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Classification of Human Epithelia and Their Neoplasms using Monoclonal Antibodies to Keratins: Strategies, Applications, and Limitations

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I. INTRODUCTION

Intermediate-sized (10 nm) filaments are found in the cytoplasm of most cell types (for reviews, see references 4, 39, 59, 72, 98, 124). These filaments are "intermediate" in diameter between microfilaments (6 nm) and microtubules (25 nm) and are composed of five types of proteins that are distinguishable immunologically and biochemically. They are (a) keratins which are present in almost all true epithelia (33, 38, 43, 117, 131, 132), (b) vimentin which is found primarily in mesenchymal cells (38, 42), (c) desmin of muscle cells (18, 73, 121), (d) glial fibrillary acid protein of glial cells (21, 22, 31), and (e) neurofilament proteins of neurons (57, 75, 114, 115). Such a tissue-specific distribution of various intermediate filament proteins is in general well preserved in neoplasms. This forms the basis for the current use of antibodies to keratins (8, 9, 16, 24, 32, 58, 61, 63, 64, 77, 81, 82, 91, 101, 103, 110–112, 134, 144, 151; for earlier

references, see reference 100) and other intermediate filaments for tumor typing (*e.g.*, for distinguishing keratin-positive carcinomas from vimentin-positive lymphomas; for recent reviews, see references 99, 100, 106).

Of the five types of intermediate filaments, keratin is the most complex. By two-dimensional gel electrophoresis, Moll *et al.* (84) have catalogued a total of 19 human epithelial keratins. Usually, a subset of two to 10 of these keratins is found in a particular epithelium (28, 34, 36, 48, 83, 84, 149). The detailed keratin composition of the epithelia varies depending on the cell type, cellular growth environment, stage of development and differentiation, and disease state (reviewed in references 39, 54, 84, 128). The fact that keratin composition varies so greatly among different epithelia raises the possibility that detailed analyses of keratins may allow the "fingerprinting" of various carcinomas.

Significant progress has been made during the last few years concerning the immunologic and biochemical characterization of keratin proteins. In this paper, we will review the recent data which indicate that all human epithelial keratins can be divided into an acidic and a basic subfamily, and that most acidic keratin forms a "pair" with a specific basic keratin, as defined by coexpression. We will also demonstrate that the expression of most keratins follows well-defined "rules" that are mainly related to three factors: (a) epithelial cell type (simple *versus* stratified) and, among stratified epithelia, (b) the differentiation program (*i.e.*, the skin, corneal, or esophageal "type" of differentiation), and (c) state of cellular growth ("normal" *versus* "hyperproliferative"). The existence of these rules suggests that the detection of specific keratins may indeed be of diagnostic value in terms of dividing epithelial tissues into various "classes" depending on their cellular origin and morphologic and/or growth features. Finally, in view of the increasing use of monoclonal antikeratin antibodies as a diagnostic tool (25, 50, 52, 53, 68, 105, 138, 141, 143, 146), we will discuss the strategies for making new hybridoma antibodies highly specific for individual keratins and the applications and limitations of such antibodies in tumor diagnosis.

II. KERATIN SUBFAMILIES

Recent results have indicated that all human epithelial keratins can be divided into an acidic (type I) and a (neutral-to-) basic (type II) subfamily according to their charges (10, 30, 113), immunoreactivities (30, 137), mRNA hybridization (46, 55, 56, 65), peptide-mapping patterns (84, 113), amino acid sequences, and relationship to wool keratins (20, 55, 56, 125). Figure 1*a* shows the one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) patterns of the water-insoluble, cytoskeletal proteins from five representative human epithelia (abdominal epidermis, cornea, esophagus, cultured epidermal cells, and cultured mesothelial cells) that, as a set, contain almost all known human epithelial keratins (84, 128). Figure 1, *b* and *c*, shows by immunoblotting that our AE3 and AE1 monoclonal antikeratin antibodies (148), originally prepared against human epidermal keratins by the hybridoma technique (67), react with two mutually exclusive subsets of keratins (137). To determine the charge properties of these keratins, we have analyzed them, as a total mixture, by two-dimensional (nonequilibrium) PAGE (Fig. 1*d*). Immunoblotting data demonstrate that all AE3-reactive keratins are relatively basic and that all AE1-reactive keratins are relatively acidic (Fig. 1*e* and *f*) (30, 128). These results, in conjunction with Fuchs' (46, 55, 56, 65) cDNA data and Franke's (39, 84, 113) peptide-mapping data provided the basis for the classification of all keratins into two subfamilies (128) (summarized in Fig. 2). Tissue distribution data have established that at least one member from each of these two subfamilies is present in all epithelia (65, 84, 93, 113, 137). Since two keratins are thought to be required for *in vitro* reconstitution of tonofilaments (74, 123), and since some acidic and basic keratins have been shown to form stable complexes (35), one may speculate that one acidic and one basic keratin may represent the minimum requirement for keratin filament formation.

