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# IMMUNOLOGICAL CHARACTERIZATION OF AMYLOID FIBRILS IN TISSUE SECTIONS

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## SUMMARY

The labelling of rabbit antiserum against purified and alkaline-degraded amyloid fibrils with fluorescein isothiocyanate (FITC), has enabled the detection of amyloid deposits in various tissue sections by direct immunofluorescence. There was antigenic identity between amyloid from different organs of the same patient. In addition, indirect immunofluorescence revealed individual antigenic specificity and cross-reactivity among amyloid from different individuals. A close relationship between deposits of amyloid, immunoglobulins and complement was observed when using FITC-and rhodamine-labelled antisera against various human plasma components.

## INTRODUCTION

Two morphologically distinct components of amyloid substance can be demonstrated by electron microscopy: the P-component (periodic rods) which has been shown to be antigenically identical with an α-globulin occuring in normal plasma, and the fibrils (Glenner & Bladen, 1968). The fibrils are the unique component of amyloid and account for the specific green birefringence when Congo-red-stained sections of amyloid-laden organs are examined in a polarizing microscope (Pras *et al.*, 1969; Glenner *et al.*, 1968).

Native amyloid fibrils are non-immunogenic (Ram, de Lellis & Glenner, 1968), but after degradation of isolated fibrils with alkali, by guanidine or by boiling, specific antibodies can be provoked by immunization of rabbits (Franklin & Pras, 1969; Glenner *et al.*, 1969; Husby & Natvig, 1972).

Direct immunofluorescence is a specific and sensitive method for detection of antigens in tissue sections and permits immunological characterization of the antigens *in situ*. By this technique, we were able to detect amyloid deposits in tissue specimens obtained at biopsy or necropsy. Antigenic specificity and cross-reactions among amyloid from different individuals

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were also studied. A close relationship between certain amyloid deposits, immunoglobulins and complement was observed.

## MATERIALS AND METHODS

*Tissues*

Sections from the following tissue specimens were examined: 1. Kidney biopsies from patients with various renal diseases. 2. Liver biopsies from patients with chronic liver diseases, mainly active, chronic hepatitis. 3. Lung biopsies from patients with chronic pulmonary diseases. 4. Various organs obtained at necropsy from patients with either known or suspected amyloid disease. 5. Sections from corresponding normal organs obtained at autopsy or surgery served as controls.

*Amyloid preparations*

Isolation and alkaline degradation of fibrils from amyloid-laden organs was performed as earlier described (Husby & Natvig, 1972), using the method of Pras *et al.* (1969).

*Antisera and FITC-conjugates*

Antisera were raised in rabbits by weekly intracutaneous injections of alkaline-degraded amyloid fibrils mixed with equal amounts of Freund's complete adjuvant. The antisera (anti-DAM) were absorbed with human plasma and extracts from corresponding human organs to avoid antibody activity against non-fibrillar components. The antisera were thoroughly tested by precipitation and haemagglutination techniques as described previously (Husby & Natvig, 1972).

An antiserum against DAM, from the liver of a patient with juvenile rheumatoid arthritis (TH) was chosen for conjugation because of its ability to precipitate amyloid preparations from several other patients in double immunodiffusion, as reported elsewhere (Husby & Natvig, 1972).

Antisera against anti-F(ab')$_2$, anti-IgG, anti-IgA, anti-IgM, anti-C3, anti-fibrinogen and anti-albumin were prepared and absorbed as previously described by Munthe & Natvig (1971a).

IgG was separated from the rabbit sera by passing through an anion exchange resin (DEAE-cellulose, Whatman DE 52). Labelling with fluorescein isothiocyanate (FITC) was performed to a molar F/P-ration of 1·5–3, according to Nairn (1969). Unbound dye was removed by dialysis against PBS, followed by gel filtration (Sephadex G-25). To avoid unspecific staining, the conjugates were absorbed with acetone-dried calf liver powder, or passed through a Whatman DE 52-column. The anti-F(ab')$_2$ antiserum was also labelled with rhodamine (lissamine—rhodamine B sulphonyl chloride) as described by Fothergill (1969). The conjugated antisera were tested by haemagglutination tests, double immunodiffusion and immunoelectrophoresis. The conjugates were also tested on plasma cells from myeloma bone marrow by direct immunofluorescence. The FITC-labelled anti-amyloid antiserum gave a single precipitation line in immunodiffusion against the alkaline-degraded amyloid (DAM) used for immunization, but no line against normal plasma components or extracts from normal human liver. The specificity was further shown by agglutination of red cells coated with the autologous DAM.

