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Monoclonal Antibody Studies of α-Keratin Low-sulfur Proteins

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Abstract

Monoclonal antibodies to the two families of low-sulfur proteins from wool were produced. Selection was applied to identify those hybridomas secreting antibody that was effective in the Electro-blot system. The specificities of eight different monoclonal antibodies were investigated by their binding to α-keratin low-sulfur proteins which had been subjected to electrophoresis from wool, goat hair, porcupine quill, rat hair and echidna quill, using the Electro-blot procedure. Considerable cross-reactivity was found both within the low-sulfur protein components of individual keratins from a particular species, and also between the keratins of the different species. Some antibodies were found to bind selectively to components of one family of low-sulfur proteins in wool, while others recognized determinants in both families, indicating some homology between the two families.

Extra keyword: microfibrils.

Introduction

The low-sulfur proteins of wool (SCMK-A) are a group of eight proteins that can be divided into two families, each of four homologous proteins (Crewther *et al.* 1980). The microfibrils of the wool fibre are formed by the interaction of members of these two families to give helical coiled-coil structures. Other hard α-keratins, although differing in physical form and mechanical properties, have a microfibril–matrix structure similar to that of wool and give rise to low-sulfur proteins of approximately the same molecular weight as those of wool (Gillespie and Marshall 1977). However, as no detailed chemical characterization (e.g. amino acid sequence) has been reported for the low-sulfur proteins of hard α-keratins other than wool, it is not known whether these are also divided into two interacting families of proteins.

The study of the antigenic properties of proteins is a practical approach to obtaining comparative structural information (Arnheim *et al.* 1969). Previous studies of the antigenic properties of the low-sulfur proteins of wool (as their reduced and *S*-carboxymethylated derivatives) (Frater 1968), were hampered by the extreme antigenicity of the *S*-carboxymethyl (SCM) groups as well as by the difficulty of obtaining highly purified components of the low-sulfur protein complex. Monoclonal antibodies offer a way of eliminating these problems not only because of their high specificity but also because hybridoma cell lines producing antibodies to the SCM groups can be selected and eliminated. Because of their extremely high specificity, monoclonal antibodies are especially useful in the elucidation of relationships and homologies within families of proteins of similar composition and structure (Pruss *et al.* 1981; Tseng *et al.* 1982).

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In this paper, we describe the preparation and characterization of a number of monoclonal antibodies to the low-sulfur proteins of wool and show how these can be used to demonstrate relationships between the low-sulfur proteins of various hard α-keratins namely, wool, mohair, porcupine quill, rat hair and echidna quill.

Methods

Preparation of Keratin Samples

The low-sulfur proteins of wool and other keratins were prepared by the method of Dowling and Crewther (1974). Fractionation of wool low-sulfur proteins to give purified components 5, 7c and 8c-1 was carried out by the method outlined by Crewther *et al.* (1976).

Immunization, Cell Fusion and Growth of Hybrids

Mice (Balb-C, male) were immunized with SCMK-A or purified components of low-sulfur proteins by intraperitoneal injection of 50 μg of protein in 0·2 ml of Freund's adjuvant. Three injections were given at intervals of 4 weeks, followed by a boosting dose of 50 μg protein in saline given intravenously 4–6 weeks after the last injection.

Cell fusions were carried out with polyethylene glycol 4000 by the method of Kohler (1978), using approximately 10^8 spleen cells and 2×10^7 myeloma cells. Myeloma cell lines used in the fusions were: NS-I-Ag 4/1 (Kohler *et al.* 1976), P3 × 63 Ag 8.6.5.3 (Kearney *et al.* 1979) and SP2/0-Ag 14 (Schulman *et al.* 1978). After fusion the cells were resuspended in hypoxanthine–aminopterin–thymidine medium (Littlefield 1964) and then plated into four Costar 24-well culture dishes. Wells showing growth of hybrids were assayed for antibody after approximately 14 days. Hybrids producing antibody to keratin components were cloned twice by limit dilution in 96-well culture plates containing a confluent layer of Balb-C 3T3 fibroblasts. Cell lines were grown as ascites tumours in adult Balb-C mice primed with pristane.

Assays

Antibodies to keratin were assayed by enzyme-linked immunosorbent assay (ELISA), using the urease-conjugated antibody system of Chandler *et al.* (1982).

Isotype specificity of monoclonal antibodies were determined by Ouchterlony double-diffusion immunoprecipitation of concentrated culture supernatants against specific antisera (Nordic Immunology, Tilburg, The Netherlands).

Electrophoresis

Polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970) using 10% polyacrylamide. Gels were stained with Coomassie Blue G250.