III. RULES OF KERATIN EXPRESSION: KERATIN PAIRS AS DEFINED BY COEXPRESSION

The classification scheme described raises the following questions: (a) What is the relationship between the two subfamilies? (b) Why is each subfamily composed of so many different keratins; *i.e.*, do individual keratins play unique functional roles? To address these and other related questions, we have used our AE1, AE2, and AE3 monoclonal antikeratin antibodies to (a) localize specific keratins in different layers of human epidermis (148), (b) determine the tissue distribution of various keratins (93, 127, 137), (c) study keratin expression in cultured cells (30), in epithelial diseases (92, 145, 146), and in a vitamin A-deficient *in vivo* rabbit model system (136), and (d) follow keratin expression during embryonic development (133; B. Dale and K. Holbrook, unpublished results).

Our results derived from these diverse experiments have been quite consistent and have revealed a number of "rules" governing the expression of most keratins. Moreover, our data indicate that there exist specific "keratin pairs" consisting of an acidic and a basic member. Within each pair, the two keratins have identical "size ranks" in their respective subfamilies and appear to follow similar rules for expression (128). Thus, the largest members of the two subfamilies, the acidic 56.5-kilodalton (kd) and the basic 65 to 67-kd keratins, are found mainly in keratinized epithelia and may be regarded as markers for keratinization or "skin-type" differentiation (136, 137, 148). The second largest members, the acidic 55-kd and the basic 64-kd keratins, form another keratin pair, which appears to be cornea specific ("corneal type" differentiation) (28, 36, 84; A. Schermer and T.-T. Sun, unpublished results). The acidic 51-kd and the basic 59-kd keratins are expressed in the nonkeratinized, stratified squamous epithelia of various internal organs, including esophagus, tongue, and exocervix ("esophageal type" differentiation) (7, 36, 84). The acidic 50-kd and the basic 58-kd keratins are present in

