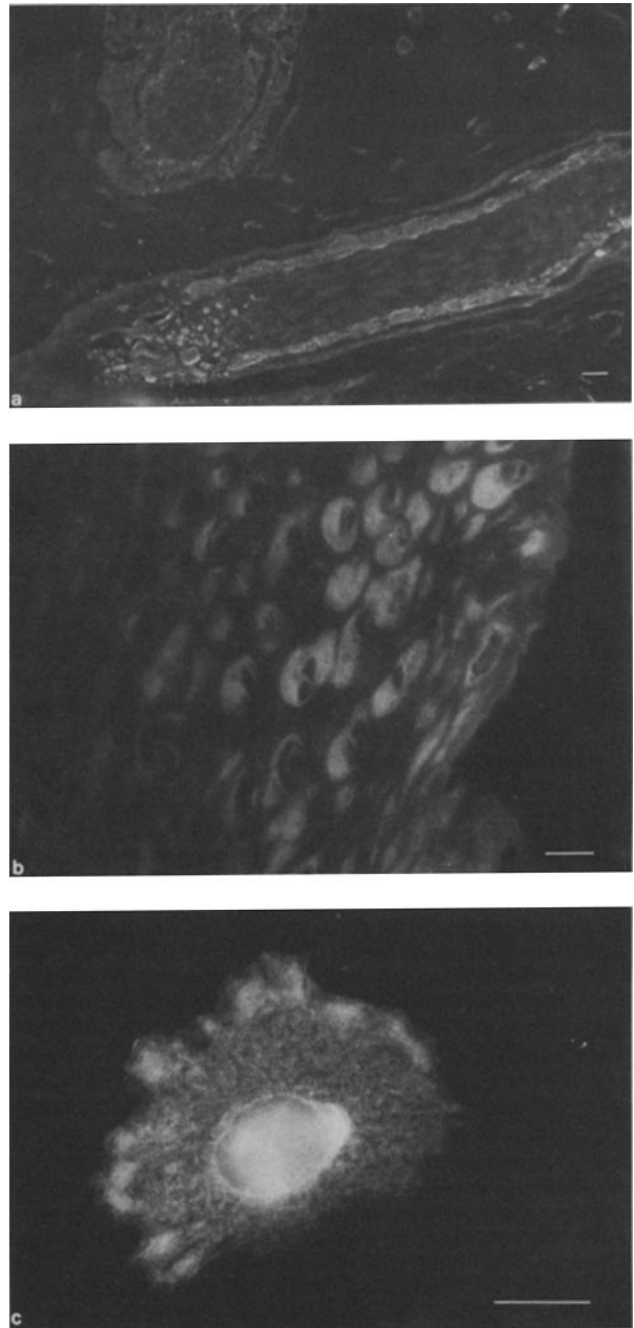
***Immunolocalization of the Binding of Anti-low-sulfur Keratin Monoclonal Antibodies in the Wool Follicle by the Indirect Immunofluorescence Technique***

The location of proteins in thin sections of sheep skin reacting with the panel of monoclonal antibodies was investigated using indirect immunofluorescent. The results are listed in Table I and shown schematically in Fig. 3. Despite the disadvantage that the monoclonal antibodies all recognize more than one protein component, the studies indicate a specific pattern of antibody binding to wool follicles. All of the seven monoclonal antibodies which specifically recognize the family 7 components bind to the cells of the developing fiber, with binding first appearing midway in the base of the follicle, above the dermal papilla (Fig. 4*a*). This binding in the fiber reaches a maximum in the region of the keratogenous zone and thereafter decreases to become negligible in the fully hardened fiber, presumably because of the exclusion of the antibodies from the more tightly packed, mature keratin microfibrils (19). The six monoclonal antibodies which specifically recognize the family 8 components are more heterogeneous than those specific for the family 7 components, and bind to cells in structures throughout the follicle. Thus, some members of the group recognize the inner and outer root sheaths, the cuticle, the cortex, and the epidermis (Fig. 4, *b-d*), whereas some are specific for the same region of the developing wool fiber as the component 7-specific antibodies described above. Those component 8-specific monoclonal antibodies which are more selective and bind only to the fiber cortex are KF29 and  $\alpha$ K1. Five monoclonal antibodies bind to the cells of the outer root sheath. One antibody ( $\alpha$ K2) binds to both the outer root sheath and to the continuation of this region into the basal layers of the epidermis (Fig. 4*c*).

The six monoclonal antibodies which bind to components from both families also recognize structures throughout wool follicles and the epidermis (Table I).

The binding of the monoclonal antibodies is typically seen as fluorescence within the cytoplasm of the cells of the target structure, indicating that the proteins recognized are intracellular.