Antibody Binding by Electro-blot Technique

After electrophoresis, protein bands were transferred electrophoretically overnight at 4°C onto nitrocellulose sheets (Bio-Rad Laboratories, California, U.S.A.) by the method of Bittner (1980) in a Bio-Rad trans blot cell. Binding of antibodies to the transferred proteins was carried out at room temperature by the method of Burnette (1981). The sheets were then washed (Burnette 1981) and incubated with rabbit anti-mouse immunoglobulin (Sera-Labs, Sussex, U.K.) for 45 min at room temperature. After washing as before, the sheets were incubated for 30 min at room temperature with 1 μCi ^{125}I-labelled protein A (the Radiochemical Centre, Amersham, U.K.). The sheets were given a final treatment for 90 min at room temperature with Sarkosyl buffer (Renart *et al.* 1979) and rinsed with water before being radioautographed for 18–64 h at −70°C using Du Pont Cronex lightning plus intensifying screens.

Results and Discussion

Antibody-producing hybridomas were obtained from most cell fusions, using spleen cells from mice immunized with wool SCMK-A or one of the purified low-sulfur proteins: components 5 and 7c. Antibody-positive clones were selected by the reactivity of culture supernatants with SCMK-A in an ELISA system. When the resulting monoclonal antibodies

were used in the Electro-blot system most gave negative results due, presumably, to low affinities for the antigen. But when the cell culture supernatants were diluted into 0·1 M sodium chloride, 0·1 M sodium borate, pH 9·0 (lacking Tween 20 and bovine serum albumin), and screened in the ELISA, a high proportion of the then positive cultures gave antibodies which were effective in the Electro-blot system. The proportion of the hybrids which were selected by this method varied between 10 and 30% of the total positive clones. The mechanism of action of this selection procedure is unknown. In other studies using a different set of immunogens (Hewish, unpublished results), the modified ELISA was found not to indicate the effectiveness of the Electro-blot technique. The selection could be related to a combination of the effects of the elevated pH and ionic strength of the buffer on the secondary structure of the immobilized antigen.

Hybrids were therefore selected by screening at pH 9 and the positive hybrids further screened for the production of anti-SCM antibodies by testing for reactivity in the ELISA against SCM-bovine serum albumin. Those cell lines producing antibodies to the SCM group were discarded. Hybrid cell lines used for further characterization are shown in Table 1. It can be seen that most subclasses of mouse immunoglobulin are represented although many are of the IgG_1 type, in accordance with the high proportion of this subclass in mouse serum (Ey *et al.* 1978).

Table 1. Characteristics of hybridoma cell lines

Cell line	Myeloma used	Immunogen	Immunoglobulin class
αK1	NSI-Ag4/1	SCMK A	IgG_1
αK2	X63-Ag8.6.5.3.	SCMK A	IgM
αK3	NSI-Ag4/1	Comp. 7c	IgG_{2b}
αK4	NSI-Ag4/1	Comp. 7c	IgG_1
αK5	NSI-Ag4/1	Comp. 7c	IgG_1
αK6	Sp2/0-Ag14	Comp. 7c	IgG_1
αK7	Sp2/0-Ag14	Comp. 5	IgG_1
αK8	Sp2/0-Ag14	Comp. 5	IgG_{2a}

The antibodies listed in Table 1 were further characterized by the Electro-blot technique using, initially, wool low-sulfur proteins. A typical result showing the specificity of antibodies αK3, αK4, αK5 and αK6 against wool low-sulfur proteins is shown in Fig. 1. Bands indicating reaction of the antibodies with most of the components staining with Coomassie Blue can be clearly seen, and the differing specificity of the different antibodies is apparent.

The reactivity of monoclonal antibodies αK1-αK8 against the low-sulfur proteins of wool, mohair, porcupine quill, rat hair and echidna quill is shown schematically in Fig. 2.

The results in Fig. 2*A* (1–8) show that the eight antibodies all have different patterns of reactivity with the wool low-sulfur proteins and therefore recognize eight different determinants. A feature of the Electro-blot patterns for all of the antibodies against all the keratins is a series of diffuse bands extended from the origin of the radioautograms almost to the region of the recognized α-keratin bands. These are not shown in Fig. 2 to simplify interpretation of the patterns. The bands staining faintly with Coomassie Blue seen toward the origin of the electrophoretograms in Fig. 2 are characteristic of all the keratins and correlate with the diffuse radioactive smear seen on the Electro-blots. The presence of such aggregated low-sulfur protein components has been shown by O'Donnell and Thompson (1964).