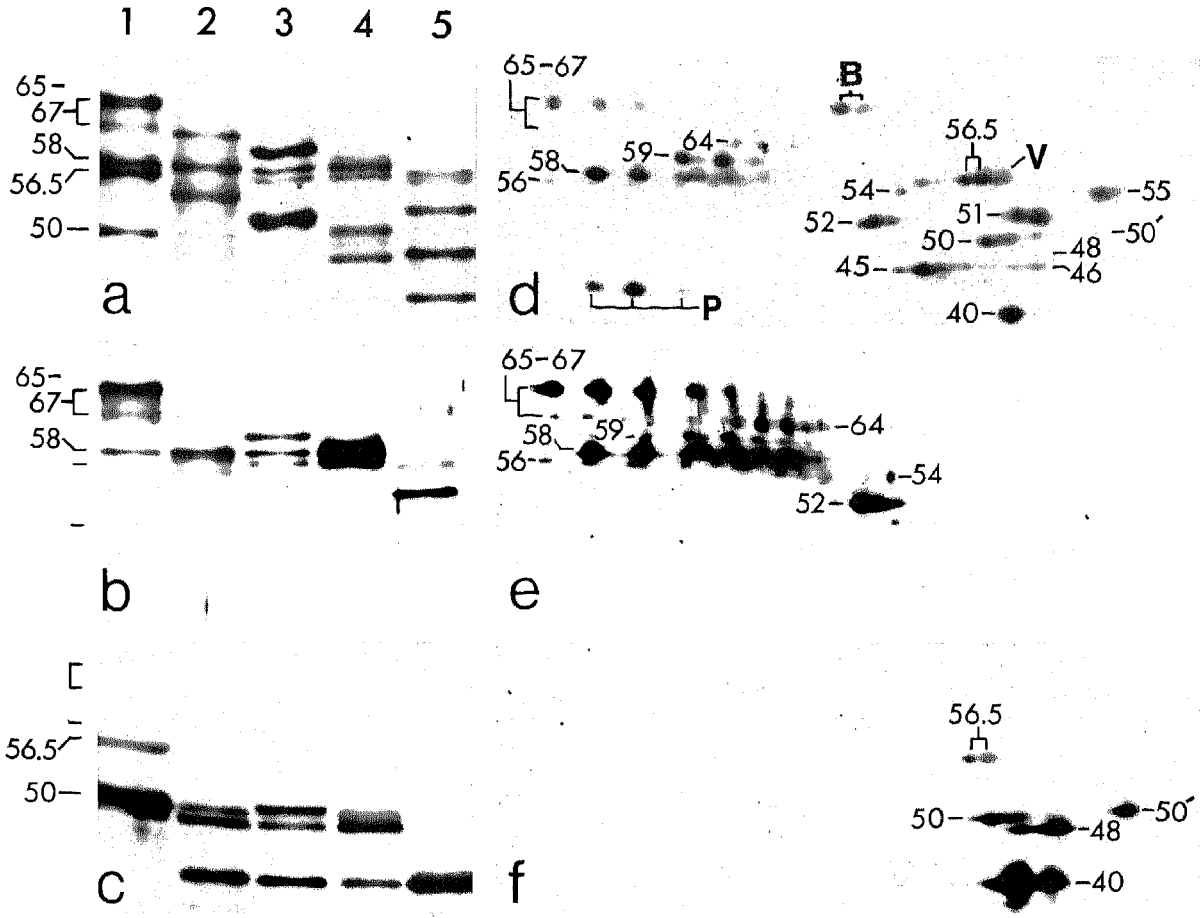

FIG. 1. One- and two-dimensional immunoblotting of human epithelial keratins with AE1 and AE3 monoclonal antikeratin antibodies. Water-insoluble cytoskeletal proteins from (*1*) abdominal epidermis, (*2*) corneal epithelium, (*3*) esophageal epithelium, (*4*) cultured epidermal cells, and (*5*) cultured mesothelial cells were analyzed individually by one-dimensional SDS-PAGE (*a* to *c*) or as a total mixture by two-dimensional, nonequilibrium pH gradient electrophoresis (*d* to *f*). Numbers to the left of SDS gels denote the molecular weights of the four major human epidermal keratins. Numbers on two-dimensional gels are molecular weights of all keratins. Charge heterogeneity of individual keratins is at least in part due to phosphorylation (*36, 51, 130*). Note that AE1 and AE3 recognize two mutually exclusive subsets of keratins and in combination recognize most human keratins. In two-dimensional gels (*d* to *f*), basic keratins are on the left and acidic ones on the right. Thus, all AE3-reactive keratins are relatively basic (*e*) and all AE1-reactive keratins are relatively acidic (*f*). *V* denotes vimentin, the mesenchymal type intermediate filament protein present in mesothelial cells (17, 149). *B* and *P* denote bovine serum albumin and phosphoglycerate kinase, respectively, which were added as standards (36, 84). Figure 1*a* and *d*, fast green; *b* and *e*, AE3 antibody stain; *c* and *f*, AE1 antibody stain.

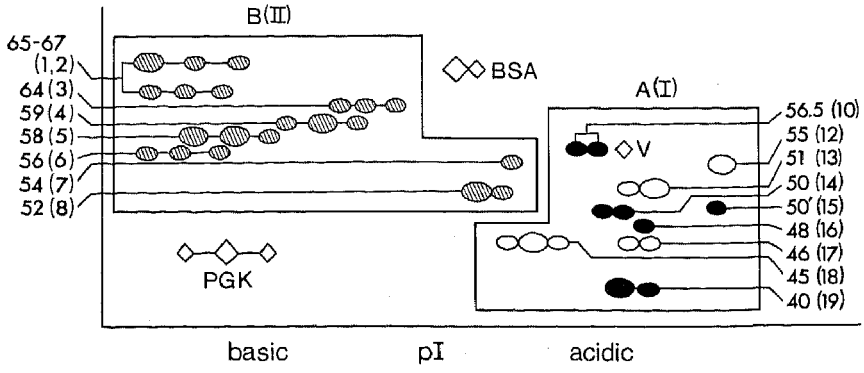

FIG. 2. Schematic two-dimensional gel diagram showing the division of human epithelial keratins into acidic (A or type I) and basic (B or type II) subfamilies. This is the schematic tracing of the actual two-dimensional gel shown in Figure 1*d*. Keratins are identified by molecular weight and catalog number of Moll *et al.* (84). All members of the basic subfamily are recognized by AE3 antibody as indicated by ◍; many members of the acidic subfamily react with AE1 antibody, as denoted by ●. Some keratins react with neither antibody, as shown by ○. For reasons mentioned elsewhere (128), keratins 9 and 11 are not included in this diagram.

