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Monoclonal Antibodies to a Subfraction of Merino Wool High-tyrosine Proteins

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Abstract

Monoclonal antibodies were prepared which react with members of the high-tyrosine type proteins from Merino wool. Specificity was confirmed by the use of Western transfer immunoassays and by enzyme-linked immunosorbent assay on purified fractions. Immunofluorescent staining of sections of wool follicles using the antibodies showed that the proteins were present in the developing wool shaft but that staining was asymmetric, indicating specific location of the proteins in the orthocortex of the fibres. Immunogold-electron microscopy confirmed that one of the antibodies bound to the keratin microfibril bundles.

Introduction

The high-tyrosine proteins of wool are a group of low molecular weight proteins (usually below 10 000), with unusual amino acid compositions, being rich in glycine (22–40 residues per 100 residues) and the aromatic amino acids, particularly tyrosine (11–20 residues per 100 residues) (Marshall *et al.* 1980). Their occurrence in wool and hair is variable, ranging from less than 1% of total protein in Lincoln wool, to over 30% in echidna quill (Fraser *et al.* 1973).

It is accepted that the high-tyrosine proteins form part of the matrix of the wool cortex, a location they share with the high-sulfur protein fraction (Gillespie and Darskus 1971; Frenkel *et al.* 1974). The evidence for this comes from diverse indirect sources, including X-ray diffraction studies of the volume of matrix present (Fraser *et al.* 1973), biochemical studies on isolated morphological components of the wool fibre (Bradbury 1973), and physical measurements of the swelling of keratins (Bendit and Gillespie 1978).

The high-tyrosine proteins have been divided into two groups on the basis of their solubility properties and amino acid composition (Marshall *et al.* 1980). This paper describes immunochemical studies of one group (referred to as type II—Marshall *et al.* 1980), of the high-tyrosine proteins. The study was carried out as an extension of a general examination of wool keratins using monoclonal antibodies (Hewish *et al.* 1984). The use of monoclonal antibodies has been found to be the only available method for applying immunochemical procedures to the study of wool proteins, since it avoids the problems of chemical modification of the proteins during extraction, considerable amino acid sequence homology between components, and cross-contamination of keratin fractions (Frater 1968; Kemp and Rogers 1970).

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Materials and Methods

Immunizations

High-tyrosine type II proteins were extracted from wool by acetone–ammonium sulfate precipitation (Marshall *et al.* 1980). Balb/c mice were primed with 100 μg of protein in Freund's complete adjuvant intraperitoneally, and 4 weeks later boosted with 100 μg of protein in Freund's incomplete adjuvant, intraperitoneally. A final (intravenous) boost of 50 μg of protein in phosphate-buffered saline (PBS) was administered 3 days prior to fusion.

Cell Fusion

Spleen cells from mice immunized with high-tyrosine type II proteins were fused with NSI myeloma cells as previously described (Hewish *et al.* 1984) using polyethylene glycol 4000 (BDH Australia, Port Fairy, Vic.). The fusion products were plated into 96-well polystyrene tissue culture trays (Corning, Oneonta, New York, U.S.A.).

Screening of Hybridoma Supernatants by Alkaline Phosphatase-linked Immunosorbent Assay

After 10 days post-fusion the culture supernatants from the wells containing hybridoma colonies were screened by alkaline phosphatase-linked immunosorbent assay against high-tyrosine type II proteins, high-tyrosine type I proteins (Marshall *et al.* 1980) and against *S*-carboxymethylated bovine serum albumin. The latter was used to detect those supernatants containing antibodies to the *S*-carboxymethyl groups present on the solubilized wool proteins as a result of the extraction process (Hewish *et al.* 1984). 200 ng of each of the antigens in 0·1 M carbonate–bicarbonate buffer, pH 9·6, was bound to several wells of a 96-well polyvinylchloride (PVC) enzyme-linked immunosorbent assay (ELISA) plate (Dynatech Laboratories, Alexandria, Virginia, U.S.A.) by incubation at 4°C for 16 h. Following washing four times with PBS–0·5% (v/v) Tween-20, culture supernatant diluted 1 : 1 (v/v) in PBS–Tween was added to the tray for 1 h at room temperature. After washing four times with PBS–Tween buffer, rabbit anti-mouse immunoglobulin (Ig) coupled to alkaline phosphatase (Sigma Chemical Co., St Louis, Missouri, U.S.A.), diluted 1 in 1000 in PBS–0·5% (v/v) Tween-20 was added to the tray for 1 h at room temperature. After washing as above, *p*-nitrophenyl phosphate [1 mg/ml in 10% (w/v) diethanolamine, pH 9·6] was added, and following a 30-min incubation at room temperature, the absorbances of the wells were read at 410 nm using a Dynatech Minireader II ELISA plate-reader.

