

Recognition of protein apparently specific to odontogenic keratocyst fluids

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SUMMARY Separate antisera were raised against keratocyst, dentigerous cyst, and radicular cyst fluids and used to analyse a range of fluids from cysts of known type. Samples were subjected to crossed immunoelectrophoresis into homologous antiserum through an intermediate gel containing antibody to whole human serum to screen out serum derived components. A major antigen, designated X, which seems to be of epithelial origin but is not a keratin, was identified in keratocyst fluids. X resolves as two bands on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with molecular weights of 81 K and 89 K and its major antigenic epitope is associated with disulphide bonds.

Of the cysts studied to date, antigen X has been found consistently and exclusively in fluids from keratocysts; its presence and detection is independent of total soluble protein concentration and thus offers real potential as a reliable marker for preoperative diagnosis.

In recent years there have been numerous studies on cysts of the oral region in general, and odontogenic cysts of the jaws in particular.¹ The continuing, and in some respects increased, interest in cysts derived from epithelial components with a role in tooth formation, can be traced back to the first appearance in the literature of the term "odontogenic keratocyst".² Unlike other cysts of odontogenic origin, the keratocyst usually tends to recur following conservative surgical treatment, and this feature alone led to its recognition as a distinct entity.³

In view of their notably more aggressive behaviour, it was inevitable that efforts should be made to devise methods to distinguish between keratocysts and other odontogenic cysts preoperatively: it was shown that the concentrations of total soluble protein in fluids aspirated from keratocysts were significantly lower (<4.0 g/100 ml) than those found in fluids from non-keratinising odontogenic cysts.⁴ Subsequently, other investigators^{5,6} confirmed this finding, and the estimation of total soluble protein in cyst fluids, accompanied by a search for keratin squames,⁷ has been undertaken as a routine preoperative diagnostic procedure.

This method is, however, not infallible and the results obtained are often of limited value because fluid aspirates are often contaminated with blood and, in some cases, the cyst in question is either frankly infected or heavily inflamed. All of these situ-

ations could produce a falsely increased estimate of protein content and thus preclude reliable preoperative diagnosis other than by conventional surgical biopsy, which is itself not without problems as inflammation can so modify the histological appearance of cyst linings as to obscure their true nature.

To overcome these obstacles attempts have been made to characterise the different cyst types using other criteria. Several investigators⁸⁻¹⁰ have analysed the proteins in cyst fluids but no one has been able to identify either a single protein or group of proteins that is characteristic of a particular cyst type. In all of these studies, however, electrophoresis of cyst fluids was performed without attempting to differentiate serum derived proteins from any others that may have been present. In this context screening gels for protein bands that are unique to any given cyst sample by direct comparison of one complex protein mixture with another is fraught with difficulty. Immunoelectrophoretic methods offer a greater sensitivity and specificity but seem to have been used on only one occasion¹¹ in the analysis of fluids from jaw cysts; this study, however, used antihuman serum alone and thus was unable to discriminate between serum derived and specifically cyst related proteins in a cyst fluid.

In an effort to overcome the limitations identified in earlier studies we used immunological techniques to search for specific components of non-serum origin in cyst fluids. It was hoped that this approach might not only provide a more reliable preoperative diagnostic

indicator but would also contribute towards a better understanding of the histogenesis and pathogenesis for odontogenic cysts.

Material and methods

CYST FLUIDS

A total of 34 fluids that had been aspirated from odontogenic keratocysts (n = 18), dentigerous cysts (n = 8), and radicular cysts (n = 8), were used in the study. Two fluids from separate keratocysts in a patient with Gorlin's syndrome were also investigated. The definitive diagnoses were established according to conventional criteria,¹ using microscopical data. In all cases the fluids had been assayed for their content of total soluble protein using the method of Lowry¹²; the ranges of total protein content were 1.82–22.0 g/100 ml for keratocysts; 5.2–17.0 g/100 ml for dentigerous cysts; and 2.9–20 g/100 ml for radicular cysts. The fluids were collected over six years and had been stored at -20°C; before use they were centrifuged at 3000 × g for 20 minutes in a Beckman TJ-6 centrifuge.

ANTISERA

Samples of the supernatants from five fluids in each cyst category (keratocyst, dentigerous, and radicular) were pooled to give representative inoculae for raising antisera. Efforts were made to select fluids that lacked signs of infection or gross contamination with blood.

