Antibody to intermediate filaments of the cytoskeleton in rheumatoid arthritis

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SUMMARY IgM antibodies against cultures of intermediate filaments (IMF) of the cytoskeleton were demonstrated by immunofluorescence in the sera of 94 (80%) of 118 patients with seropositive rheumatoid arthritis. These antibodies reacted with IMF in cultures of both human fetal fibroblasts and laryngeal carcinoma (HEp2) cells. Of 10 patients from whom paired synovial fluids were also available 8 had anti-IMF antibodies in both serum and fluid. In seronegative RA the incidence of anti-IMF was 40%, in ankylosing spondylitis 25%, in osteoarthritis 16%, and in normal subjects 14%. Only a minority of RA sera positive for anti-IMF antibodies were also positive for smooth muscle antibody. Absorption experiments suggest that in RA anti-IMF is directed at the intermediate filament protein, vimentin.

The presence in patients' sera of antibodies reactive with components of the cytoskeleton has usually been first recognised because the sera give immunofluorescent staining of smooth muscle cells in cryostat sections of tissue such as stomach. Thus the antibodies against smooth muscle (SMA) noted in chronic active hepatitis sera by Johnson *et al.*¹ proved to be directed at antigenic determinants on the actin component of microfilaments,^{2,3} while the SMA present in infectious mononucleosis sera was shown to be directed at the tubulin protein of microtubules.^{4,5} Recently Kurki *et al.*^{6,7} have found that SMA of nonactin type in the sera of patients with a variety of other conditions are directed at antigens of a third distinct cytoskeletal component of eukaryotic cells, the intermediate filaments (IMF), and Toh *et al.*⁸ demonstrated a similar anti-IMF reactivity for the SMA occurring in patients with certain acute viral infections. Yildiz *et al.*⁹ have in addition shown that the SMA present in many normal rabbit sera react with IMF in many cell types.

IMF are composed of chemically heterogeneous protein subunits of 5 known classes.¹⁰ These are desmin (in smooth, skeletal, and cardiac muscle cells), vimentin (in mesenchymal cells, including fibroblasts), keratin (in epithelial cells), neurofilament protein (in neurones), and glial filament protein (in glial cells).

Anti-IMF antibodies are readily detected in sera by immunofluorescence, giving a characteristic fine network staining pattern on cultured cells of different types. Linder *et al.*^{11,12} have reported that most rheumatoid arthritis (RA) sera also contain anti-IMF antibodies demonstrable in this way. Rheumatoid patients usually have a normal (12%) or only slightly raised incidence of SMA,¹³ but antibodies reactive with IMF proteins other than desmin would not be expected to stain smooth muscle cells in cryostat tissue sections. We report here that 80% of patients with established diagnoses of rheumatoid arthritis have antibodies against intermediate filaments of antivimentin specificity.

Materials and methods

Sera and synovial fluid. Sera from 118 patients with definite or classical seropositive RA (according to ARA criteria) and with latex titres $\geq 1:320$ were obtained from the serum bank of the Bone and Joint Research Unit, the London Hospital Medical College. These sera had been stored at -20°C for a period of 6-36 months. For 10 of these patients stored synovial fluid was also available.

Also examined were stored sera from 12 seronegative RA patients, 32 patients with ankylosing spondylitis, 60 patients with SLE, and 25 with osteoarthritis. Sera from 50 normal subjects, 25 male and 25 female (age range, 30 to 65), were

obtained from the London Hospital Blood Transfusion Service Unit by kind permission of Dr B. T. Colvin, Dr A. L. Turnbull, and Professor G. C. Jenkins.

Cell lines and tissue culture. Established laryngeal carcinoma cell lines (HEp2) were kindly made available by Professor L. H. Collier, the London Hospital, and human skin fibroblast (HSF) cell cultures by Dr C. Stern and Miss G. Cambridge, of the Royal Postgraduate Medical School, Hammer-smith. Subsequent subcultures and preparation of cell monolayers on glass slides for immunofluorescence were as described elsewhere.¹⁴

Treatment of cells with cytotoxic drugs. Monolayer cultures of HSF and HEp2 cells on glass slides were incubated at 37°C with colchicine (Sigma), 0.5 µg/ml of culture medium, for 12 hours; vinblastin (Eli-Lilly) 10 µg/ml for 4 hours; or cytochalasin B (Sigma) 10 µg/ml for 30 minutes in a humidified atmosphere containing 5% CO₂ and 95% air. Before fixing, monolayers were briefly rinsed once with phosphate buffered saline (PBS) pH 7.2 at 37°C.