Hybridoma colonies secreting antibodies to the high-tyrosine type II proteins only were cloned by limit dilution and resulting monoclonal antibodies in the form of culture supernatants were used for immunochemical and immunohistological characterization of the high-tyrosine type II protein.

Isotype Determination by ELISA

The isotype of each monoclonal antibody was determined by ELISA using the standard procedure as described above. Antibody binding was detected by affinity-purified rabbit antisera to each murine immunoglobulin subclass (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM, IgA, BJκ_1, BJλ) (Mallinckrodt Australia, Melbourne), diluted 1 in 500 in PBS-Tween and added to the plate for 1 h at room temperature. After washing as above, goat anti-rabbit Ig conjugated to horse radish peroxidase (Bio Rad Laboratories, Richmond, California, U.S.A.) was diluted 1 in 200 in PBS–Tween-20 and added to each well and incubated and the plate washed as above. The substrate used was *o*-phenylenediamine, 0·34 mg/ml in 0·1 M citrate buffer, pH 6, to which 0·075% (v/v) H_2O_2 was added. The absorbances were read on a Dynatech Minireader II at 490 nm.

Western Transfer Immunoblotting

Sodium dodecyl sulfate (SDS)–12·5% (w/v) polyacrylamide gel electrophoresis of partially purified preparations of low-sulfur keratin proteins containing high-tyrosine type II proteins was carried out according to the method of Laemmli (Laemmli 1970). The proteins were transferred to nitrocellulose membranes (0·45 μm pore size, Schleicher and Schull, Dassel, W. Germany) in a transfer buffer consisting of 0·025 M Tris-HCl, 0·192 M glycine, 20% (v/v) methanol, pH 7·4, for 16 h at room temperature according to the method of Towbin *et al.* (1979). After blocking the nitrocellulose membranes with a skim milk solution, BLOTTO (Johnson *et al.* 1984) immunoblotting was performed using monoclonal antibodies in the form of undiluted culture supernatants. After 1 h incubation at room temperature followed by four 10-min washes in BLOTTO, goat anti-mouse Ig conjugated to horseradish peroxidase (Bio-Rad) diluted 1 in 2000 in BLOTTO was added to the nitrocellulose strips. Incubation and washing was as

for the first antibody. Binding of individual monoclonal antibodies to the keratin proteins was visualized using 0·3% (w/v) solution of 4-chloro-1-naphthol in 20 ml methanol, to which 0·06% (v/v) H_2O_2 in 100 ml Tris-buffered saline, pH 7·4, had been added.

Characterization of Binding by ELISA on Separated Components of High-tyrosine Type II Proteins

Components of high-tyrosine type II proteins were separated on quaternary aminoethyl cellulose chromatography at pH 10·5 (Marshall *et al.* 1980). The seven components, designated, α, β, γ, δ, λ, θ, and μ, respectively, were coated onto wells of a 96-well PVC ELISA tray in 0·1 M bicarbonate buffer at a protein concentration of 4 μg/ml. The binding of each monoclonal antibody [in the form of culture supernatants diluted 1 : 4 in PBS–0·05% (v/v) Tween-20] to each component was assessed by alkaline phosphatase-linked immunosorbent assay as described above.

