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TECHNIQUES

AN IMMUNOFLUORESCENCE MIXED STAINING TECHNIQUE FOR THE DETECTION OF IgG-RHEUMATOID FACTOR AND IgG-β_{1C} COMPLEXES IN TISSUES

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SUMMARY

A mixed immunofluorescent staining method for the detection of immune complexes in the tissues is described.

This method demonstrated the presence of IgG-rheumatoid factor and IgG-β_{1C} complexes in synovial membrane from patients with rheumatoid arthritis.

INTRODUCTION

Immunofluorescent staining of serial sections has been employed for the separate detection of immunoglobulins, complement components and rheumatoid factor (RF) in several tissues, such as the synovial membrane (Mellors *et al.*, 1959; Rodman *et al.*, 1967; Bonomo, Tursi & Gillardi, 1968) and synovial fluid leucocytes (Rawson, Abelson & Hollander, 1965; Vaughan *et al.*, 1968) in rheumatoid arthritis (RA), the kidney in glomerulonephritis and systemic lupus erythematosus (Dixon, Feldman & Vazquez, 1961; Andres *et al.*, 1966; Koffler *et al.*, 1967), the myocardium in rheumatic fever (Kaplan *et al.*, 1964), and the blood vessels of cutaneous vasculitis (Cochrane, Hawkins & Kniker, 1967; Parish, 1969). These data indirectly suggest the deposition of immune complexes (IC) in the tissues investigated, but the simultaneous presence of the complex components has not yet been demonstrated.

The purpose of the work described in this paper was to develop a mixed staining method suitable for detecting the presence of IgG-RF and IgG-β_{1C} complexes in tissues. By the use of two contrastingly labelled reagents, two proteins could be simultaneously demonstrated in the same tissue section. Synovial membrane obtained from patients with RA was used as substrate in this study. Therefore, a particular modification of the method was devised to avoid the possible reaction between the RF present in the synovia from seropositive patients and the IgG globulin of the rabbit antisera employed.

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MATERIALS AND METHODS

Synovial tissue

Specimens of synovial membrane were obtained by needle biopsy from three seropositive and two seronegative patients with RA. Specimens of normal synovia were obtained during various orthopaedic procedures. The specimens were embedded and subsequently treated as previously described (Bonomo *et al.*, 1968).

Immunological reagents

All the reagents employed were conjugated with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TMRITC) (B.B.L. Baltimore) according to the method of Riggs *et al.* (1958), at a ratio of 0·05 mg of dye to 1 mg of protein. The conjugates were freed from excess fluorescein or rhodamin by gel-filtration through Sephadex G-25 (for FITC) or G-50 (for TMRITC) (Pharmacia, Uppsala). Before use the conjugates were absorbed with mouse liver powder. The following conjugates were used:

(1) *Human monoclonal IgM immunoglobulin (H-IgM).* An immunoelectrophoretically pure human IgM was obtained by gel-filtration on Sephadex G-200 from a patient with Waldenström's macroglobulinaemia, whose serum showed a strong anti-human IgG activity (F_{II}-tanned cell agglutination: 1:80,000). The isolated IgM was conjugated with FITC and diluted to give a final F_{II}-tanned cell agglutination titre of 1:1240. No reactivity was found against rabbit IgG by the sensitized sheep cell and tanned cell agglutination methods.

This reactant with anti-IgG activity was used instead of rabbit anti-human IgG antiserum, to detect human IgG, either in the aggregated form or in antigen-antibody combination, according to Lachmann *et al.* (1962), and McCormick (1962). The labelled H-IgM showed a strong reactivity with strands of aggregated human F_{II}.

(2) *Aggregated rabbit-IgG (R-IgG).* Immunoelectrophoretically pure rabbit IgG was obtained by ammonium sulphate precipitation and subsequent filtration through Sephadex G-200 from pooled normal rabbit sera, and conjugated with TMRITC and heat-aggregated at 63°C for 30 min. It did not show any natural antibody activity against human IgG. To detect RF, R-IgG instead of human F_{II} was used in order to avoid the reaction of the H-IgM reactant with the latter. Indeed preliminary mixed staining procedures with contrastingly labelled human and rabbit IgG showed that the great majority of RF present in the synovial tissues of our cases reacted with both human and rabbit IgG.