various quantities in all keratinocytes (the major cell type of stratified epithelia) (36, 84, 92, 93, 137, 149). The acidic 48-kd and basic 56-kd keratins are characteristic of hyperproliferative keratinocytes, both *in vivo* and *in vitro* (80, 88, 145). The acidic 46-kd and the basic 54-kd keratins are found mainly in simple, but also occasionally in stratified, epithelia (36, 84, 85, 149). The acidic 45-kd and basic 52-kd keratins form the smallest keratin pair and are present as major components only in simple epithelia (36, 84, 137, 149). These rules, which govern

the expression of human epithelial keratins, are summarized in Table 1.

IV. WITHIN EACH KERATIN PAIR THE BASIC MEMBER IS ALWAYS LARGER THAN THE ACIDIC MEMBER BY APPROXIMATELY 8 KD: A MODEL

A striking feature of the so-called "keratin pairs" as defined by coexpression is that, within each pair, the basic member is always larger than the acidic member by approximately 8 kd (Table 1); the structural significance of this consistent size difference is not yet clear. Figure 3 shows a schematic arrangement of all keratins according to their subfamilies and molecular weights (128). In the acidic subfamily, the 40-, 45-, 46- 48-, 50-, and 56.5-kd keratins are arranged from the smallest to the largest, in ascending order, and are connected with a vertical line to indicate a possible evolutionary relationship (128, 137). Both the 51-kd keratin of the internal stratified epithelia and the 55-kd cornea-specific keratin are connected to the 50-kd keratin; they are placed off the vertical line, however, to indicate that these two keratins, like the 56.5-kd keratin, probably represent markers for advanced states of keratinocyte differentiation. The keratins of the basic subfamily are similarly arranged according to a molecular weight scale that is 8 kd higher but otherwise identical with that used for the acidic subfamily. This arrangement thus provides a schematic demonstration that (a) keratins of the two subfamilies are closely related in terms of their relative size distribution, (b) keratins with identical size ranks in the acidic and basic subfamilies always form a pair and follow similar rules of expression, and (c), as mentioned earlier, within each keratin pair, the basic member is always larger than the acidic one by about 8 kd (Fig. 3).

V. CLASSIFICATION OF EPITHELIA ACCORDING TO THEIR KERATIN COMPOSITION

Since this model is constructed mainly based on rules of keratin expression (Fig. 3; also see Table 1), it allows one to deduce the gross keratin composition of most adult epithelia according to their tissue type and histologic and growth conditions. Conversely, it also enables one to classify epithelia and their neoplasms according to their keratin composition, as will be outlined (Fig. 4; for our earlier versions of this scheme, see references 30, 127, 128, 137).

A. Stratified Squamous Epithelia and Their Neoplasms

These epithelia express mainly the larger keratins (above the horizontal line in Fig. 3). Since none of these larger keratins have been found so far in any simple epithelia or adenocarcinomas (84, 86, 92, 149), their

Table 1. Classification, Immunoreactivity, and Rules of Expression of Human Epithelial Keratins[a]

Keratin subfamily												
Acidic (type I)					Basic (type II)					B-A mol wt × 10^{-3}	Markers for	Convenient source of antigen
Mol wt × 10^{-3} (catalog no.)	pI	Antibody			Mol wt × 10^{-3} (catalog no.)	pI	Antibody					
		aIF	AE1	AE3			aIF	AE1	AE3			
56.5 (10)	5.3	+	+	−	65–67 (1, 2)	6–8	+	−	+	8	Skin-type differentiation	Normal epidermis, epidermal callus
55 (12)	4.9	+	−	−	64 (3)	7.5	+	−	+	9	Corneal-type differentiation	Corneal epithelium
51 (13)	5.1	+	−	−	59 (4)	7.3	+	−	+	8	Esophageal-type differentiation	Esophageal epithelium
50/50′ (14/15)	5.3/4.9	+	+	−	58 (5)	7.4	+	−	+	8	Keratinocytes	Epidermis
48 (16)	5.1	+	+	−	56 (6)	7.8	+	−	+	8	Hyperproliferative keratinocytes	Various cultured keratinocytes; psoriatic epidermis
46 (17)	5.1	+	−	−	54 (7)	6.0	+	−	+	8	Simple and some stratified epithelia	HeLa, cultured mesothelial cells
45 (18)	5.7	+	−	−	52 (8)	6.1	+	−	+	7	Mainly simple epithelia	HeLa, cultured mesothelial cells
40 (19)	5.2	+	+	−							Mainly simple epithelia	Mesothelial cells

