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EPIDERMAL NUCLEAR IgG DEPOSITION IN NORMAL SKIN: CHARACTERIZATION BY ANTI-RIBONUCLEOPROTEIN IgG-Fab FRAGMENTS

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Abstract. Epidermal nuclear IgG deposition in clinically normal skin may occur in patients with scleroderma or scleroderma-like features. In order to evaluate the mechanisms of the fixation, Fab fragments of anti-RNP IgG antibodies, obtained after papain digestion, were incubated for increasing times with various substrates: human skin, human mononuclear cells, cultured human fibroblasts and rabbit lip. Our results showed that anti-RNP IgG-Fab fragments could penetrate most of the living cells of human skin and rabbit lip and, to a lesser degree, mononuclear cells and poorly cultured fibroblasts. No ability to fix was found either with anti-RNP IgG-Fc fragments or with anti-nDNA/DNP IgG-Fab. It was concluded that anti-RNP IgG could penetrate viable epidermal and non-epidermal cells and that surface Fc receptors must play a minor role in the cellular penetration of antibodies.

Key words: Immunofluroescence; Ribonucleoproteins; Anti-RNP-IgG; Epidermis; Mononuclear cells

Epidermal nuclear IgG deposition in a speckled (particulate) pattern on normal skin as observed by direct immunofluorescence (DIF) is an easily detectable marker for a subset of connective tissue diseases characterized by antibodies to extractable nuclear antigen (ENAs) (3, 6, 11, 12). This finding usually reflects high serum concentrations of antibodies to ENA (7) and is most commonly seen in patients with systemic scleroderma or scleroderma-like features. The purpose of this work was to elucidate the mechanisms by which antibodies to nucleic acids can penetrate viable cells.

PATIENTS AND METHODS

Four groups of patients were selected.

Group I: five patients with circulating and fixed anti-RNP antibodies (speckled pattern, indirect IF titre: 10^{-4}). All had systemic scleroderma.

Group II: three patients with circulating RNase-resistant anti-ENA antibodies (speckled pattern, indirect IF titre: 10^{-4}). Two had systemic lupus erythematosus and one had severe Raynaud's phenomenon.

Group III: three patients with circulating anti-DNA/DNP antibodies (homogeneous pattern, indirect IF titre: 10^{-3}) and no epidermal nuclear fixation. All had systemic lupus erythematosus.

Group IV: three patients with non-monoclonal hyperglobulinemia G and an absence of circulating antinuclear antibodies (ANAs). All had liver cirrhosis.

Determination of circulating ANAs was assessed by an indirect IF technique, using *Crithidia luciliae* as a substrate for the detection of double-stranded DNA antibodies (1).

Determination of anti-ENA antibodies was performed according to Tan's technique (14). An absence of precipitin line after treatment with RNase and trypsin was indicative of anti-RNP antibodies. Absence of significant change in precipitation after digestion of ENA with RNase was interpreted as RNase-resistant and termed Sm.

Isolation and fragmentation of IgG-Fab and Fc fragments: IgG were isolated on a DEAE-A 50 cellulose column in phosphate buffer 0.01 M, pH 6.5, and had a titre of anti-RNP activity of $1:10^{-4}$ at a protein concentration of 11.8 ± 2.3 g/l. Fragmentation of IgG into Fab and Fc portions was performed with papain (6 hrs incubation, 37°C). Non-fragmented IgG were separated on a Sephadex G 100 column (phosphate buffer 0.002 M, pH 8). The purity of the fragments, enhanced by incubation with glutaraldehyde polymerized anti-Fab and Fc sera, was controlled by immunoelectrophoresis and Ouchterlony analysis in sequential dilutions (Figs. 1, 2).

Cells and tissues used as substrates. Human mononuclear cells isolated on a layer of Ficoll metrizoate and incubated at once with the various antisera or cultured for 72 hrs with 25 μg/10^6 cells/ml of phytohemagglutinin-P (PHA-P Difco); human diploid fibroblasts (strain Wi 38) used after a 3-day culture in Eagle's minimal essential medium; and normal human skin (1 mm^3 chunks collected from the periphery of excised benign tumours) and rabbit lip.

Incubation procedures. The substrates were incubated in Barski's tubes with whole serum, IgG, Fab and Fc fragments for 10 min, 30 min, 1 h, 2 h and 72 h at 4°C, room temperature and 37°C. Supernatants from each experiment were tested for the presence of ANAs.

IF studies. After incubation, the substrates were washed and snap-frozen. Human skin and rabbit lip were mounted in O.C.T. and sectioned at 4 μm in a cryostat.