Indirect Immunofluorescence

Frozen cryotome sections of sheep skin 6 μm thick were placed onto gelatin-coated glass microscope slides, dried for 2 h at 4°C and exposed to anti-high-tyrosine type II monoclonal antibodies in the form of undiluted culture supernatant for 45 min at room temperature. After three washes in PBS–Tween-20 the sections were exposed to rabbit anti-mouse Ig antiserum conjugated to fluorescent isothiocyanate (Silenus Laboratories Melbourne, Australia) diluted 1 in 20 in PBS–0·05% (v/v) Tween-20 for 45 min at room temperature. After three washes in PBS–Tween-20, the sections were mounted in 90% (v/v) glycerol in PBS, pH 8·0, containing 1 mg/ml *p*-phenylenediamine and examined under incident ultraviolet light illumination with excitation at 450 nm and emission observed at 510 nm. Controls of culture supernatant containing antibodies which did not recognize wool protein were routinely included.

Immuno-electron Microscopy

Biopsies of sheep skin were fixed in 1% (w/v) 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_4Cl 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, London Resins, Basingstoke, U.K.), 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 (Roth *et al.* 1978).

Monoclonal antibody supernatants were partially purified by precipitation with 50% (w/v) saturated ammonium sulfate at 4°C and diluted to 50 μg of protein per millilitre (as estimated by absorbance at 280 nm) in PBS containing 1% (v/v) Tween-20, and 3% (w/v) bovine serum albumin. Grids containing ultra-thin sections were inverted over a drop of antibody for 10 min at room temperature. After washing (3 × 5 min) in PBS–Tween the grid was placed onto a drop of rabbit anti-mouse Ig for 10 min at room temperature. After washing as above, the grid was transferred to a drop of protein A–colloidal gold (14 nm particles), 1/20 in PBS. The sections were washed with distilled water, stained in 2% (w/v) uranyl acetate followed by lead citrate and examined in a JEOL transmission electron microscope.

Results

Monoclonal Antibodies

From two cell fusions, four monoclonal antibodies specific for the type II proteins were raised and characterized. These four monoclonal antibodies were designated HiT 11, HiT 12, HiT 96 and KF 22. They were of the IgG_1 subclass (HiT 11, HiT 12, HiT 96) and of the IgG_{2b} subclass (KF 22), all with kappa light chains, as determined by peroxidase-linked immunosorbent assay isotyping.

Western Transfer Immunoblotting

When high-tyrosine proteins were subjected to electrophoresis and transferred to nitrocellulose the resulting immunoblots exhibited poor resolution and low antibody binding. However, in Western transfers of low-sulfur keratin proteins, each of the four monoclonal antibodies bound to high-tyrosine protein bands present as

contaminants in the protein preparations, but not to any of the higher molecular weight low-sulfur keratin proteins (Fig. 1). In fact, the low-sulfur proteins appear as pale bands in the background staining of the nitrocellulose, presumably because the high local protein concentrations exclude antibodies from non-specific binding sites. Although it is not known why this procedure was superior, it demonstrated that the antibodies were specific for the high-tyrosine type II proteins. One possible explanation is that the different treatment of the protein fractions during the