(3) *Rabbit anti-human β_{1C} globulin serum (R-anti β_{1C}).* The antiserum was prepared in rabbits by immunization with human β_{1C} absorbed to zymosan as described by Stratton (1966), and labelled with TMRITC. The conjugate gave a single characteristic β_{1C}–β_{1A} line when analysed by immunoelectrophoresis.

Immunofluorescent staining methods

Four-micron sections were cut from the frozen tissue blocks in a cryostat at −18°C and allowed to dry on slides at room temperature. No difference was found in staining between fixed and unfixed preparations, therefore the fixation step was subsequently omitted.

As already pointed out by McCormick (1963) the possibility exists of unwanted reactions between the IgG globulin of the rabbit antisera under study and the RF present in synovia of seropositive patients with RA. In order to avoid such a possibility, probably due to the

occurrence of some denaturation of the rabbit IgG, the sections were extensively treated with unlabelled heat-aggregated rabbit-IgG before the application of rabbit antisera. This pretreatment produced complete inhibition of the RF reactivity in companion serial sections when stained with aggregated labelled rabbit-IgG.

Single staining procedures

Because immunoglobulins, RF and complement were distributed unevenly in some of the specimens, it was necessary to cut several serial sections from each block. Single staining with each labelled reactant was first performed in order to identify the location of immunoglobulins and complement before applying the mixed staining procedures to the complete series of the sections.

Mixed staining procedures

A direct mixed staining procedure was performed by exposing the sections to a mixture of equal volumes of two contrastingly labelled reagents, as follows:

(a) FITC labelled H-IgM plus TMRITC labelled R-IgG, to detect deposits of IgG and RF;

(b) pretreatment with unlabelled R-IgG and subsequent staining with FITC labelled H-IgM plus TMRITC labelled R-anti β_{1C}, to detect deposits of IgG and β_{1C}.

Inhibition procedures

Direct inhibition in both single and mixed staining procedures was performed by staining control sections with the conjugate(s) after prior treatment with the corresponding unlabelled protein(s).

To perform cross-inhibition in the direct mixed staining each of the conjugates under study was replaced in turn by the corresponding unlabelled protein, which was applied twice for 1 hr before the mixed staining.

Under these conditions complete or virtually complete inhibition was obtained. By these criteria the fluorescent staining reactions reported in this study were considered specific.

The positive fluorescent staining and the control procedure were performed on single alternate sections throughout the entire series.

Microscopy

A Leitz Laborlux microscope was used with a Philips 150 mercury vapour light source. The sections stained with either FITC conjugate, TMRITC conjugate or both (intermediate shades of yellow) were observed using a UG1-2 mm exciter filter plus a BG 38-4 mm filter, an oil-immersion dark-field condenser 1·20 A, and an UV 2·5 mm barrier filter.

Further differentiation of green, red and mixed staining was achieved by sequential observation of the same preparation with either Kodak Wratten filter No. 58 (green) or 23A (red), according to the method of selective filtering by Cebra and Goldstein (1965).

Pictures were taken with a Leica camera exposing Agfacolor CT18 colour film for 1–5 min. Prints on Polacolor type 108 were obtained directly from transparencies using a Polaroid MP-3 land Camera. To correlate the fluorescence microscopy findings with those of light microscopy, serial sections from either paraffin embedded or frozen blocks were stained with haematoxylin and eosin.

RESULTS

With the method described it was possible to demonstrate simultaneously the concurrent presence of IgG and RF in the synovial membrane of seropositive cases of RA, and of IgG and β_{1C} in both seropositive and seronegative patients.

Yellow (mixed) staining was taken as indicating the presence of two components, one—the IgG— reacting with the FITC H-IgM and the other —RF or β_{1C}— reacting with TMRITC R-IgG or TMRITC R-anti β_{1C} respectively. Further confirmation was achieved by using the method of selective filtering on the same fluorescent immune deposits (Fig. 1) as well as the cross-inhibition procedure. The localization of the IC was extracellular (interstitial or/and vascular) and cytoplasmic (phagocytic cells).