Antisera were produced by three consecutive injections, at three weekly intervals, of pooled fluid from each cyst type into New Zealand white rabbits using Freund's incomplete adjuvant. The IgG fractions of the resultant antisera were then prepared according to a standard method.¹³

Antisera against whole human serum proteins and human keratin were obtained from Dakopatts Ltd (Denmark).

PREPARATION OF HUMAN KERATIN

Keratin was prepared from human skin callous material using the method of Sun and Green.¹⁴ Briefly, this entailed cutting callous from a foot into small pieces followed by partial homogenisation in Tris-hydrochloric acid buffer using a teflon tissue grinder. The insoluble material was washed in buffer and then

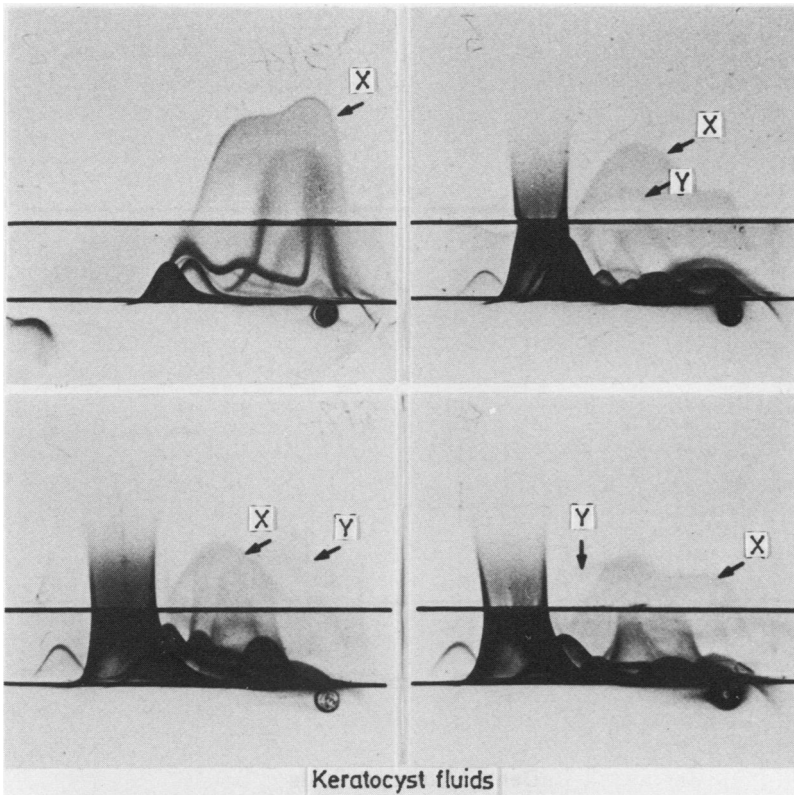


Fig 1 Four examples of keratocyst fluids analysed by crossed immunoelectrophoresis into homologous antiserum through intermediate gel containing antiwhole human serum immunoglobulin. Antigens X and Y are not of serum origin.

solubilised in 8 M urea and 0.1 M β -mercaptoethanol overnight at room temperature. Residual insoluble material was then removed by centrifugation and the supernatant dialysed for 48 hours against three changes of Tris buffer.

CROSSED IMMUNOELECTROPHORESIS

Crossed immunoelectrophoresis was performed essentially according to the method of Eckersall and Beeley¹⁵ but with an intermediate gel containing antiserum raised against whole human serum (50 μ l/ml). Samples of keratocyst fluids were diluted 1/10 and those from dentigerous and radicular cysts 1/20 in Tris-hydrochloric acid buffer (0.05 M pH 7.5) for use.

In some experiments keratocyst fluids were mixed with varying amounts of human keratin preparation and portions of the mixture were examined by crossed immunoelectrophoresis into antikeratocyst fluid serum, antikeratin antibody, or a combination of both antisera. Keratocyst fluids and the keratin preparation were also examined by "tandem CIE" into the above sera.

COLUMN CHROMATOGRAPHY

Samples (0.5 ml) of a keratocyst fluid were chromatographed on a column of Sepharose CL-6B, equilibrated, and eluted with 0.05 M phosphate buffer, pH 7.0, containing 0.1% sodium azide. Fractions (1 ml) were collected and 10 μ l portions electrophoresed by the method of Weeke¹⁶ into 1% agarose containing antikeratocyst fluid serum through an intermediate gel containing antihuman serum antibody.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Samples of cyst fluids (5 μ l), Sepharose column fractions (75 μ l), or human keratin preparation (10 μ l) were heated at 100°C in the presence of 1% (w/v) SDS for five minutes and electrophoresed on 9% polyacrylamide-SDS gels. The electrophoretic method and conditions used were as described by Russell¹⁷ but with the exception that β -mercaptoethanol was usually omitted from the sample buffer.