Fixation of cultured cells and indirect immunofluorescent staining. Monolayers were fixed in absolute acetone at -20°C for 5 minutes and air dried. Drops of sera at 1:10 dilution were then placed on the monolayers and allowed to react for 45 minutes at 37°C. The slides were washed in PBS for 30 minutes and stained with anti-Ig or with Ig class-specific anti-immunoglobulin FITC conjugates (Nordic Immunological Supplies) at a dilution of 1:20. After a 30-minute wash in PBS the preparations were mounted in glycerol in phosphate buffer (pH 7.2) and examined by transmitted light with a Reichert microscope equipped for transmitted dark-ground illumination. The light source was a 100 W quartz halogen lamp, primary filter Balzer FITC-3, secondary filter Ilford 110.

Absorption of sera. Lyophilised purified 3T3 vimentin was a gift from Professor W. W. Franke, Cancer Research Centre, Heidelberg, and human callus prekeratin from Dr D. Skerrow, Department of Dermatology, University of Glasgow. Vimentin was dissolved in PBS (1 mg/ml) and insoluble prekeratin suspended in PBS at an approximate concentration of 1 mg/ml.

The anti-IMF antibody activity of 17 RA sera was titrated in doubling dilutions to the end point of fluorescent staining. Each serum was diluted to 4-fold its end-point titre and absorbed with an equal volume of the vimentin solution or the prekeratin suspension at 4°C overnight.

Separation of IgM rheumatoid factor (IgM RF) from rheumatoid sera. IgM RF was obtained by

passing 1 ml of RA serum through a 20×2 cm² column of Degalan beads coated with heat aggregated IgG at a flow rate of 18 mg/hour in 0.5 M borate running buffer containing 0.15 M NaCl (pH 8).¹⁵ The isolated IgM RF was eluted with 0.2 molar citrate buffer containing 0.15 M NaCl, pH 4.5, concentrated 100 times with Amicon B15 concentrator (Amicon Corp, Lexington, Mass 02173, USA) and tested for anti-IMF activity on monolayer cells.

IgM RF fractions isolated in this way were positive in a mixed reverse (solid phase) antiglobulin haemadsorbance test with anti-IgM coated cells as indicator.¹⁶

Cryostat sections of tissue blocks. Tissue blocks of normal rat liver, kidney, and stomach were snap frozen and stored at -70°C. Cryostat sections (6 µm) were air dried under a fan, fixed in cold acetone (-20°C), and stained by indirect immunofluorescence with patients' or normal sera at screening dilutions of 1:10.

Results

Many of the rheumatoid sera tested by immunofluorescence with anti-whole Ig conjugate gave a filamentous network pattern of cytoplasmic staining on cultured human skin fibroblasts (Fig. 1), and on

Fig. 1 Human fetal skin fibroblasts showing pattern of intermediate filament staining given by a rheumatoid serum. Indirect immunofluorescence; patients' serum 1:10, anti-human IgG FITC conjugate 1:20. (×240, enlarged×3).

HEp2 cells a filament network staining pattern rather differently orientated was also often seen (Fig. 2). When the fibroblast cultures had been pre-treated with colchicine or vinblastine, the stained filaments appeared as perinuclear coils (Fig. 3). Pre-treatment with cytochalasin B did not change the staining pattern, which was thus of the character described for intermediate filaments.¹⁷

Of the 118 sera from seropositive rheumatoid

arthritis tested 94 (80%) gave fluorescent staining of IMF. In 8 of the 10 RA synovial fluid/serum pairs antibody was demonstrated in both serum and synovial fluid. Five (41%) of 12 seronegative rheumatoid arthritis patients had a similar antibody. In other arthritides the incidence was lower (Fig. 4) and in SLE and osteoarthritis patients was not significantly different from that in blood donors (14%). Fig. 5 shows that titres measured with anti-Ig conjugate were highest (up to 1/320) in the rheumatoid sera.

The use of class-specific conjugates showed that of 48 positive RA sera tested all had IgM anti-IMF antibody, and in 12 IgG antibody was present also (Fig. 6).

Since most of the RA sera tested stained intermediate filaments in both fibroblast and HEp2 cells, 17 such positive sera were separately absorbed with vimentin and prekeratin as described in 'Methods'. Absorption with vimentin removed from all 17 sera the ability to stain intermediate filaments in both fibroblasts and HEp2 cells. Absorption with keratin abolished the staining of both fibroblasts and HEp2 cells by seven of these 17 sera.

Fig. 2 HEp2 cell treated as in Fig. 1. The intermediate filament network stained has a somewhat different pattern. ($\times 240$, enlarged $\times 4$).