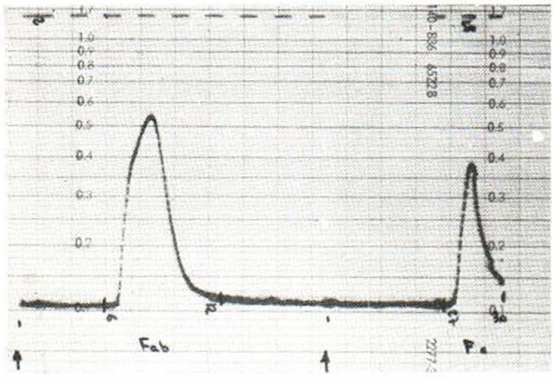

Fig. 1. Anti-RNP IgG fractionation after papain digestion in a DEAE-A 50 cellulose column. Fab isolation in phosphate buffer 0.002 M, pH 8; Fc isolation in phosphate buffer 0.3 M, pH 8.

Smears and tissue sections were then incubated for 30 min with fluorescein-conjugated antisera obtained from Behringwerke AG and our laboratory by conventional methods. We used goat anti-human Igs (dilution 1 : 100), rabbit anti-human IgG (dilution 1 : 30) and rabbit anti-IgG Fab and Fc fragments (fluorescein/protein ratio, μg/mg: 2.3). The sustrates were washed for 20 min in PBS, pH 7.2, and mounted in 0.1 M glycerin buffer. The same method was applied on prefixed cells (air drying for smears, acetone–alcohol fixation for tissues). Specimens were examined with a Leitz Orthoplan incident light fluorescence microscope using a xenon light source and BG38 and K510 filters. The percentage of FITC-labelled cells was established on a minimum of 100 cells.

RESULTS

Labellings were only obtained with anti-RNP IgG-Fab fragments from group I patient sera. They were first noticeable after 10 min incubation at 37°C, increased gradually and were optimal at 1 hr for each substrate used. The percentages of labelled nuclei were as follows (Group I): 18±7% on mononuclear cells (lymphocytes and macrophages), 42±11% on PHA-P transformed mononuclear cells, 86±5% on human skin and 79±3% on rabbit lip. The figures were very low on fibroblasts (2±0.5%). A sharp decline in ANA activity was noted in the supernatant of each group I serum when incubated with human skin and rabbit lip. An absence of nuclear labelling was found with IgG-Fab fragments in group II (non-RNP-ENA), group III (dsDNA/DNP) and group IV, as well as with IgG-Fc fragments in all groups, whatever substrate was used. The pattern of nuclear fluorescence was always speckled (particulate) (Fig. 3). A homogeneous pattern was observed only on prefixed cells with dsDNA/DNP IgG-Fab fragments. Membrane and intracytoplasmic fluorescence seldom occurred and was not correlated to intranuclear fluorescence. No evidence of basement membrane fluorescence was present in any of the incubation studies with human skin or rabbit lip. It was also found that the 4 subclasses of anti-RNP IgG were responsible for the nuclear binding and that IgM, IgA or C3 were not participating in the immune reaction. The viability of the cells, which is the most important point in establishing the significance of our findings, was assessed by Trypan Blue exclusion (death rate of the cells below 5% at 1 hr incubation) and by the absence of passive penetration of antibodies in group III and group IV patients' sera.

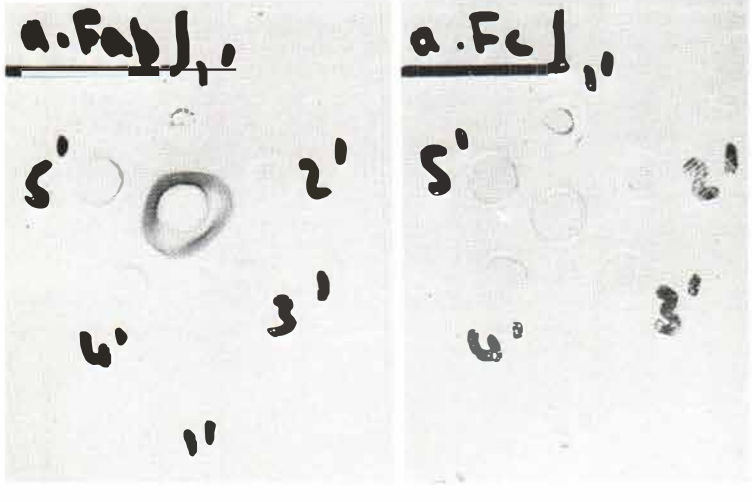

Fig. 2. Immunodiffusion study showing the reaction with an anti-human Fab serum. The precipitation line is continuous among the five sera from group I. No precipitation is observed with the Fc fragments.