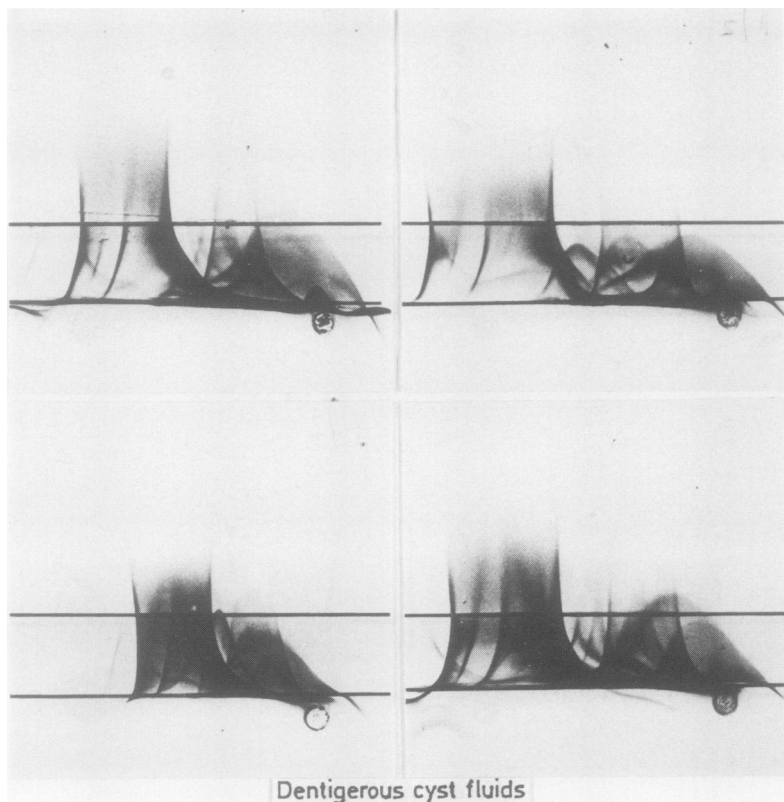


Fig 2 Four examples of dentigerous cyst fluids analysed by crossed immunoelectrophoresis into homologous antiserum through intermediate gel containing antiwhole human serum immunoglobulin. No antigens are evident in upper part of gel.

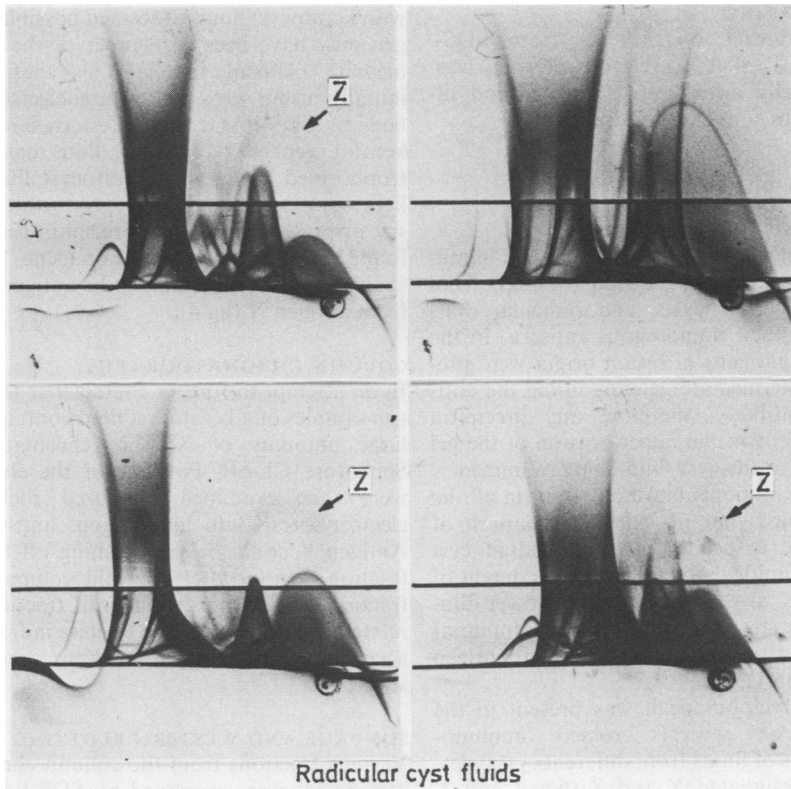


Fig 3 Four examples of radicular cyst fluids analysed by crossed immunoelectrophoresis into homologous antiserum through intermediate gel containing antiwhole human serum immunoglobulin. Antigen Z is not of serum antigen.