[a] Keratins are identified by their molecular weight and Franke's catalog number (84), pI (isoelectric point), and reactivities (positive or negative) with aIF antibody, as well as AE1 and AE3 monoclonal antikeratin antibodies. The rules of expression are listed in the column labeled "Markers for." Note that there are keratin markers for simple *versus* stratified epithelia, differentiation programs, and hyperproliferative state of keratinocytes. Also note that there are no tumor- or disease-specific keratin markers (88, 145; *cf.* 147). The last column indicates some convenient tissue sources of particular keratin antigens. (Adapted from references 19, 30, 127, 128, 137.)

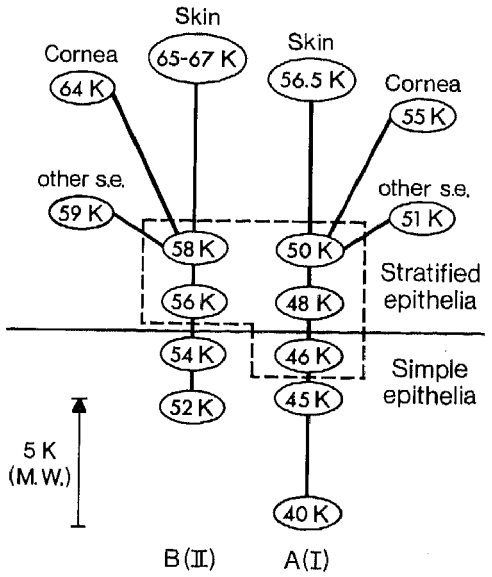

FIG. 3. A unifying model of keratin expression. Keratins of the acidic (type I) and basic (type II) subfamilies are arranged vertically according to their sizes (see the 5000-dalton scale). Keratins below the horizontal line are mainly expressed by simple epithelia (except the 46-kd keratin which is present in HeLa and hyperproliferative keratinocytes) (84, 149), and those above the line are unique to stratified epithelia. Dashed box encloses the keratins commonly expressed by all stratified epithelia in neoplasms, hyperproliferative diseases, and in culture. (Note the presence of the 46-kd but not the 54-kd keratin of the 46-/54-kd "pair".) This model is identical with an earlier one (128) except that, based on the SDS-gel results shown in Figure 1*a*, we have revised the molecular weight of the major acidic esophageal keratin (no. 13) from "54,000′" to "51,000."

Epithelial Type	Differentiation Program	Culture and Some Diseases
A. Simple epithelia ("small" keratins)		
B. Stratified epithelia (50K/58K and other "large" keratins)	skin-type (56.5K/65-67K); corneal-type (55K/64K); esophageal-type (51K/59K)	"Hyperproliferative/de-differentiated" keratinocytes (48K/56K; 46K)
C. Complex epithelia ("small" and "large" keratins)		

FIG. 4. Classification of human epithelia and carcinomas according to their keratin composition. This scheme emphasizes that epithelial tissues can be classified according to their keratins, whose expression depends on (a) epithelial type (simple, stratified, or complex), and, among stratified epithelia, (b) the differentiation program (skin, corneal, or esophageal type), and (c) the culture or disease state. On a tissue level, keratinocytes can sometimes express more than one pair of differentiation markers. For instance, rabbit corneal or esophageal epithelia undergoing partial keratinization as a result of vitamin A deficiency express the 56.5-/65- to 67-kd skin-type markers (136) in addition to their normal differentiation markers (equivalent to human 55-/64- and 51-/59-kd keratins, respectively). "Small keratins" refers to the 40-, 45-, and 46-kd acidic and 52- and 54-kd basic keratins; "large keratins" refers to the 48-, 50-, 51-, 55-, 55-, and 56.5-kd acidic and 56-,58-, 59-, 64-, and 65- to 67-kd basic keratins (see Table 1 and Fig. 3).

presence constitutes rather strong proof that the cell in question is actually keratinocyte derived.