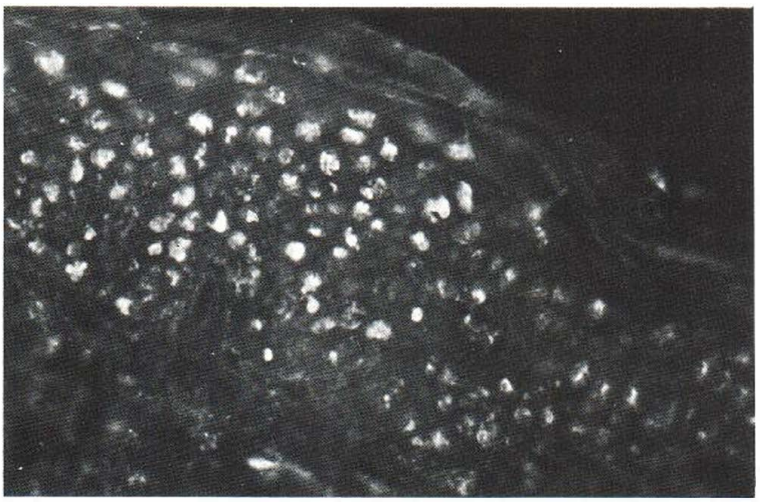

Fig. 3. Epidermal nuclear speckled fluorescence with anti-RNP IgG-Fab fragments (normal human skin, 1 h incubation).

COMMENT

Nuclear IgG deposition on DIF of clinically normal skin is a well-known phenomenon in systemic scleroderma and scleroderma-like features (3, 6, 8, 11, 12). It always correlates with high titre serum ANAs and especially with antibodies directed against a saline-soluble (extractable) nuclear antigen fraction that is known to have two distinct antigenic sites, RNP and Sm (10). Direct IF cannot differentiate between anti-RNP and anti-Sm antibodies (11).

The presence of Igs in viable nuclei is a puzzling biological phenomenon which has not so far been extensively studied. A penetration of Igs in nuclei has been demonstrated on organ culture of monkey skin by Shu and Beutner (13), on normal human skin specimens by Izuno (9) and on circulating mononuclear cells by Alarcon Segovia et al. (2). A passive penetration of high concentrations of circulating ANAs during the surgical procedure or when specimens are prepared for DIF has been suggested to explain this unique phenomenon (5). This hypothesis must be ruled out on the following grounds, however. When using similar techniques for processing the biopsies, positive reactions are only found with anti-ENA sera and never with anti-nDNA/DNP sera; negation of a previously positive reaction may occur in the same patient, depending on the specificity of the ANAs; complement deposits have been found, which means that a complete immune reaction may take place in vivo (3). We showed that the nuclear labelling of epidermal and non-epidermal cells was an in vivo phenomenon and that only anti-RNP IgG were involved in the reaction. Alarcon Segovia et al. (2) have suggested that the anti-RNP IgG may penetrate viable human mononuclear cells via their Fc receptors. Our study did not confirm these results, as an active penetration was shown with anti-RNP IgG Fab fragments both on mononuclear cells and on keratinocytes which are known to lack Fc membrane receptors. Tissues and cells incubated in identical conditions did not allow the penetration of non-anti RNP IgG-Fab fragments, whereas prefixed specimens did so. These data do not imply that anti-RNP IgG penetrate living cells, but suggest that our incubation procedures do not damage the cell membranes and do not allow the passive penetration of antibodies. Furthermore, Galoppin et al. (4) demonstrated the simultaneous uptake of tritiated thymidine, [^{14}C]leucine and anti-RNP IgG on trypsin isolated keratinocytes in vitro.

The mechanisms of penetration of IgG into the nucleus of viable cells remain speculative. Cell membrane permeability may be altered by inflammatory processes induced by circulating immune complexes, while antibodies to soluble proteins of nuclear origin may be markers of such complexes (15), but no direct evidence has been brought forward to support this hypothesis. Aging of the cells may permit IgG to reach the nucleus but it cannot explain the selective penetration of anti-RNP antibodies. Ultraviolet radiation was also found unable to elicit an in vivo epidermal immunoglobulin fixation among patients with high concentrations of circulating ANAs (3). The modification of the

cytoplasmic and/or nuclear membrane induced by PHA transformation on mononuclear cells was an enhancing factor in the penetration of ANAs and it may partly explain one of the steps in the pathway to the nucleus. High concentrations of highly diffusible antibodies against soluble fractions of ribonucleic material may be able to penetrate the cytoplasm and bind the antigen on the porous nuclear membrane without interfering with the protein synthesis mechanisms. This hypothesis may explain the absence of clinical alteration of the skin involved with the anti-RNP IgG interaction and the unique particulate fluorescence pattern.

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