The conjugated anti-DAM antiserum was diluted in PBS to a final protein concentration

of 0·3 mg/ml before application on the tissue sections, and in this dilution there was a precipitating activity of 1/6 units (Beutner, Holborow & Johnsen, 1967). The F/P-ratio was estimated, as described by The & Feltkamp (1970), to 2·1. In a few experiments, immunofluorescence was performed by the indirect technique using FITC-labelled goat anti-rabbit-IgG (Munthe & Natvig, 1971b).

Normal rabbit IgG was also labelled with FITC and used as control conjugate.

*Fluorescence microscopy*

Tissue specimens were quick-frozen, and cryostat sections (4 $\mu$) were fixed in cold acetone or 90% alcohol and washed in PBS. After washing, the sections were treated with fluorescein isothiocyanate (FITC) or lissamine-rhodamine B conjugates as described by Munthe & Natvig (1971b).

Double staining was performed by first treating the sections with rhodamine-labelled anti-$F(ab')_2$, and after thorough washing with PBS, the same sections were treated with FITC-labelled anti-DAM.

Stained sections were inspected in a Leitz Orthoplan Microscope. UV light source was a HBO 200 W burner. Filter combinations were for FITC: 1·5-mm BG 12, AL 490 nm and secondary filter K 530. Rhodamine: 4-mm BG 38, 2-mm BG 36, AL 546 nm, TK 580 nm and as secondary filter K 580.

Microphotographs were taken by incident light through a Leitz vertical illuminator with an Orthomat camera.

For demonstration of specific green birefringence of amyloid, Congo-red-stained sections (Puchtler, Sweat & Levine, 1962) were also examined in a Leitz Orthoplan microscope equipped for polarization.

## RESULTS

*Immunohistochemical detection of amyloid*

Amyloid deposits, detected in six out of 120 kidney biopsies tested, were mainly localized to the glomerular tufts as large globular bodies, often confluent to fill the whole glomerular area (Fig. 1); the tubules, vessels and interstitial tissue also often contained fluorescent amyloid material. Examination by conventional histochemistry (polarization microscopy after Congo-red staining), confirmed the findings in five of these cases, and the sixth was 'possible amyloidosis'.

Of thirty liver biopsies, none showed specific immunofluorescence after treatment with FITC-labelled anti-DAM, and all were Congo-red negative.

One of the four lung biopsies showed massive amyloid infiltration in one part, while another part of the section contained small, linear deposits among the basement membranes of mucous glands, as shown in Fig. 2, and these findings were confirmed by Congo-red staining.

Furthermore, in each of the twenty-four organ specimens, including kidney, liver (Fig. 3), spleen, heart, thyroid, adrenals and pancreas (Fig. 4) obtained from eight necropsies, amyloid deposits were demonstrated by immunofluorescence. Except for one specimen (cardiac muscle from the patient TH), the same tissues also gave a positive Congo-red reaction.

The distribution within the tissues of amyloid deposits as shown by immunofluorescence