1. *Normal Epithelia.* As mentioned earlier, the 50-/58-kd keratins are present in various quantities in almost all keratinocytes. This keratin pair is supplemented by one other pair depending on the differentiation program: the keratinized epidermis contains the 50-/58-kd plus the 56.5-/65- to 67-kd keratinization (or skin type) markers, corneal epithelium expresses small amounts of 50-/58-kd plus two major 55-/64-kd corneal type markers, and esophageal, tongue, and cervical epithelia make small quantities of 50-/58-kd plus two major 51-/59-kd esophageal type markers (84, 128)

2. *Abnormal or Cultured Epithelia.* A common keratin pattern consisting mainly of 50-/58-, 48-/56-, and 46-kd keratins (box in Fig. 3) is expressed by almost all stratifed squamous epithelia in neoplasms (84, 86, 88, 92), hyperproliferative diseases (80, 145), and cell culture (28, 48, 129, 149, 150). This may reflect a "dedifferentiated" (marked by a loss of the top three keratin pairs) and "hyperproliferative" (marked by a gain of the 48-/56- and 46-kd keratins) state of the epithelia. Partial dedifferentiation can lead to a complex keratin pattern consisting of some remaining differentiation markers and some hyperproliferation markers (86, 88, 145). Finally, some neoplasms and cultured cells of keratinocyte origin can also express small quantities of the smaller "simple epithelial" keratins (86, 149, 150).

B. Simple Epithelia and Their Neoplasms

By analyzing microdissected epithelial tissues and various cultured epithelial cells, Franke, Moll, and their coinvestigators (39, 84) as well as Rheinwald and their coworkers (149) have established that, in general, simple epithelia express two to four of the smaller keratins (below the horizontal line in Fig. 3). The 45- and 52-kd proteins (45-/52-kd pair) are the only major keratins in hepatocytes (27, 34) and pancreatic acini (86). Intestinal epithelia (small intestine and colon) express the 45-/52-kd pair plus a 40-kd component (44, 84, 113). Most other simple epithelia (pancreatic duct, gall bladder, mesothelium, endocervix, endometrium, oviduct, lung alveoli) express the 45-/52-kd pair as well as the 40- and 54-kd keratins (11, 36, 84, 86, 87, 137, 149). HeLa cells, which are presumably derived from an adenocarcinoma of cervical glands, express the 45-/52-kd and 46-/54-kd pairs (13, 25, 40, 60, 84).

The keratin patterns of simple epithelia are usually quite stable and undergo only minor, if any, change in culture or in malignancy (27, 84). Because similar keratin patterns are often shared by several simple epithelia, such patterns are useful for establishing the simple epithelial nature (see, *e.g.*, reference 60) but rarely the precise cell origin of an adenocarcinoma.

C. Complex Epithelia

Complex epithelial structures such as glandular, transitional, and pseudostratified columnar epithelia usually express a mixture of "simple" and "stratified" types of keratins (Fig. 4) (36, 84, 88, 137, 149).

VI. STRATEGIES OF MONOCLONAL ANTIKERATIN ANTIBODY PRODUCTION

The fact that we can now predict with a reasonable degree of confidence the gross keratin composition of most adult epithelia attests that keratin analysis should yield useful information concerning the cell type, the differentiation program, and the growth state of an epithelium or its neoplasms (Fig. 4). Although experimentally one can obtain keratin data from biochemical analysis, in practice immunohistochemical staining of tissue sections using a panel of well-characterized monoclonal antibodies that are specific for individual keratins is much more convenient and, therefore, potentially more useful to pathologists.

Although antibodies that recognize multiple keratins have proven useful for studying keratin classification and keratin expression, they may not be ideal for fine typing of carcinomas (however, for the different AE1-staining patterns of various epithelial diseases, see references 119, 146). Therefore, we will discuss in this section some general principles regarding the production of monoclonal antibodies that are highly specific for small subsets or individual keratins.

A. IMMUNOGEN

Since different epithelia express different keratins, care must be taken in choosing a proper source of keratin antigens. Thus, one may use keratins of the epidermis, corneal epithelium, or esophageal epithelium, respectively, as immunogens in order to produce antibodies against the keratins characteristic of these three "types" of stratified epithelia (Table 1). Following the same principle, one may use keratins from hyperproliferative stratified epithelia, such as psoriatic epidermis or cultured keratinocytes (107), as the immunogen for generating antibodies to the 48-/56-kd hyperproliferative marker keratins. Finally, to make antikeratin antibodies specific for simple epithelia, one can immunize with keratins of HeLa or cultured mesothelial cells (17, 149). Some of these convenient sources of keratin antigens are listed in Table 1.

Both native and SDS-denatured keratins have been used successfully as the immunogens. Although native keratins (particularly reconstituted filaments) (123, 130) appear to be somewhat more antigenic than denatured keratins, the former frequently elicit antibodies that fail to react with SDS-denatured keratins, which are used in both immunoblotting and immunoprecipitation. This can complicate the determination of the keratin specificity of the antibody (90). Therefore, we prefer to use SDS-denatured keratins as the immunogen.