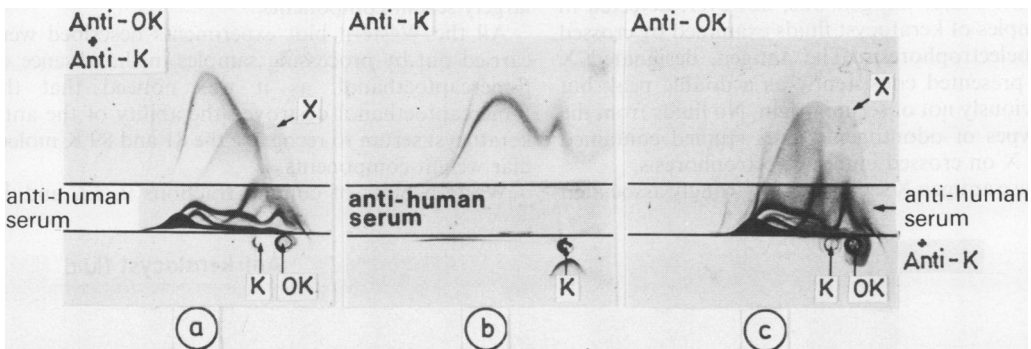


Fig 4 a) Tandem crossed immunoelectrophoresis of keratin preparation (K) and keratocyst fluid (OK) into mixture of their homologous antisera through intermediate gel containing antiwhole human serum immunoglobulin. Antigen X and keratin peaks in upper gel do not fuse, indicating that they are dissimilar antigenically.
 b) Crossed immunoelectrophoresis of keratin preparation (K) into homologous antiserum through intermediate gel containing antiwhole human serum immunoglobulin.
 c) Tandem crossed immunoelectrophoresis of keratin preparation (K) and keratocyst fluid (OK) into antikeratocyst fluid serum through intermediate gel containing mixture of antikeratin serum and antiwhole human serum immunoglobulin. Feet of keratin appear in intermediate gel indicating that antikeratocyst fluid serum has weak antikeratin activity but that this is separate and distinct from its anti-X activity.

“WESTERN” BLOTTING

Electrophoretic transfer of proteins from SDS-polyacrylamide gels—that is, Western blotting was performed essentially according to the method of Burnette,¹⁸ but with some modifications.¹⁹

Results

CROSSED IMMUNOELECTROPHORESIS

Figs 1–3 show typical examples of the crossed immunoelectrophoresis patterns produced by fluids from keratocysts, dentigerous cysts, and radicular cysts when run against their homologous antisera. In the system used here antigens of serum origin were precipitated in the intermediate gel containing the anti-human serum antibody; therefore any precipitin peaks appearing only in the upper portion of the gel had to be specific to the cyst fluid being examined.

Various serum components were present in all the cyst fluids examined, but no particular pattern of antigens could be associated with individual cyst types. Keratocyst fluids had a much lower content of serum components, as exemplified by the lower dilution factor necessary for the crossed immunoelectrophoresis and the heights of the serum antigen peaks produced (fig 1).

An indistinct precipitin peak was present in the upper portion of several crossed immunoelectrophoresis gels of fluids from different cyst types. These antigens, designated Y and Z (figs 1 and 3, respectively), have not been studied further, so it is not yet known whether they are identical, but neither could be identified as a constituent of all examples of any particular cyst type.

Another major antigen was, however, detected in all examples of keratocyst fluids examined by crossed immunoelectrophoresis. The antigen, designated X (fig 1), presented consistently as a double peak but was obviously not of serum origin. No fluids from the other types of odontogenic cysts studied contained antigen X on crossed immunoelectrophoresis.