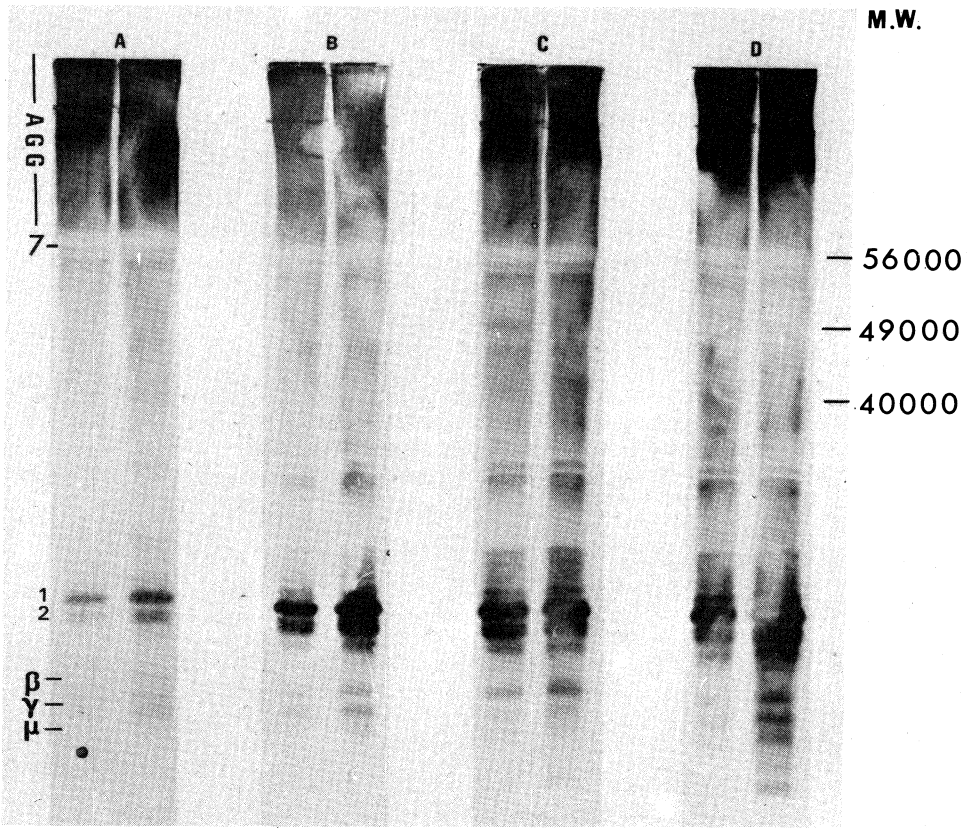

Fig. 1. Western transfer immunoblots of monoclonal antibodies to type II high-tyrosine proteins. Wool low-sulfur proteins were subjected to electrophoresis on a 12·5% (w/v) acrylamide gel, transferred to nitrocellulose and probed with antibodies: *A*, HiT 96; *B*, HiT 12; *C*, KF 22; *D*, HiT 11. The two tracks in each case contain different loadings of protein. *Agg*, aggregated components; *7*, region of the 'component 7' family of low-sulfur keratin protein (Crewther *et al.* 1980). The molecular weights (M.W.) were estimated from the position of the low-sulfur protein components. 1 and 2 are the proteins indicated on Fig. 2. Positions corresponding to the major protein bands in the β, γ, and μ fractions shown in Fig. 2 are also indicated.

extraction process affects their relative abilities to function in Western transfer assays. In addition, these monoclonal antibodies showed heavy binding to the region of keratin aggregates of low-sulfur proteins, above the components 7 family, which have previously been shown to be strongly immunoreactive (Hewish *et al.* 1984). The binding of the high-tyrosine components is strongest to two of the higher

molecular weight bands of the high-tyrosine proteins. Minor differences in the binding of the four monoclonal antibodies to lower molecular weight components can also be seen (Fig. 1).

ELISA on Separated Components

The alkaline phosphatase-linked immunosorbent assay performed on the purified components of the high-tyrosine type II proteins, α, β, γ, δ, λ, θ, μ (Table 1) confirmed the results of the Western transfer immunoblots. Reactivity was confined to the fractions, α, β and γ, which contain the higher molecular weight components, as illustrated by SDS–12·5% (w/v) polyacrylamide gel electrophoresis (Fig. 2). The reactivities of the four monoclonal antibodies to each of the purified components appear to be virtually identical in this assay. Antibody BV724, included as a control, does not recognize wool keratin proteins (Table 1).

Table 1. Binding of anti-high-tyrosine type II monoclonal antibodies to components separated by QAE-cellulose chromatography as determined by alkaline phosphatase-linked immunosorbent assay

Optical density values measured at 410 nm

Antibody No.	Fraction α	β	γ	δ	λ	θ	μ
HiT 11	0·70	0·36	0·20	0·15	0	0	0
HiT 12	0·89	0·53	0·27	0·20	0·15	0	0
HiT 96	0·74	0·37	0·22	0·13	0	0	0
KF 22	0·74	0·36	0·22	0·15	0	0	0
BV 724 (control)	0·01	0·00	0·02	0·01	0	0·01	0