Fig. 3 Vinblastine treated fibroblast showing perinuclear coils of intermediate filaments stained with a rheumatoid serum as in Fig. 1. ($\times 240$, enlarged $\times 4.5$).

Fig. 4. The incidence of anti-IMF antibody (detected with anti-Ig conjugate) in rheumatoid arthritis and other connective tissue diseases. RA+ = seropositive rheumatoid arthritis. RA- = seronegative rheumatoid arthritis. AS = ankylosing spondylitis. SLE = systemic lupus erythematosus. OA = osteoarthritis. Figures in brackets denote number of patients' sera tested.

Fig. 5 Titres of anti-IMF antibody (determined with anti-Ig conjugate) in rheumatoid arthritis and other connective tissue diseases.

Fig. 6 IgM and IgG anti-IMF in rheumatoid and other sera positive for anti-IMF with anti-Ig conjugate.

IgM RF fractions isolated from 3 RA sera with anti-IMF activity gave no staining of either human skin fibroblasts or HEP2 cells.

On cryostat sections of rat tissue blocks 9 of 57 RA sera positive for anti-IMF antibody stained smooth muscle cells in the SMA-V distribution at 1:10 dilution, and 1 gave SMA-G staining.¹⁸

Discussion

These results demonstrate a high incidence (80%) of IgM anti-IMF antibodies in patients with established seropositive rheumatoid arthritis. In other arthritides and in normal sera both the incidence and the titres of antibody were much lower. The fine branching pattern of filaments stained in the cytoplasm of HSF is identical with the radial arrangement, particularly abundant around the nucleus, described for intermediate filaments of vimentin type by Franke *et al.*,¹⁷ and quite unlike the cable pattern of staining of microfilaments seen with antiactin antibody.¹⁹ The lack of effect of pretreatment of the cells with cytochalasin B on the staining of the filaments confirms this, and the effect of vinblastine or colchicine pretreatment in causing rearrangement of the filaments into a coiled perinuclear formation due to microtubule loss induced by these drugs¹⁷ further indicates that the cytoskeletal component with which rheumatoid sera reacts is the intermediate filaments.

As well as fibroblasts many of the RA sera stained IMF in HEP2 cells. This at first suggested the presence of two anti-IMF antibodies of different specificities, one against the vimentin protein of fibroblast IMF, the other against the prekeratin protein of epithelial cells (since HEP2 is derived from a laryngeal carcinoma). The absorption results, however, did not support this conclusion, since absorption with vimentin removed the ability to stain both cell types. Absorption with keratin, on the other hand, abolished the ability of only 7/17 sera to stain both cells. Although vimentin is a predominant IMF protein in fibroblasts and prekeratin in epithelial cells, vimentin and certain keratins can coexist in epithelial cells, and vimentin and desmin may likewise coexist in both muscle and nonmuscle cells,^{17 20} while most cells grown in culture contain IMF of vimentin type.²¹

In the light of these findings, together with the observations of Toh *et al.*⁴ and Yildiz *et al.*⁹ on the cross-reactivity of human and rabbit anti-IMF antibodies with cells of different types, it seems likely that rheumatoid sera contain mixtures of antibodies reactive with vimentin alone, with vimentin and keratin, and less often (the SMA positive sera) with desmin also.

The reason why anti-IMF autoantibodies are so frequently present in rheumatoid arthritis is at present not clear. There is a good deal of evidence associating SMA in human sera with viral or bacterial infection,²²⁻²⁴ and where SMA has been shown to have anti-IMF activity the association is particularly with viral infections.⁸ Many of the rheumatoid arthritis sera in which we have found

antibodies against IMF are negative for SMA. Relevant to this is the observation of Linder *et al.*²⁵ that, while 67% of a group of infectious mononucleosis patients they studied had anti-IMF antibodies, in these patients also there was no correlation with the presence of SMA. In infectious mononucleosis it therefore appears that anti-IMF antibody occurs independently of antibody with anti-smooth muscle specificity, which in this disease, according to Mead *et al.*⁵ is directed at tubulin protein. Thus the relatively low incidence of SMA in RA sera does not exclude the possibility of a viral cause for the presence of anti-IMF antibody reactive with vimentin. Various mechanisms postulated to explain how virus might enhance the immunogenicity of the cytoskeleton are discussed by Allison and Denman²⁶ and Toh.²⁷ As the accompanying paper shows, the vimentin component of the cytoskeleton is prominently represented in some of the mesenchymal cells of the rheumatoid synovial membrane.

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