Because antigen X seemed to be strongly associated

with keratocyst fluids, it seemed possible that the antigen may have been a member of the keratin intermediate filament family. This thesis was tested initially using crossed immunoelectrophoresis but later by SDS-PAGE and Western blotting. Human keratin prepared from skin callous material was electrophoresed along with keratocyst fluids into anti-keratocyst serum, but no reproducible effect on the size or presentation of the precipitin peak X could be found. Further experimentation using “tandem CIE” showed epithelial keratins to be antigenically distinct from antigen X (fig 4).

COLUMN CHROMATOGRAPHY

In an attempt to further characterise this novel antigen samples of a keratocyst fluid containing relatively large amounts of X were chromatographed on Sepharose CL-6B. Portions of the eluted fractions were then examined by fused rocket immunoelectrophoresis into homologous antiserum (Fig 5). Antigen X could be seen coming off the column in fraction 6 (6 ml after the void volume), peaking in fraction 17 and persisting until fraction 22. Serum related components began to elute in fraction 12 and, accordingly, fractions 8–11 were used for further study.

SDS-PAGE AND WESTERN BLOTTING

Various fractions from the column chromatography experiment were examined by SDS-PAGE. In fractions 8–11 two protein bands were visible, which had molecular weights of 81 000 and 89 000 daltons (81 and 89 K); later column fractions contained numerous other protein bands, which were presumably largely serum components.

All the Western blot experiments described were carried out by processing samples in the absence of β -mercaptoethanol, as it was noticed that the β -mercaptoethanol destroyed the ability of the anti-keratocyst serum to recognise the 81 and 89 K molecular weight components.

Western blots of column fractions 9, 12, and 15

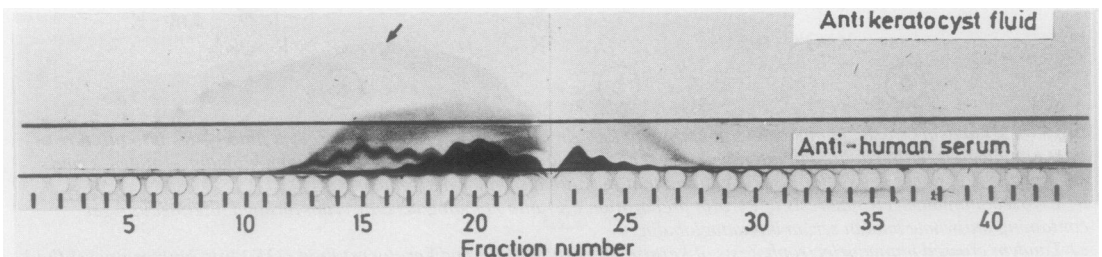


Fig 5 Fused rocket immunoelectrophoresis of column chromatography fractions (Sepharose CL-6B) from keratocyst fluid into homologous antiserum through intermediate gel containing anti-whole human serum immunoglobulin. Arrow indicates antigen X which is distributed between fractions 6 and 22.

showed that both the 81 and 89 K bands were the principal antigens recognised by the antikeratocyst fluid serum but were not detected by antihuman serum antibody (fig 6). Probing Western blots of fraction 9 and the human callous derived epithelial keratins with antikeratin antibody showed that the 81 and 89 K components were distinct from the keratins both

antigenically and by virtue of their molecular size. The principal epithelial keratins identified by this method had molecular weights of 57 K, 63 K, and 69 K, but a family of minor constituents was also present between 45 K and 69 K molecular weight (data not shown). The principal keratins present in the keratocyst fluid had the same molecular weights as the main epithelial keratins in the callous preparation.

Having identified the molecular size of the antigen X complex, attempts were made to survey a range of keratocyst fluids for the presence of the antigen by the method of Western blotting using antikeratocyst serum that had been absorbed with human serum (fig 7). The 81 K and 89 K proteins were present in all keratocyst fluids screened except one, which had a single major antigen of molecular weight 77 K. The two fluids obtained from separate keratocysts in a patient with Gorlin's syndrome also contained the characteristic 81 K and 89 K material. A few keratocyst fluids seemed to contain minor quantities of other bands recognised by the antikeratocyst serum; their relation to the 81 and 89 K, however, is unknown at present, and some of them are probably of serum origin as the antikeratocyst serum also has some antihuman serum activity.