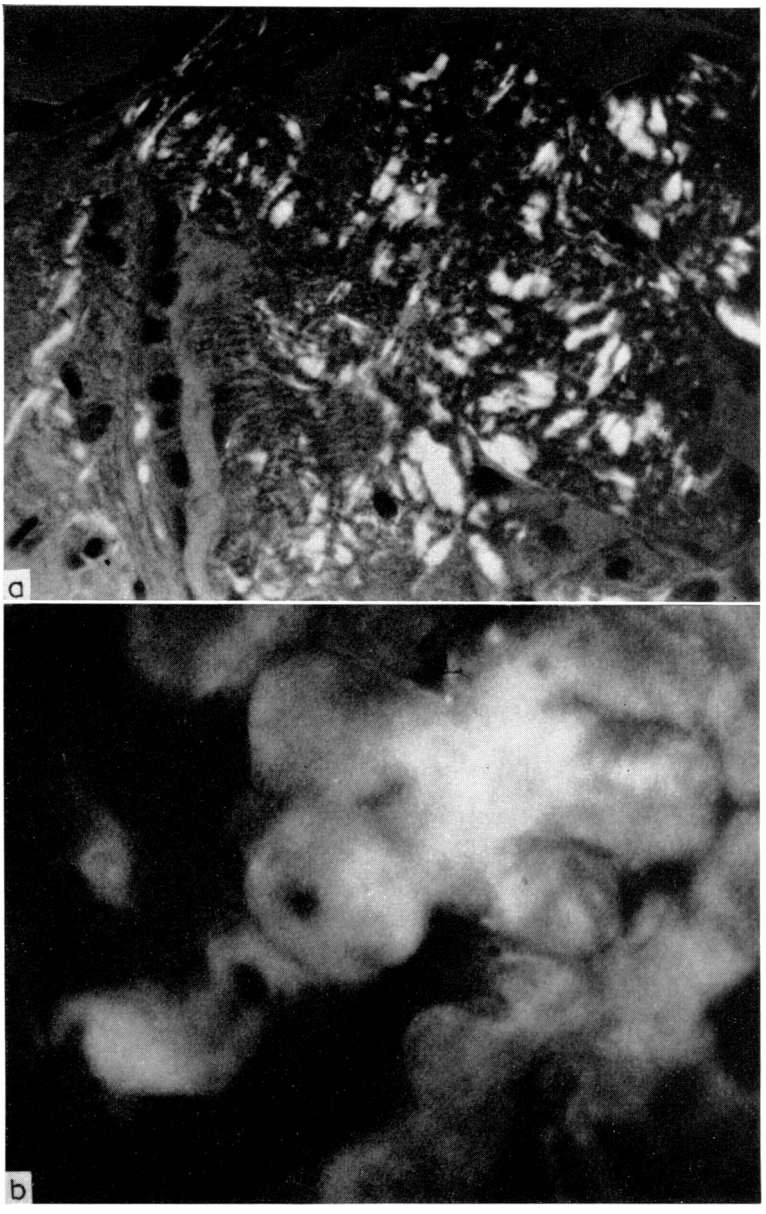


FIG. 1. Amyloid-laden kidney. (a) Section from biopsy stained with Congo-red and photographed in polarized light. Birefringent material in large amounts in the glomerulus (× 800). (b) Section from the same kidney biopsy stained with FITC-labelled anti-DAM. Staining with rhodamine-labelled anti-$F(ab')_2$ showed identical deposits of immunoglobulins. This demonstrated deposits of both amyloid and immunoglobulins in the same areas of the same glomerulus by double immunofluorescence staining (× 800).