Localization of Binding by Indirect Immunofluorescence

In longitudinal cryotome sections of Merino wool follicles it was noted that binding of each of the four anti-high-tyrosine type II monoclonal antibodies appeared well above the level of the follicle bulb. This is in comparison to the binding observed for the low-sulfur proteins (French and Hewish 1983) in which many anti-low-sulfur keratin monoclonal antibodies bound to cells in the mid-level of the follicle bulb. In addition, the binding was seen to be asymmetric in the fibre. In transverse sections, the monoclonal antibodies preferentially localized to cells on one side of the wool fibre (Fig. 3*a*). By histochemical staining using methylene blue, a dye known to stain the cells of the orthocortex (Orwin *et al.* 1984), it was shown that the anti-high-tyrosine type II monoclonal antibodies bind to orthocortical cells (Fig. 3*b*). Pretreatment of the skin sections with trypsin (1 mg/ml in PBS) did not affect the pattern of antibody binding. This procedure has been seen to improve the binding of antibodies excluded by lack of penetration into hardened fibre regions (D. Hewish and P. French, unpublished data). Control sections treated with normal mouse serum or culture supernatants containing antibodies which did not recognize keratin components showed no antibody binding.

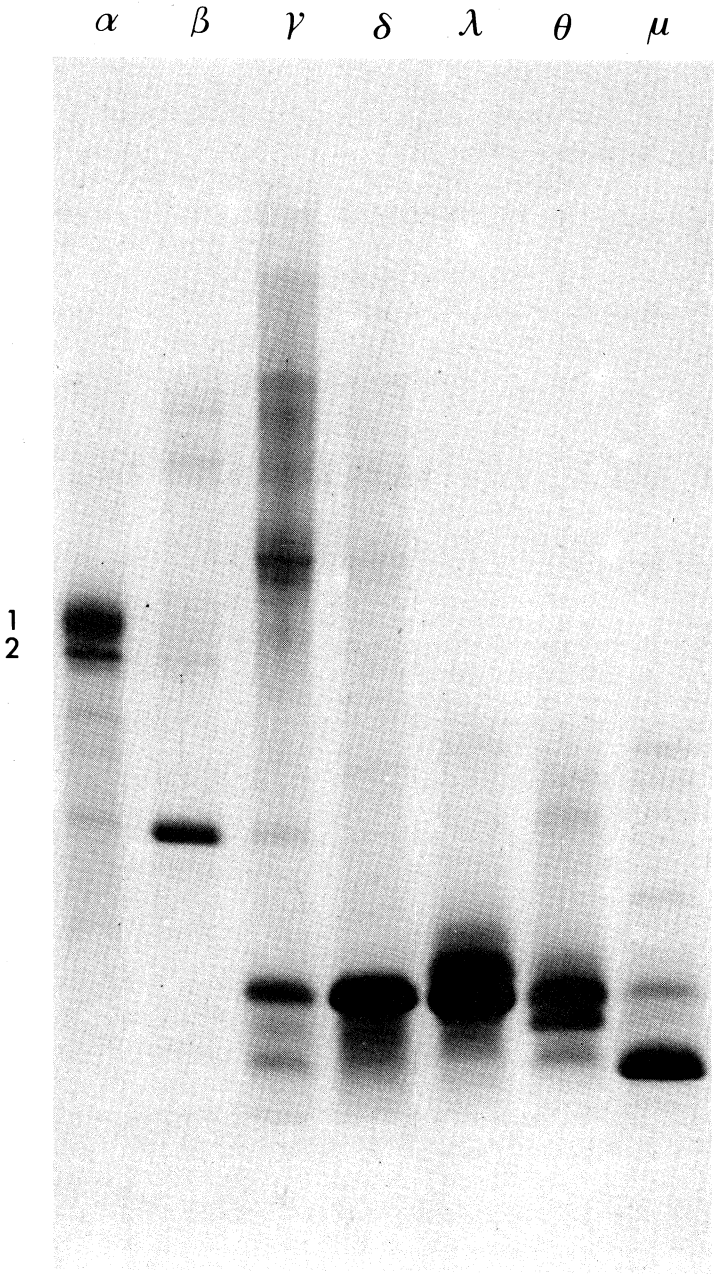

Fig. 2. SDS–polyacrylamide gel electrophoresis of high-tyrosine type II fractions. The gel concentration was 15% (w/v) acrylamide. Protein fractions were obtained by QAE-cellulose chromatography. The gel was stained with Coomassie blue. 1 and 2 are the proteins indicated on Fig. 1. Molecular weight markers were not included because the small size of the proteins made such comparisons unreliable.