Discussion

THE NATURE OF X

This investigation identified the presence of a major antigen in the fluid aspirated from odontogenic keratocysts; in crossed immunoelectrophoresis, as used here, the X complex presented as a characteristic "bimodal" peak and seemed not to be derived from serum. On the basis of positive staining with periodate Schiff reagent the antigen is a glycoprotein and in SDS-PAGE resolves as two bands with molecular

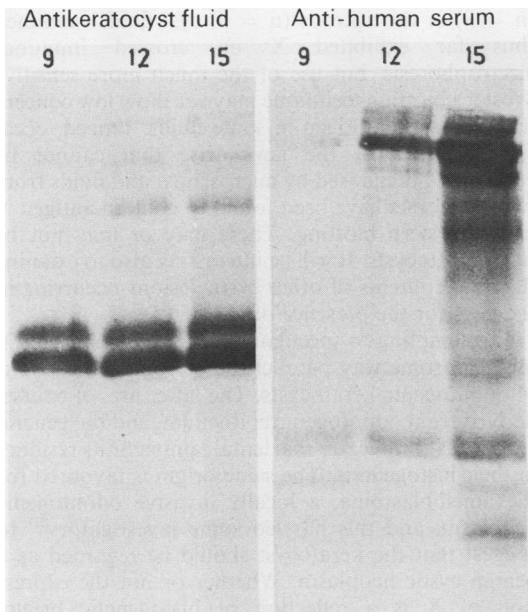


Fig 6 Western blot of column chromatography (Sephacose CL-6B) fractions 9, 12, and 15 from keratocyst fluid probed with antikeratocyst fluid or antiwhole human serum immunoglobulin and located with a peroxidase conjugated second antibody.

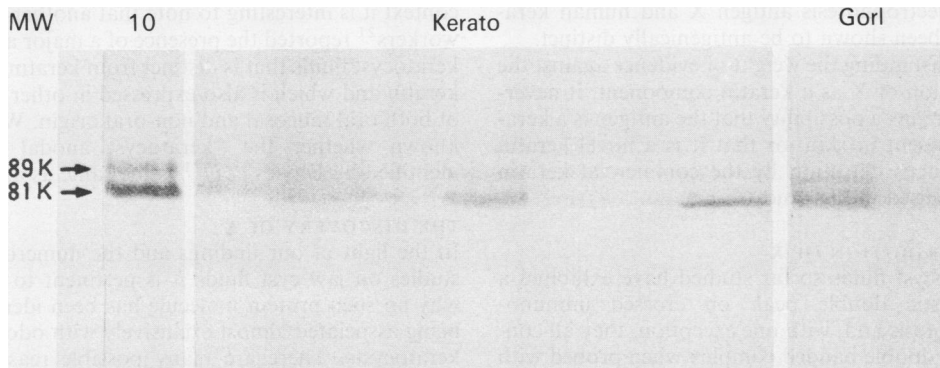


Fig 7 Western blots of keratocyst fluids (7), Gorlin's syndrome (Gorl) cyst fluids (2), and fraction 10 of keratocyst fluid separated by column chromatography on Sepharose CL-6B. Antigen X is depicted as two bands 81 K and 89 K.

Blots were probed with antikeratocyst fluid serum absorbed with whole human serum by affinity chromatography and bands were located with peroxidase conjugated second antibody.

weights of 81 and 89 K. Although the charge mobility of this complex has yet to be determined, its migration characteristics in the electrophoretic system used here are compatible with an isoelectric point below 8.6.

As indicated previously, the inclusion of β -mercaptoethanol in the SDS sample buffer destroys the ability of the antikeratocyst serum to recognise both the 81 and 89 K components which comprise antigen X; this observation strongly suggests that the major antigenic epitope on the "X complex" is related to disulphide bonds, as these bonds are cleaved in the presence of reducing agents such as β -mercaptoethanol.

In view of this antigen's striking association with keratocysts and its presumed content of disulphide bonds it seemed very likely that X could be a member of the keratin family. Evidence to the contrary, however, has come from several sources. Firstly, the antikeratocyst antibody displayed activity against members of the keratin family, which was distinct from that associated with the recognition of X; this antikeratin activity was demonstrable against both a range of keratocyst fluids and the skin callous keratin preparation. Notably, the antibody activity against keratin that was present in our antikeratocyst serum was not affected by reducing agents while, in contrast, the anti-X activity was abolished under reducing conditions. Additional support for the non-keratin identity of X comes from the results obtained with the commercial antihuman keratin antibody, which is raised against a skin callous preparation and, though it consistently recognised members of the keratin family in keratocyst fluids and our own callous derived keratin sample under both reducing and non-reducing conditions, it always failed to detect antigen X under any conditions. Similarly, by tandem crossed immunoelectrophoresis antigen X and human keratins have been shown to be antigenically distinct.