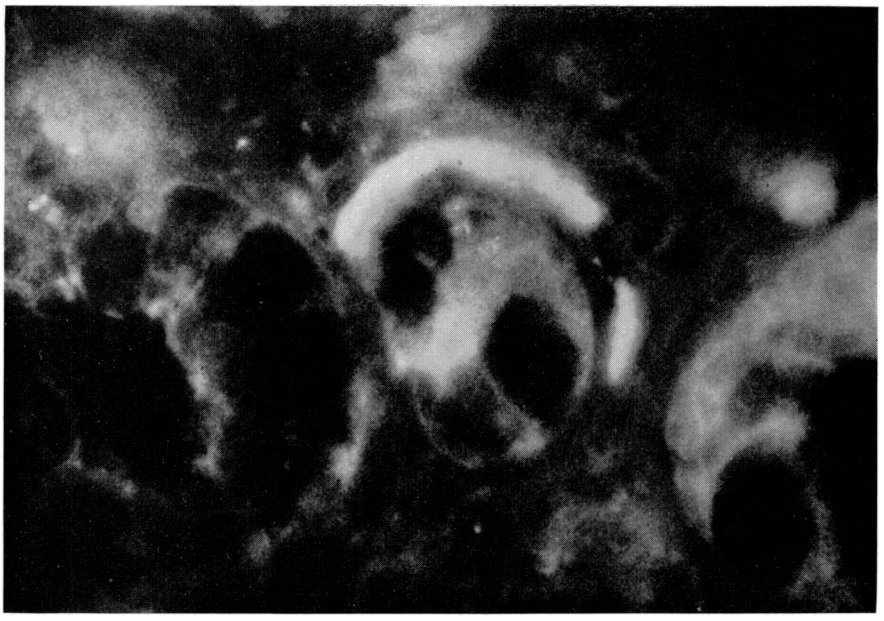

FIG. 2. Minor amyloid deposits surrounding mucous glands in a bronchus of a patient with pulmonary amyloidosis, showing fluorescence after staining with FITC-labelled anti-DAM (× 800).

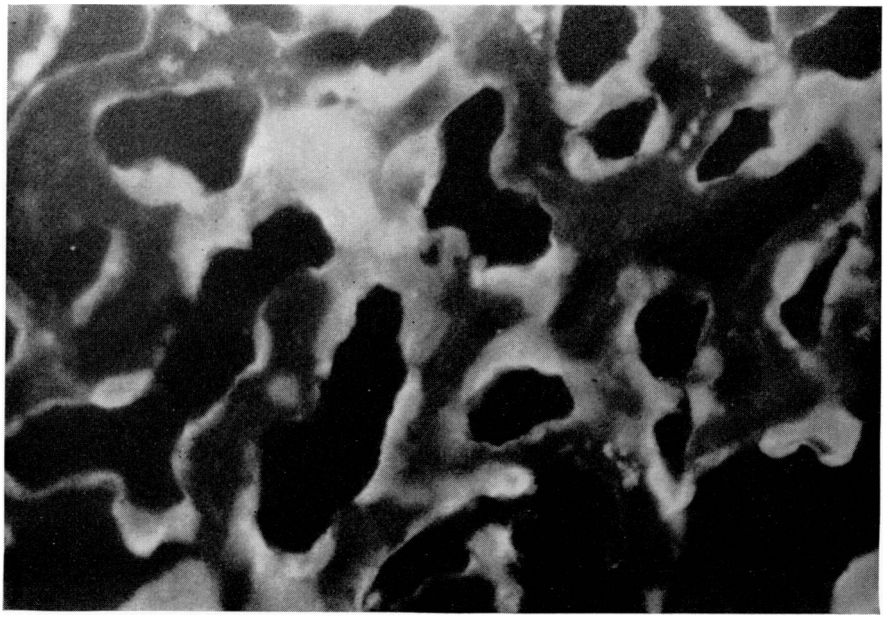

FIG. 3. Section from liver (autopsy) from a patient with generalized secondary amyloidosis, stained with FITC-labelled anti-DAM. Large, fluorescent amyloid deposits clearly demonstrated (× 400).