B. SCREENING

Because keratins are in general quite immunogenic, it is not uncommon to obtain after each fusion numerous wells showing antikeratin activities by either ELISA (78) or immunohistochemical staining of tissue sections. To identify those wells containing antikeratin antibodies with the desired specificity, one may assay the supernatants of each well against the keratins of two different epithelia chosen according to their keratin composition (Fig. 3; Table 1). For example, to isolate an antibody specific for some of the simple epithelial keratins (below the horizontal line in Fig. 3), one may test the supernatants against keratins of HeLa (simple) *versus* normal epidermal (stratified) cells. Hybridoma cells from those wells showing positive reactions to HeLa but not to epidermal keratins can then be selected and cloned. We have applied this kind of strategy successfully to obtain antibodies specific for a 64-kd corneal and a 51-kd esophageal keratin (Fig. 5) (A. Schermer, unpublished results). In these two cases, we have assayed the hybridoma supernatants against corneal or esophageal keratins *versus* epidermal keratins and then cloned cells from the few wells that show selective reaction with corneal or esophageal keratins (*arrows* in Fig. 5).

To select for antibodies that work well in SDS-PAGE immunoblotting, we routinely use SDS-denatured, instead of native, keratin antigens to coat our ELISA plates.

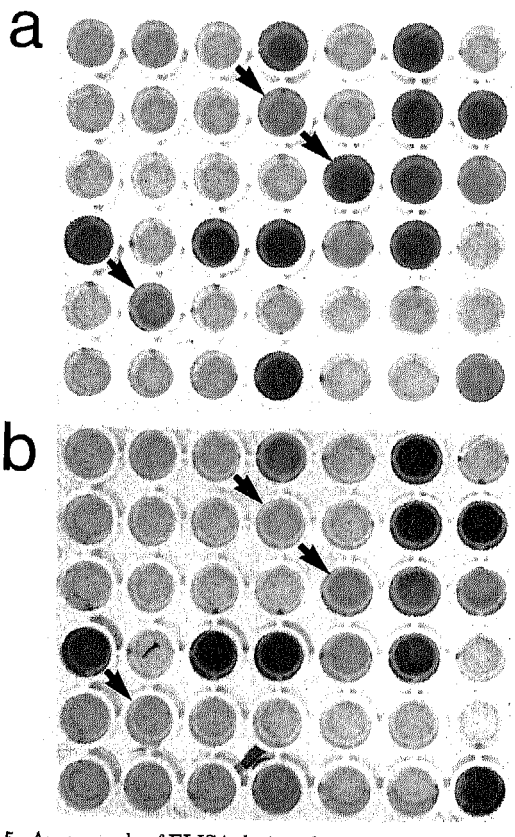

FIG. 5. An example of ELISA designed for detecting highly specific antikeratin antibodies. Spleen cells from a BALB/c mouse immunized with human esophageal epithelial keratins were fused with P3 X 63 Ag8.653 mouse myeloma cells. Supernatants from individual wells were assayed against SDS-denatured keratins of (*a*) esophageal epithelium (containing 50-/58- and 51-/59-kd keratin pairs) and (*b*) epidermis (containing 50-/58- and 56.5-/65- to 67-kd keratin pairs). Note that many antibodies cross-react with both skin and esophagus, suggesting that they are either broadly reactive or specific for keratins common to skin and esophageal epithelia (50/58 kd). However, media from a few wells (*arrows*) react preferentially or only with esophageal keratins. These antibodies turn out to be specific for the 51- and/or 59-kd esophageal-type keratins.

C. Ascites Fluid *Versus* Conditioned Medium

Monoclonal antibodies can be produced in large quantities in the form of ascites fluids or sera from hybridoma-bearing mice. We have found, however, that occasionally these body fluids contain autoimmune antikeratin antibodies which, if unsuspected, could lead to confusing results. Therefore, we prefer to utilize hybridoma-conditioned tissue culture media as a source of monoclonal antikeratin antibodies, at least during the initial stages of antibody characterization.

D. Tissue and Cell Specificity

To determine whether a given antibody is keratin specific, one may use it to stain frozen sections of various organs to ascertain that the antibody reacts exclusively with epithelial cells. In addition, the antibody should decorate a network of cytoplasmic filaments in cultured cells that express any of the keratin(s) recognized by the antibody. This filament network should show connections with desmosomal cell-cell junctions (if desmosomes are present) (38, 43, 131, 132), and the staining pattern should not be affected significantly by pretreating the cells with cytochalasin B or colcemid (97, 131). It should be noted, however, that keratins may lose their filamentous configuration and become aggregated in mitotic cells (15, 37, 69) or in cells that have been pretreated with a mixture of cytochalasin and colcemid (66).