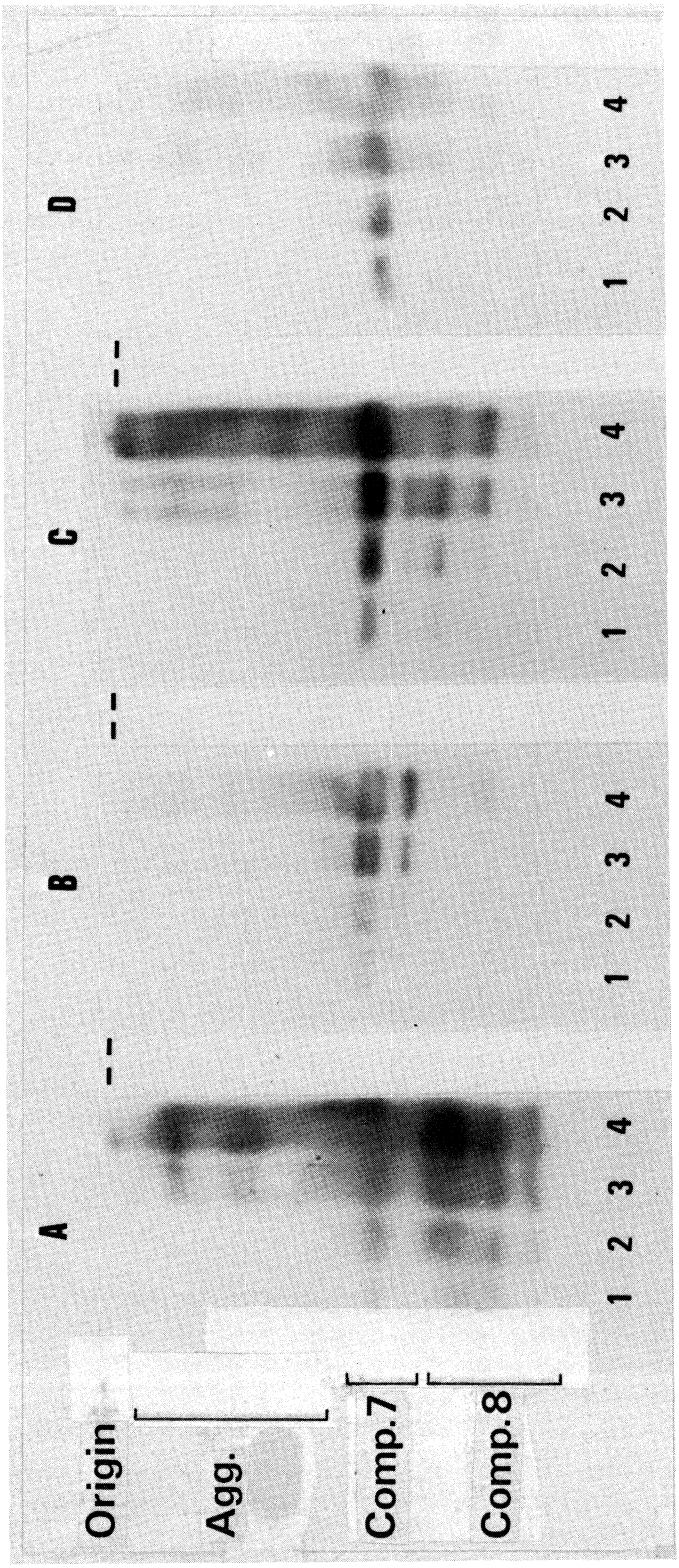

Fig. 1. Electro-blot immunoassays of the binding of monoclonal antibodies αK3 (*A*), αK4 (*B*), αK5 (*C*) and αK6 (*D*), to wool SCMK-A. Tracks 1–4 in each case contain increasing quantities of SCMK-A. The areas corresponding to the keratin component families 7 and 8 are indicated. 'Agg.' designates the region containing aggregated components.