One of the monoclonal antibodies binding to the family 7 components (K2F2) binds very strongly to component 5 as indicated by Western Transfer (Fig. 2*d*) and was found to recognize trichohyalin granules in the inner root sheath of the wool follicle (Fig. 4*a*). Trichohyalin is a protein which is chemically distinct from keratin in that it is deficient in cystine and contains the amino acid citrulline (23), and is considered to be a keratin precursor (20). This also binds to human epithelial cells, both in culture and in biopsies of squamous epithelium of the cervix (Fig. 5, *b* and *c*). In the case of human



*Figure 5.* Indirect immunofluorescence using monoclonal antibody K2F2. (a) Longitudinal section of wool follicle in keratogenous zone. (b) Section of biopsy of normal human cervical squamous epithelium. (c) Human epithelial kidney cell. Bars, 10  $\mu$ m.

epithelial kidney cells, binding can be seen to a filamentous network in the cytoplasm of the cells.

***Localization of the Ultrastructural Binding of Anti-low-sulfur Keratin Monoclonal Antibodies in the Cortex of the Wool Fiber by the Immunogold Technique (16)***

The immunogold technique confirmed many of the observations from the indirect immunofluorescence technique and demonstrated that the binding of the monoclonal antibodies

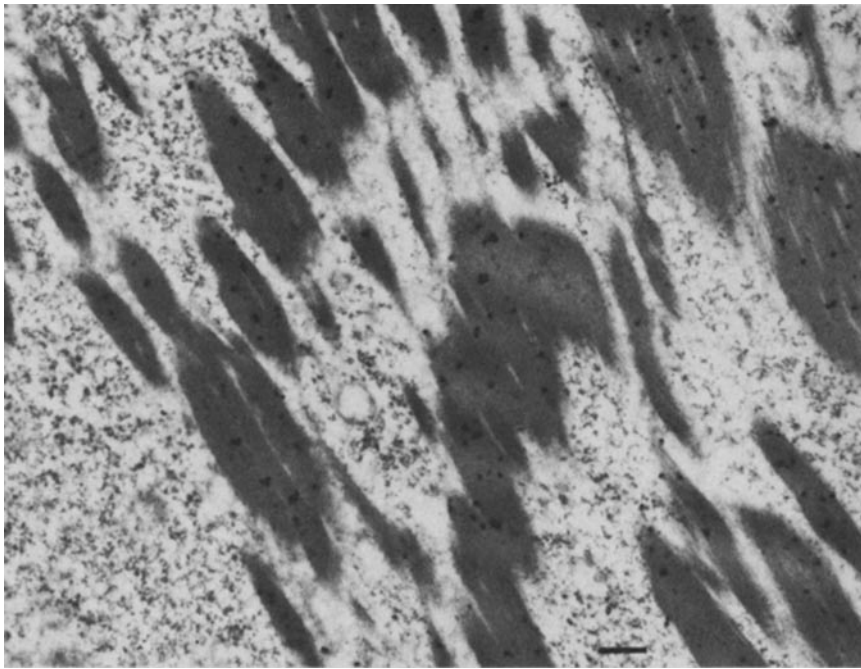
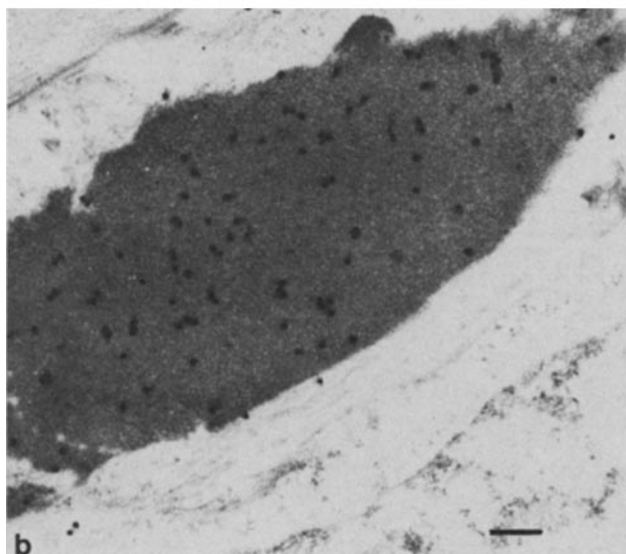
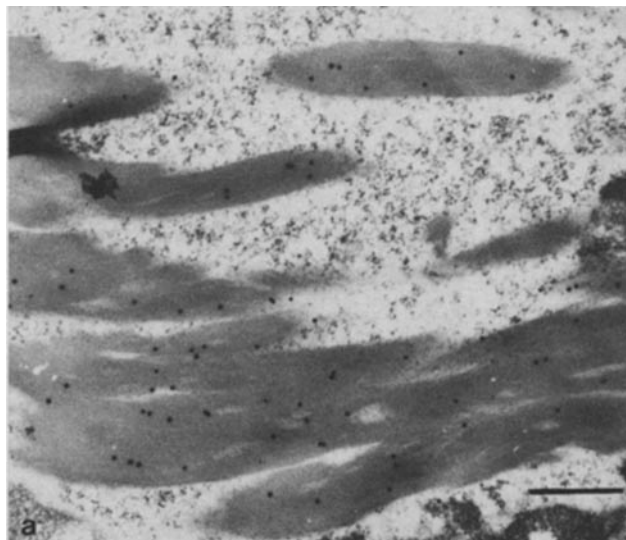