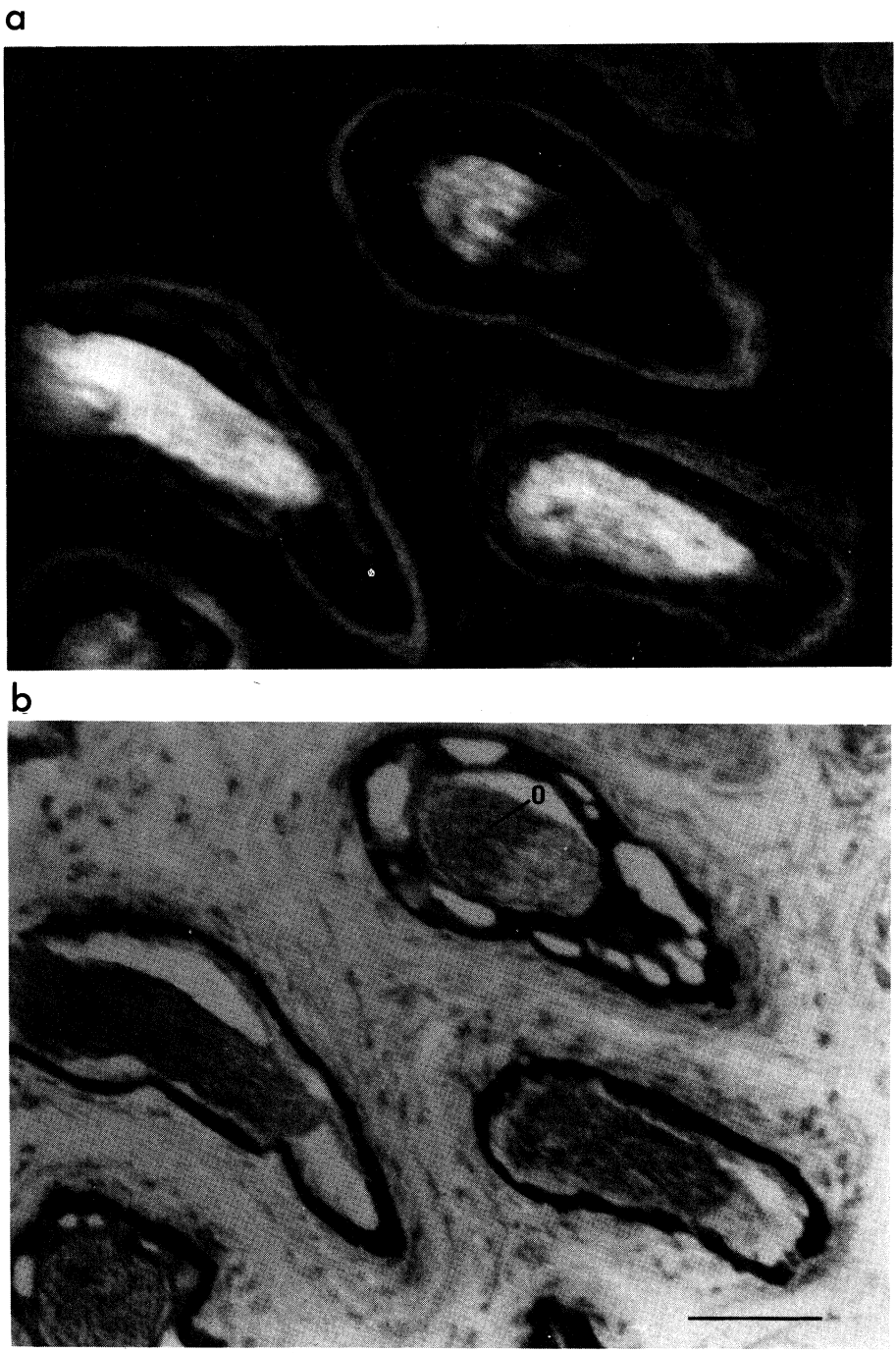

Fig. 3. Immunofluorescence of sheepskin sections treated with antibody HiT 96. (*a*) Oblique sections of wool follicles showing fluorescence of the orthocortex of wool follicles. (*b*) The same section as (*a*) stained with methylene blue to show preferential dye uptake in the follicle orthocortex (*O*). Scale bar 40 μm.