E. Keratin Specificity

The keratin specificity of an antikeratin antibody should be established by immunoblotting or immunoprecipitation against all known human epithelial keratins obtained from a set of five or more representative human epithelia (*e.g.*, skin, cornea, esophagus, cultured human epidermal cells, and cultured mesothelial cells; Fig. 1) (128). This information is crucial because of the extreme complexity of keratins as a group; any unsuspected cross-reaction with other keratin species can lead to misinterpretation of immunohistochemical staining results.

It is important to realize when considering a source of epidermal keratins that, although callus or stratum corneum keratins are readily available and have been used extensively as immunogens, they are partially degraded and are, therefore, not suitable for immunoblotting (10, 48, 122, 148). To obtain intact keratins, one can extract fresh (unfrozen) human epidermis first with an aqueous buffer to remove the water-soluble proteins and then with 9 M urea or 1% SDS without a reducing agent (148). This will solubilize the keratins of epidermal viable layers (equivalent to the so-called "prekeratin" fraction isolated by Matoltsy (79) using a pH 2.6 citrate buffer) but not the disulfide-cross-linked keratins of stratum corneum (5, 6, 122, 130, 148).

Usually, immunoblotting requires the transfer of proteins from duplicate polyacrylamide gels to two separate sheets of nitrocellulose paper, one of which is stained for proteins and another stained immunochemically with the antibody (135). The patterns of the two parallel blots are then compared to determine which keratin(s) has reacted with the antibody. Alternatively, one can perform a single transfer, stain the same paper first with fast green to reveal all proteins (148) and then with antibody by the peroxidase-antiperoxidase or other peroxidase-related techniques (126). With diaminobenzidine as a substrate, keratin bands reacting with the antibody will appear dark brown. This procedure allows rapid, unambiguous determination of the immunoreactivities of individual keratins, even in a "crowded" region of the gel containing numerous other potentially reactive keratins (30, 148).

To resolve the keratins adequately, it is sometimes necessary to perform immunoblotting after two-dimensional gel electrophoresis in which the first dimensional separation should be nonequilibrium pH gradient electrophoresis (96); isoelectric focusing (95) is frequently unsatisfactory because it results in the loss of almost all neutral-to-basic keratins.

The two-dimensional polyacrylamide gel (nonequilibrium pH gradient electrophoresis-SDS) patterns of keratins can sometimes be complicated by the tendency of some acidic and basic keratins to form complexes that are stable in 8 to 9.5 M urea and, therefore, remain as aggregates during the first dimensional gel electrophoresis (35, 149). These complexes can be recognized because (a) they dissociate during the second dimensional SDS-PAGE, resulting in stacks of horizontal "smears" which extend from the original spots in the direction toward the opposite charge (35, 149), (b) they tend to diminish with increasing concentrations of urea during the first dimensional separation (35), and (c) they are immunologically identical with the original spot (30).

A technical problem often encountered during keratin immunoblotting relates to the widespread contamination of chemical reagents and laboratory glassware by human stratum corneum keratins (94). It has been estimated that each person sheds daily up to one billion stratum corneum cells (containing 300 mg of keratins) into the environment (45; A. Kligman, personal communication), making human epidermal keratins one of the most abundant protein contaminants in the laboratory. These contaminant bands can be recognized because they have the following characteristics. (a) During SDS-PAGE, they usually appear as two broad bands of 63 to 65 kd (as a major component) and 55 to 57 kd (minor), which are identical with the size ranges of stratum corneum keratins (10, 47, 148). (b) Consistent with the fact that stratum corneum keratins are cross-linked by intermolecular disulfide bonds, these contaminant bands are more prominent in reduced samples (94). (c) These contaminants can be detected easily, sometimes even in "blank" lanes of SDS-slab gels, by immunoblotting using antiintermediate filament (aIF) (102), AE3 (137), 34BE12 antibodies (52, 53), and some conventional antikeratin antisera (94, 152). Although these bands are usually barely detectable by Coomassie blue staining, they become heavily stained after the more sensitive antikeratin staining, thus obscuring the immunoblotting results. This may explain the detection of a "66-kd" component and other "keratin-like" proteins in extracts of various epithelial cells as well as nonepithelial cells including fibroblasts (52, 53, 102, 137, 152).