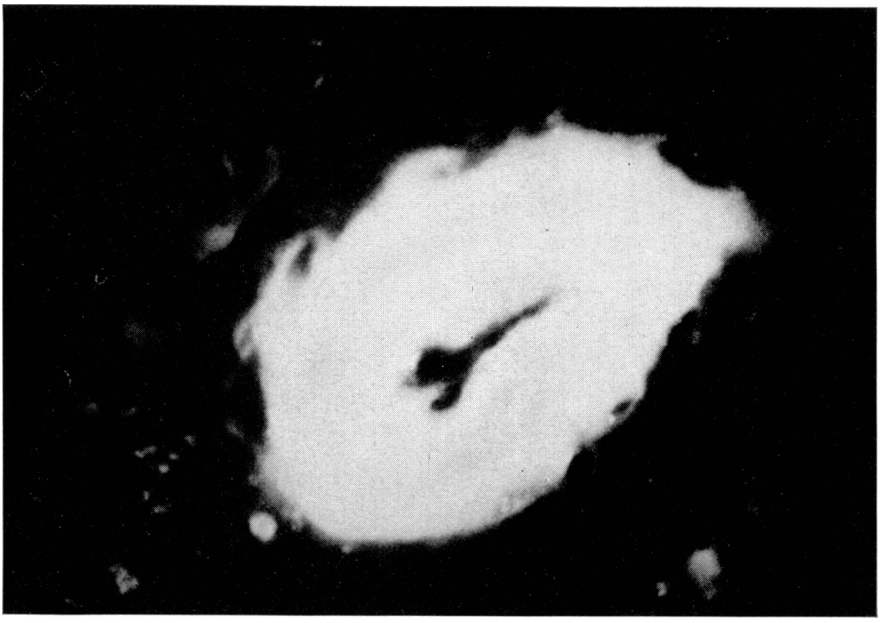

FIG. 4. Massive infiltration in a vessel wall of pancratic tissue, stained with FITC-labelled anti-DAM (× 800).

corresponded well with areas specifically stained by Congo-red, although immunofluorescence generally revealed wider extent of the amyloid (Fig. 1).

In unstained sections, amyloid deposits exhibited a weak, white-grey autofluorescence which was easily distinguishable from the apple-green FITC-fluorescence of the specifically stained amyloid.

In no case was amyloid detected histochemically without being also demonstrated by immunofluorescence. Except for an occasional faint, diffuse stromal staining, no immunofluorescence was obtained in the sections from non-amyloid organs. FITC-labelled normal rabbit IgG did not show any binding to the amyloid deposits. In blocking experiments, the specific fluorescence of the fluorochrome-labelled anti-amyloid antiserum was prevented by pretreatment of the sections with unlabelled anti-amyloid antiserum but not by pretreatment with normal rabbit serum. Absorption of the FITC-labelled anti-DAM with autologous native or alkaline-degraded amyloid fibrils also completely prevented the fluorescence.

*Antigenic specificity of amyloid fibrils*

There was a considerable variation in the strength of the specific immunofluorescence within the different organs, and there was no correlation with the size of the amyloid deposits. The strongest staining was obtained when sections from various organs from the patient TH, whose liver was the source of the amyloid preparation used for immunization, were examined. These findings indicated that the strength of the staining might be influenced by individual antigenic specificities of the amyloid fibril proteins. To investigate this further, we employed the indirect immunofluorescence technique with unlabelled rabbit antisera

TABLE 1. Indirect immunofluorescence with anti-DAM antisera absorbed with autologous or homologous DAM

| Tissue | DAM preparation used for absorption | Antiserum | |
|---|---|---|---|
| | | Anti-DAM Ho | Anti-DAM Kl |
| Ho | PBS (Ctr.) | +++ | + |
| Ho | DAM Ho | − | − |
| Ho | DAM Kl | ++ | − |
| Kl | PBS (Ctr.) | +(+) | +++ |
| Kl | DAM Ho | − | + |
| Kl | DAM Kl | − | − |

against DAM preparations from two different patients. Tissue sections from these two patients were examined with rabbit antiserum against the autologous and the homologous amyloid respectively. The strongest staining was shown with the autologous antisera. By using a grading of 1+ to 3+, a 3+ reaction was obtained with autologous antiserum while the homologous antiserum only gave a 1+ reaction (Table 1). After mixing the two antisera, the strength of the fluorescence was equally strong (3+) in both tissues.

By absorbing each anti-amyloid antiserum with different DAM preparations, the specific staining was prevented by autologous DAM, while some staining of autologous DAM still was obtained when homologous DAM was used for absorption (Table 1).

*Relation of amyloid to immunoglobulins and complement deposits*

Sections from the six amyloid-positive kidney biopsies were also treated with FITC-labelled antisera against the following human plasma proteins: IgG, IgM, IgA and C3. For control, anti-fibrinogen and anti-albumin were also used. The results are shown in Table 2. In two of the six biopsies the amyloid deposits were intimately associated with the immunoglobulins and complement. In two cases only immunoglobulin was seen in addition to amyloid. Of the four biopsies with immunoglobulins closely related to amyloid deposits,