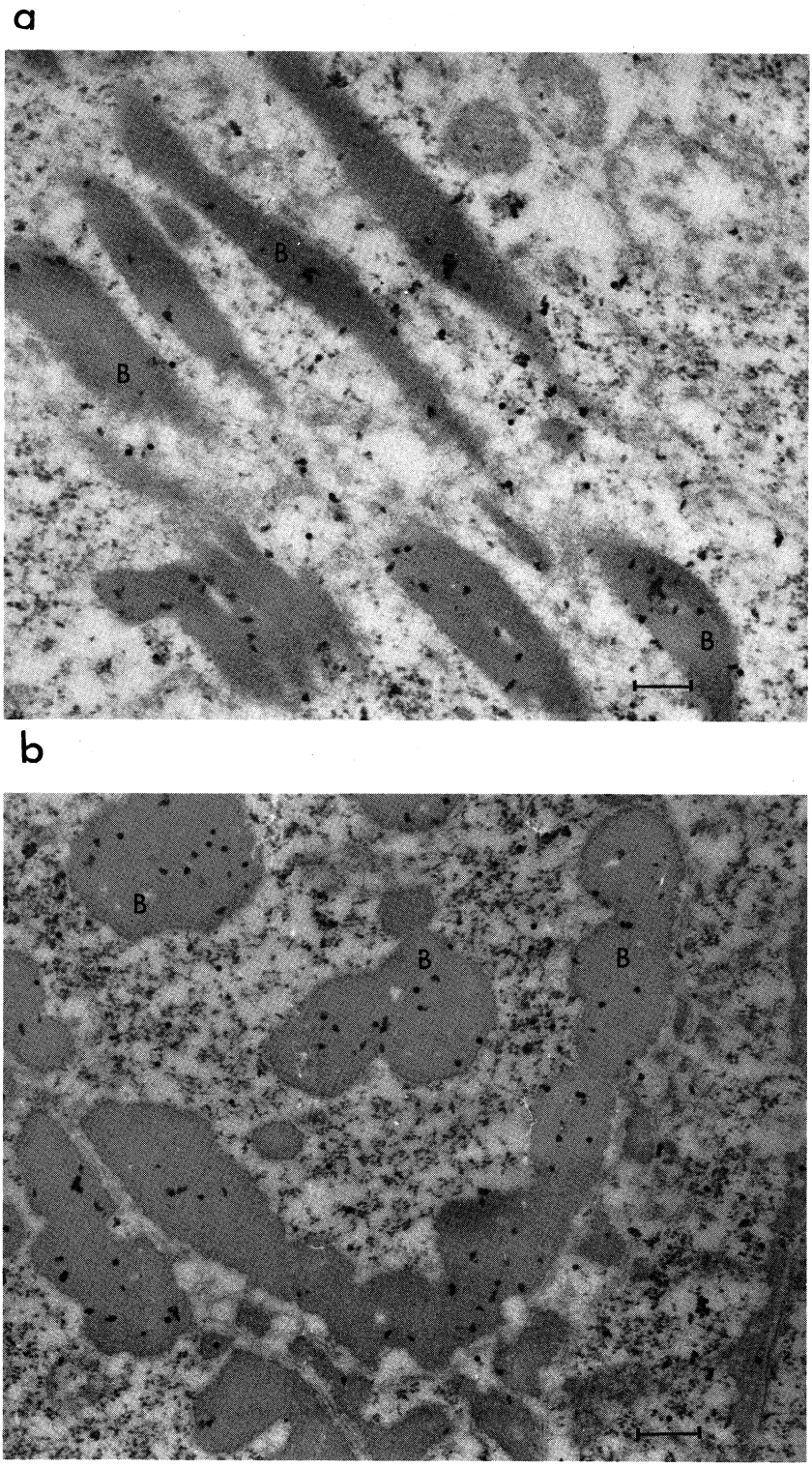

Fig. 4. Immunogold-electron microscopy of antibody HiT 96 binding to a section of wool follicle orthocortex. (*a*) Keratin microfibril bundles (*B*) in cross-section. (*b*) Keratin microfibril bundles cut in longitudinal section. Scale bar 100 nm.