Figure 6. Immunogold electron microscopy of section of wool follicle cortex treated with antibody  $\alpha$ K4. Longitudinal section of keratin microfibril bundles. Bar, 100 nm.



which recognize the family 7 components bind to keratin bundles in the cortex of the wool fiber (Fig. 6). The binding of K2F2 was also clearly seen to occur both on keratin filament bundles and on amorphous electron dense granules in the inner root sheath, assumed to be trichohyalin granules (23) (Fig. 7).

### Discussion

The low-sulfur proteins of wool are a group of closely related proteins with high helical content (8). There is considerable ultrastructural and chemical evidence that they constitute the microfibrils of the wool cortex (2, 8, 13, 24) and that proteins with similar amino acid sequences form filamentous structures in cells of other keratinous tissues (for example, epidermis) as well as the intermediate filaments of mammalian cells (26, 27). It appears that the filamentous proteins of wool and hair are specialized forms of a much more widespread group of filament proteins (6).

The high specificity of monoclonal antibodies has allowed more precise studies of the antigenic relationships between the different wool low-sulfur proteins. As previously reported (16), the high degree of homology between the proteins leads to recognition of multiple components by each antibody. Subdivision of the proteins into two families, the component 8 family and the component 7 family, predicted from amino acid sequence studies (6), is reflected in the pattern of antibody binding. This subdivision apparently corresponds to the distinction between type I and type II intermediate filament proteins, respectively, which have similar molecular weight ranges to the two wool keratin families and have also been found to possess characteristic immunological epitopes (15, 29, 30). Type I and II intermediate filament proteins have been shown to possess some common antigenic determinants

Figure 7. Immunogold electron microscopy of sections of wool follicles using monoclonal antibody K2F2. (a) Longitudinal section of keratin bundles in the cortex. (b) Section of inner root sheath showing trichohyalin granule. Bar, 100 nm.



(3, 12, 21) as do the wool low-sulfur protein families.

The homology with intermediate filaments is illustrated by the antibody which exhibits the greatest selectivity for wool proteins, K2F2. This antibody recognizes the greatest range of cells other than those of the wool cortex. The antibody was found to recognize structures in cultured cells, in biopsy samples of human squamous epithelium from the cervix, and trichohyalin granules in the inner root sheath of wool follicles. To date, there has been no suggestion of homology between trichohyalin and cortical keratin or intermediate filament components. It is possible that K2F2 recognizes an epitope which is rare in low-sulfur proteins, but more common in distantly related proteins.

The immunofluorescent localization of low-sulfur proteins confirms previous results obtained with polyclonal antisera (9–11, 19) but greater precision has been possible through the use of monoclonal antibodies. Reaction of the antibodies with cells in the cortex of the developing fiber was observed, as expected, although binding was lost in upper levels of the follicles as the fibers hardened, as previously reported (11, 19). This phenomenon may be caused by loss of penetration of the antibodies into the hardened, highly cross-linked cortex. Binding of antibodies to hardened fibers can be restored by mild protease digestion of the fibers (Hewish, D. R., unpublished observations), which presumably opens the fiber structure to allow penetration of antibody.

The strong binding of some antibodies, particularly those recognizing the component 8 family, to parts of the wool follicles which do not become incorporated into the wool fiber is surprising. We assume that the explanation for this phenomenon, as for K2F2 above, may be cross-reactivity of the antibodies with related proteins in the follicle structures, and possibly with intermediate filament proteins in the follicle cells. Some of the monoclonal antibodies described here, although reacting with isolated wool keratins, show little or no binding to the wool cortex. Possibly these antibodies recognize determinants which are poorly exposed, or of altered conformation, in the native fiber structure, but are more accessible in intermediate filaments in the associated cells. Protein structural studies (13) have shown that proteins of both families are present in the microfibrils, so the lack of reactivity must be due to an immunological phenomenon, rather than differential location of the proteins within the follicle.

The authors wish to thank Dr. Stuart Craig for advice and assistance with the immunogold technique; Professor Ban-Hok Toh, Alfred Hospital, Melbourne for carrying out the intermediate filament immunofluorescence; and Dr. Andrew Ostor, Royal Women's Hospital, Melbourne, for provision of pathology slides. We are also grateful for the excellent technical assistance rendered by Miss Silvana Ciccostoto and Miss Rosalia Bruzzese.

This work was supported by the Australian Wool Corporation in the form of a Commonwealth Scientific and Industrial Research Organization Postgraduate Fellowship for P. W. French.

Received for publication 11 June 1985, and in revised form 18 November 1985.

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