TABLE 2. Immunofluorescent tracing of immunoglobulins and complement in amyloid-positive kidney biopsies

| Patient | Diagnosis | Amyloid | IgG | IgM | IgA | C3 | Fibrinogen | Albumin |
|---|---|---|---|---|---|---|---|---|
| F.L. | Ankylosing spondylitis | + | − | − | − | − | + | + |
| E.J.L. | Ankylosing spondylitis | + | − | − | − | − | − | − |
| M.H. | Juvenile rheumatoid arthritis | + | − | + | − | + | + | + |
| T.K. | Ulcerative colitis | + | − | + | − | − | + | − |
| H.F. | 'Collagenosis' Nephrotic syndrome | + | + | + | − | + | − | − |
| A.O. | Rheumatoid arthritis | + | − | + | − | − | + | − |

three had IgM and one both IgM and IgG. To demonstrate the localization of amyloid and immunoglobulins together in the same glomerulus of a kidney biopsy, we utilized double immunofluorescent staining which showed both amyloid and immunoglobulins localized to the same areas of the glomerulus (see Fig. 1).

In four cases, fibrinogen was demonstrated in relatively small amounts, mainly in the interstitial tissue, and in two cases albumin was detected, but neither of these two proteins seemed to have any specific morphological relationship to amyloid.

## DISCUSSION

Until recently, it has not been possible to obtain specific antisera against purified amyloid fibrils, since native fibrils are non-immunogenic (Ram *et al.*, 1968). However, degradation by strong alkali developed by Pras *et al.* (1969), by guanidine (Glenner *et al.*, 1969) or by boiling (Pick, Lavi & Joshua, 1970), renders the amyloid immunogenic, permitting the raising of specific antisera in rabbits. Immunologic studies of such sera have given evidence of cross-reactivity among degraded amyloid fibrils from different individuals (Franklin & Pras, 1969; Glenner *et al.*, 1970; Cathcart, Skinner & Cohen, 1971; Husby & Natvig, 1972) and individual antigenic specificity of amyloid (Glenner *et al.*, 1970; Husby & Natvig, 1972).

Recently, Cathcart *et al.* (1971) demonstrated specific binding of anti-amyloid antisera to amyloid deposits in organs from other individuals by using indirect immunofluorescence. In our study, we have employed direct immunofluorescence, which may be more specific although less sensitive than the indirect method. The sensitivity proved to be sufficient even for detection of small amounts of amyloid. According to Missmahl (1968), Congo-red-staining with polarizing microscopy is the most reliable method for detection of amyloid. However, by immunofluorescence, we detected amyloid deposits with a comparable sensitivity to that of the Congo-red technique.

The findings in our studies indicate that there are common antigenic determinants between native amyloid fibrils in tissue sections from different patients. In addition, individual antigenic specificities of amyloid from single patients was demonstrated. This is in accordance with our previous demonstrations of individual antigenic specificity and cross-reactivity between various DAM preparations using immunodiffusion and haemagglutination inhibition techniques (Husby & Natvig, 1972). There are probably varying degrees of cross-reactivity between different amyloid fibrils. Therefore, for routine purposes, antisera against amyloid preparations from a number of different patients should perhaps be pooled before routine detection purposes.

Many immunofluorescence studies describe findings of immunoglobulins and/or complement in amyloid deposits (Vasques & Dickson, 1956; Lachman *et al.*, 1962; Vogt & Kochem, 1960; Letterer, Caesar & Vogt, 1960). Pick & Osserman (1968) demonstrated immunoglobulins, complement, albumin and other plasma proteins in the surrounding area of the amyloid deposits. Our findings with double immunofluorescence staining indicate that both immunoglobulins and complement were localized in the same area of the glomeruli as the amyloid deposits. This intimate relationship has been demonstrated previously (Husby & Natvig, 1972) showing that apparently highly purified amyloid fibrils still contained small amounts of IgG. Whether this phenomenon is a pathogenetic factor, or merely secondary, remains to be decided. The recent report by Glenner *et al.* (1971) demonstrating chemical

identity of a purified guanidine-denatured amyloid protein and κ light chain, and our findings of antigenic cross-reactivity between certain amyloid preparations and light chains (Husby & Natvig, 1972) is compatible with the theory that amyloid may consist of insoluble aggregates of immunoglobulin light chain fragments. This may explain the frequent findings of amyloid associated with immunoglobulins and immune complexes. The possibility that other immunoglobulin fragments and chains or other proteins may participate in amyloid formation is not, however, excluded.

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