Localization of Binding by Immunogold Staining

In Figs 4*a* and 4*b* binding of antibody HiT 96 to keratin bundles in the orthocortex of sectioned developing wool fibres can be seen by the localization of protein A-coated gold particles (dark dots) in the region of the bundles. Control sections treated with an irrelevant antibody at equivalent antibody concentration showed no such specific localization. The association of gold particles with the keratin bundles, based on a count of gold particles over the keratin bundles compared with the rest of the photographic area, was highly significant statistically ($P < 0{\cdot}001$).

Discussion

It would appear from the above results that the process of producing a monoclonal antibody specific for the high-tyrosine type II family of wool proteins results in only one particular protein or group of proteins being recognized. The four monoclonal antibodies described above appear to have very similar patterns of binding to components of this protein family, both by ELISA on separated components, in which all bind most strongly to component α, and by Western transfer, in which the binding of all antibodies is strongest for the higher molecular weight components. These results may mean either that the reactive components contain no amino acid sequence homology with the other solubilized wool keratin protein families, or that the unrecognized components are not immunogenic in mice. The latter explanation is possible as these components are all of lower molecular weight than the reactive α components (less than 10 000) and thus may produce only a weak immune response. Marshall *et al.* (1980) showed that the subfraction of extracted high-tyrosine type II proteins which the monoclonal antibodies bind to most strongly (namely α and β) constitute a minor proportion of the type II high-tyrosine proteins, having a higher molecular weight and containing less of the amino acids glycine and tyrosine than the other type II high-tyrosine proteins. Thus these components, although part of the extracted type II high-tyrosine proteins, are atypical members of this group. It must be pointed out, therefore, that the present antibodies react with a subset of the type II proteins and the localization data may only be pertinent to this subset.

It is clear from the above results that the monoclonal antibodies bind asymmetrically to the wool fibre in the follicle. By differential histological staining, it was found that the proteins recognized by the antibodies are located in the orthocortex of the hardening fibre. The orthocortex of the fibre differs from the paracortex by several characteristics. Firstly, in the amount and distribution of non-keratinous material surrounding the keratin microfibrils (there is more in the orthocortex); secondly, there appears to be a larger amount of matrix relative to microfibrils in the paracortex (Bradbury 1973). The proteins of the orthocortex have a lower cystine content than those of the paracortex. Thirdly, the orthocortex is more accessible to penetration by dyes, both acidic and basic (Bradbury 1973). Horio and Kondo (1953) have correlated the asymmetry of the wool fibre cortex with crimping characteristics.

One explanation for the differential binding of the monoclonal antibodies to the orthocortex may be simply that the paracortex is not accessible to penetration by antibody molecules. Two observations would appear to disprove this hypothesis. Firstly, in other studies, monoclonal antibodies raised to the low-sulfur keratin proteins were shown to bind to the cortex of the wool fibre, in the same region of the follicle, with no asymmetry of localization (French and Hewish 1985).

Secondly, the use of trypsin to increase penetration of antibodies does not affect the differential binding of the monoclonal antibodies to the orthocortex. Further support for the above hypothesis comes from Frenkel *et al.* (1974), who concluded from amino acid analyses of the cells of each half of the cortex, that cells of the orthocortex contain most of the high-tyrosine proteins, whereas much of the high-sulfur protein is located in cells of the paracortex.

The high-tyrosine type II proteins in wool are separated from the low-sulfur keratin proteins by an acetone–ammonium sulfate fractionation (Marshall *et al.* 1980). This process, however, does not yield absolutely pure preparations of either protein family, as the anti-type II monoclonal antibodies bind to the high-tyrosine proteins present in low levels in low-sulfur protein preparations, including the high-tyrosine proteins present in the high molecular weight aggregates of the low-sulfur proteins, but not to individual components of the low-sulfur proteins. We conclude from these results that the high-tyrosine type II components α and β recognized by the monoclonal antibodies are present in aggregations of keratin proteins formed *in vitro*. This may be an artefact of the solubilization process, or it may indicate an association with the low-sulfur keratin proteins in the microfibrils of the wool fibre cortex. Immunogold staining of ultra-thin sections of the cortex show that these antibodies bind to keratin microfibril bundles. These bundles consist primarily of low-sulfur keratin proteins (Fraser *et al.* 1972), and this tends to support the above inference of the specific association of the two protein types.

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