Notwithstanding the weight of evidence against the identification of X as a keratin component, it nevertheless remains a possibility that the antigen is a keratin not present in skin, or that it is a novel keratin which escapes detection by the commercial keratin antiserum used in this study.

THE DISTRIBUTION OF X

All keratocyst fluids so far studied have exhibited a characteristic double peak on crossed immunoelectrophoresis and, with one exception, they all contained the double banded complex when probed with the homologous antiserum in Western blots. The only exception presented as a single band of molecular weight 77 K, which could well represent a degradation product of the usual 81 and 89 K complex, as in crossed immunoelectrophoresis the same

fluid also showed the typical double peak associated with X.

It remains to be seen whether, by studying larger numbers of cyst fluids than those reported here, the preferential occurrence of antigen X in keratocysts can be confirmed. In this context, however, we have yet to find a keratocyst fluid (26 studied to date) that does not contain the X antigen on crossed immunoelectrophoresis. In contrast, none of the radicular ($n = 9$) or dentigerous ($n = 9$) cyst fluids examined thus far exhibited X on crossed immunoelectrophoresis, but use of the much more sensitive Western blotting technique may yet show low concentrations of the antigen in some fluids. Indeed, occasionally cysts of the jaws arise that cannot be confidently diagnosed by microscopy, and fluids from two such cysts have been found to contain antigen X using Western blotting. These may or may not be from keratocysts. It will be interesting also to examine the fluid contents of other cystic lesions occurring in the jaws for the presence of X.

It is tempting to speculate that the expression of X might in some way parallel the biological behaviour of odontogenic keratocysts. The latter are, of course, derived from odontogenic epithelium, and the general consensus¹ implicates the dental lamina or its residues in their histogenesis. The same origin is favoured for the ameloblastoma, a locally invasive odontogenic neoplasm, and this has led some investigators²⁰ to suggest that the keratocyst should be regarded as a benign cystic neoplasm. Whether or not the expression of X is a reflection of histogenetic origin, differentiation potential or biological behaviour remains to be determined; indeed, its origin from epithelium is as yet unsubstantiated. Preliminary evidence from immunohistochemical studies, however, suggests that X may be epithelially derived. In this context it is interesting to note that another group of workers²¹ reported the presence of a major antigen in keratocyst fluids that is distinct from keratin and prekeratin and which is also expressed in other epithelia of both oral mucosal and non-oral origin. We do not know whether the "keratocyst anodal protein" identified by Davies *et al*²¹ is the same as antigen X.

THE DISCOVERY OF X

In the light of our findings and the numerous other studies on jaw cyst fluids it is pertinent to consider why no such protein molecule has been identified as being associated almost exclusively with odontogenic keratocysts. There are many possible reasons why past attempts to detect markers of individual cyst types have proved unsuccessful; certainly the search for a single component that may not be quantitatively dominant over numerous other components is fraught with difficulty. Also, and with respect to

keratocysts in particular, the amounts of proteolytic activity in their fluids²² will almost certainly complicate the picture. Furthermore, the almost universal use of β -mercaptoethanol in SDS-PAGE electrophoresis sample buffer may well reduce the chances of recognising several constituents, especially those composed of subunits.

To a large extent the use of immunological methods avoids many of the problems inherent in the traditional approach to the analysis of cyst fluids. A family of components can be identified readily even if several are present in the form of degradation products of a parent molecule. The approach adopted in this study also offers the potential to discriminate unequivocally between serum derived and lesion related components. Although immunological methods have been used before,¹¹ the investigation was confined to an examination of the serum constituents of cyst fluids.

THE DIAGNOSTIC POTENTIAL OF X

The identification of antigen X offers a real prospect for improvement in the reliability of preoperative diagnosis for odontogenic keratocysts. Of the keratocyst fluids assayed by crossed immunoelectrophoresis to date, the presence of X has been universal, notwithstanding total soluble protein concentrations ranging from 1.86–22.0 g/100 ml; by this criterion alone, 80% of the fluids would not have been diagnosed as coming from a keratocyst—that is, a total soluble protein <4.0 g/100 ml. We hope to substantiate the reliability of antigen X as a diagnostic marker for keratocysts and to further characterise its nature, origin, and distribution.

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