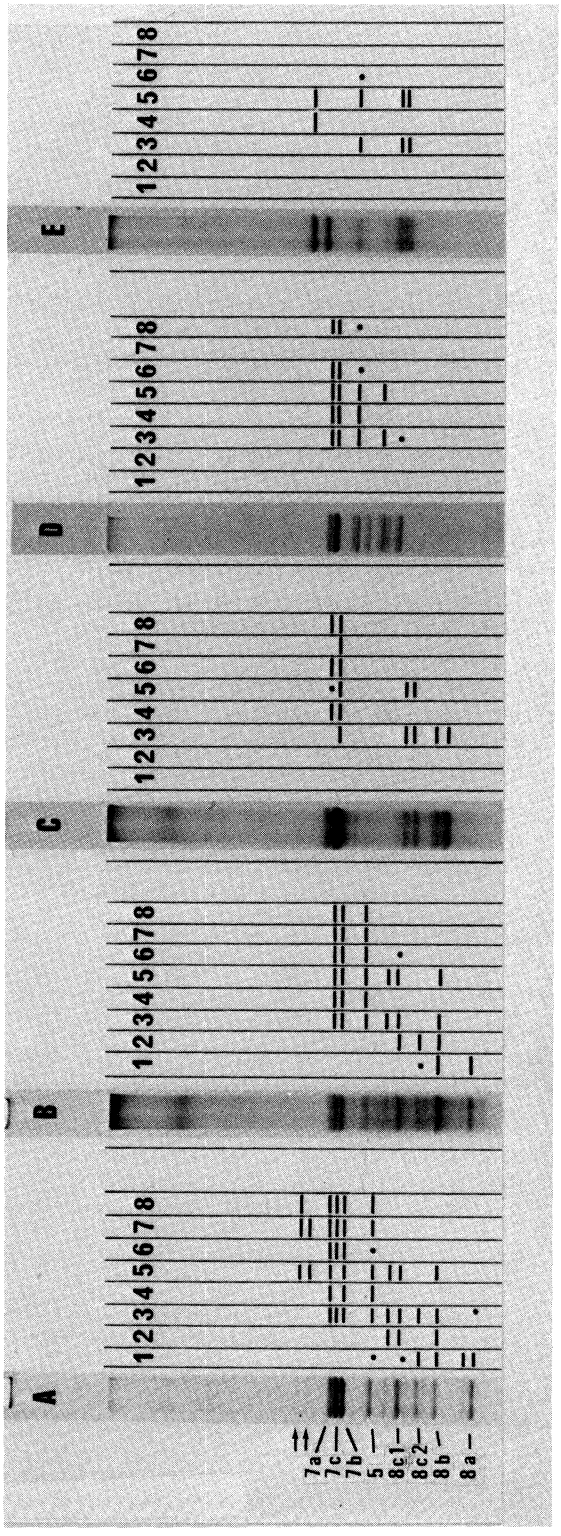

Fig. 2. Schematic representation of antibody binding to SCM low-sulfur proteins of (*A*) wool; (*B*) mohair; (*C*) porcupine quill; (*D*) rat hair; and (*E*) echidna quill. The binding of monoclonal antibodies αK1–αK8 are shown in tracks 1–8 respectively for each keratin. The photographs show Coomassie Blue staining of the electrophoretograms of the keratins. The components of wool SCMK-A are indicated at the left of the figure, and the arrows indicate two previously unrecognized components. Solid lines in tracks 1–8 denote the binding of an antibody to a particular component, whilst weak but consistently observed binding is indicated by dots. None of the assignments given in the figure are based on a single experiment but are the results of several replicates.

The amino acid compositions, peptide maps and partial amino acid sequences of the wool low-sulfur proteins, together with studies of their interactions in solution, suggest that they can be divided into two families of proteins, designated component 7 (made up of components 7a, 7b, 7c and 5) and component 8 (components 8a, 8b, 8c-1 and 8c-2) as indicated in Fig. 2 (Dowling *et al.* 1979, Crewther *et al.* 1980). The amino acid sequence data now available for component 8c-1 (Dowling, Crewther and Inglis, personal communication), component 7c (Sparrow and Inglis 1980, Sparrow, unpublished data) and component 5 (Gough, McMahon, Sparrow, and Inglis, unpublished data) are consistent with this classification, but suggest that there is significant homology between the two families of proteins (Dowling *et al.* 1983). The patterns of reactivity of the monoclonal antibodies described here are also consistent with both the subdivision into two families of proteins and the homology between proteins within each family. Thus, antibodies αK1 and αK2 recognize essentially only proteins of the component 8 family while antibodies αK4, 6, 7 and 8 react only with members of the component 7 family. Antibodies αK3 and αK5 react with members of both classes of proteins. Faintly staining bands between components 5 and 8c-1 and above 8a are also recognized by some of the antibodies. These correspond to modified forms of components 8c-1 and 8a (Crewther *et al.* 1980). Two other faintly staining bands (arrowed in Fig. 2) are also recognized by antibodies αK7 and 8, indicating that these are members of the component 7 family.

The reactivity of αK1–αK8 with the other α-keratins studied here has the same general characteristics as for that with wool, although, with increasing evolutionary distance A → E (Fig. 2), fewer and fewer determinants are recognized.

Because αK1 and αK2, specific for the component 8 family, recognize determinants on only wool and mohair low-sulfur proteins and not those of the more unrelated species, the data do not show unequivocally that porcupine quill, rat hair and echidna quill low-sulfur proteins can be divided into two families homologous with those of wool. Nevertheless, the behaviour of porcupine quill and rat hair with antibodies αK3 and αK5 (which recognize a determinant common to both families in wool) compared to that with antibodies αK4, 6, 7 and 8, which recognize component 7 determinants only, suggests that two similar families also exist in these other keratins. Echidna quill possesses fewer components than the other α-keratins and those bind insufficient of the antibodies for confident determination of their family relationships.

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