DISCUSSION

It should be pointed out that the H-IgM reagent is particularly suitable for detecting IgG because of its rheumatoid anti-IgG properties. The specificity of the RF for IgG in an aggregated form or in antigen–antibody complex is well established (Edelman, Kunkel & Franklin, 1958). Furthermore, the very strong and specific reactivity of the pure monoclonal IgM against human IgG allows its use at high dilutions, as compared with the usual rabbit anti-human IgG antisera. This provides an essentially complete elimination of non-specific staining.

In agreement with the findings of Hijmans, Schuit & Klein (1969), the order in which the conjugates were applied appeared to be critical. In fact, it was found that in some instances the results of the sequential mixed staining procedure were not comparable with those of the direct mixed staining, probably because of competition between reactants; therefore the latter technique was preferred throughout the whole investigation.

Pretreatment of sections with aggregated unlabelled rabbit-IgG was required before performing the direct mixed staining when a rabbit anti-serum was one of the two contrastingly labelled reagents. The R- anti β_{1C} antiserum, as well as normal pooled rabbit globulin, rabbit anti-bovine serum albumin and rabbit anti-sheep erythrocyte antisera, clearly reacted to varying degrees with the RF present in the synovia of seropositive cases of RA and, when used unlabelled in inhibition procedures, inhibited the staining by the labelled aggregated rabbit-IgG.

RF is presumed to react with rabbit-IgG denatured or aggregated in some way (Edelman *et al.*, 1958). Such denaturation is likely to occur in rabbit antisera during their preparation (ammonium sulphate precipitation, conjugation etc.) and their successive ageing (freezing and thawing). The possibility of a reaction between RF and the rabbit antisera to be used, should therefore be borne in mind when studying substrates in which RF cross-reacting with both human and rabbit IgG is present or suspected.

The use of antisera obtained from other animals (goat, horse) is also possible only when preliminary testing demonstrates the absence of reactions between RF and the IgG of these antisera. This was not the case with our seropositive patients whose sera reacted with some or all of the IgG tested (rabbit, horse and goat IgG), as shown by the tanned cell agglutination method. This finding is in agreement with the wide cross-reactivity of RF versus the IgG of several mammals (Butler & Vaughan, 1964).

In conclusion the technique described seems useful in detecting IC in synovial membrane as well as in other tissues. It permits both an evaluation of their distribution and localization,

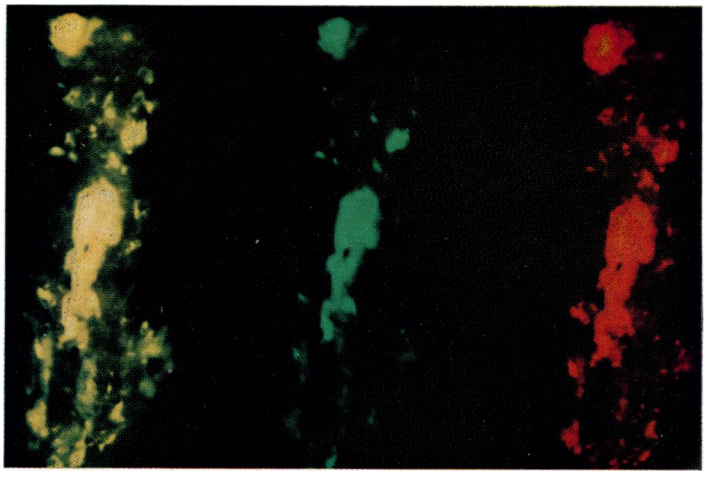

FIG. 1. Synovial membrane from a patient with rheumatoid arthritis. Direct mixed staining immunofluorescence procedure: FITC-conjugated human IgM with anti-human IgG activity plus TMRITC-conjugated aggregated rabbit-IgG. Original magnification: ×250. Interstitial deposition of IgG-Rheumatiod Factor (RF) complex in masses of lumpish aspect (1A). Selective filtering of the same field with Kodak Wratten No. 58 (1B) showing the presence of IgG and Kodak Wratten 23A (1C) showing the presence of RF.

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and an approximate measure of the relative proportions of the two components of the IC present.

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ABBREVIATIONS

FITC, fluorescein isothiocyanate.
TMRITC, rhodamine isothiocyanate.

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