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ULTRASTRUCTURAL LOCALIZATION AND CHARACTERIZATION OF A RIBOSOMAL ANTIBODY DETECTED BY IMMUNOFLUORESCENCE IN SYSTEMIC LUPUS ERYTHEMATOSUS

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SUMMARY

The ribosomal antibody detected by tissue immunofluorescence in about 1% of SLE patients was conjugated with peroxidase and its antigen localized by immuno-electronmicroscopy, using rat stomach as substrate. The antibody stained ribosomes on rough ER, single ribosomes and polyribosomes, but not membranes. Gastric chief cells reacted most intensely; ribosomes in plasma cells, lymphocytes and eosinophils seen between gastric cells were also positive, thus confirming earlier immunofluorescence studies which showed that all tissues react in relation to their ribosomal content. Nucleolar ribosomes were unreactive.

The ribosomal antigen was resistant to glutaraldehyde, formaldehyde, acetone, ether, ethanol, methanol and detergents such as deoxycholate. Digestion of the sections with RNase did not diminish the immunofluorescence. Trypsin could not be used on sections but is known from previous CFT studies to destroy the ribosomal antigen. It was concluded that this antigen is a ribosomal protein unlike other tissue autoantigens of which several studied so far are lipoprotein in nature.

INTRODUCTION

Antibodies detected by immunofluorescence (IFL) on tissue sections were found in a small proportion of patients with systemic lupus erythematosus (SLE). These antibodies were non-species-specific and reacted with all organs tested. The distribution of IFL staining in various tissues was related to the known content of ribosomes in each cell type. The ribosomal nature of the antigen was demonstrated by absorption of the IFL with cytoplasmic subfractions and by complement fixation with purified ribosomes. It was thought that the antigen is a ribosomal protein since ribosomal RNA was ineffective in absorbing out the

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fluorescence and gave negative results by CFT (Homberg, Rizzetto & Doniach, 1974). In the present paper we report the ultrastructural localization of the antibodies by the immunoperoxidase technique and further attempts to characterize the antigen biochemically.

MATERIALS AND METHODS

Standard sera

Serum from a 23-year-old female patient (SL) with systemic lupus erythematosus showing ribosomal IFL to a titre of 320 and CFT to 256, and from a 67-year-old female patient (Rou) with systemic sclerosis having ribosomal IFL to a titre of 40 and CFT to 16, were used for all experiments. An additional two sera were tested for precipitins in agar: An (F 23, SLE) and Jo (M 57, collagenosis).

Preparation of ribosomes

Rat liver microsomes were prepared as described in De Duve *et al.* (1955) and ribosomes were obtained after solubilization of the microsomal membranes with 0·5% deoxycholate (DOC) according to Arora & deLamirande (1968). Protein content of the subfractions was estimated by the method of Lowry *et al.* (1951).

Fixatives and enzyme treatments

To assess the effect of fixatives on the antigen, rat liver and other tissue sections were treated with 0·5% glutaraldehyde, 1% formaldehyde, acetone, ether, ethanol and methanol for 10 and 30 min at 4°C followed by a 30-min wash in phosphate-buffered saline (PBS) before testing by immunofluorescence. Rat pancreas was easier to cut in the cryostat after formaldehyde fixation. Fixed or unfixed sections were digested with RNase A (Sigma type 1) at a concentration of 0·5 and 1·0 mg/ml in PBS at room temperature for 1 or 2 hr, and tested by immunofluorescence. Digestion with trypsin (0·1 mg/ml) on tissue sections was attempted for 10 and 30 min.

Immunofluorescence and precipitin tests

The sandwich technique was carried out on composite blocks of human thyroid and stomach, and rat liver and kidney, with added sections of rat pancreas. Anti-γ and anti-IgG–FITC conjugates were used. The Ouchterlony test was done in 1% agar at pH 8·6 using a concentrated suspension of ribosomes containing approximately 1 mg protein/ml. The serum and antigen wells were refilled once at 12 hr.

Immunoperoxidase staining and preparation for electron microscopy (EM) IgG–peroxidase conjugates. The standard serum SL was chromatographed on a DEAE column equilibrated with 20 mM phosphate buffer (pH 6·5) and the IgG fraction collected. After dialysis against 0·1 M phosphate buffer (pH 6·8) 5 mg of IgG were mixed with 15 mg of peroxidase (Avrameas & Terninck, 1971) and conjugation was effected by stirring with 0·05 ml of aqueous 1% glutaraldehyde for 2 hr at room temperature (Avrameas, 1969). The mixture was then dialysed overnight against PBS at 4°C and spun at 25,000 *g* to remove precipitates. The peroxidase (Px) conjugate of ribosomal antibody (Px-R) could be stored at 4°C for at least 3 months without loss of activity. Normal IgG was prepared and similarly conjugated (Px-N).

Fixation. The effects of fixation on antigenic and morphological preservation were

investigated with 1% and 2% formaldehyde (freshly prepared from paraformaldehyde) in 0·1 M phosphate buffer (pH 7·4) containing 0·25 M sucrose. Blocks were fixed for 40 min at 40°C. At light microscopy level 5 μm cryostat sections were treated with 1 : 10 dilutions of Px-R and Px-N applied to sections of fixed and unfixed blocks.

Tissue. For EM stomach was selected as a suitable substrate in view of the high antigen content in the chief cells of gastric mucosa, as shown by previous immunofluorescence studies (Homberg *et al.*, 1974). Strips of stomach wall 1 × 0·5 cm were placed in fixative for 2 min and the muscle layer was then stripped to facilitate penetration. After a further 38 min in fixative and a 1-hr wash in sucrose phosphate buffer, the strips were snap-frozen in isopentane with a solid CO_2–acetone mixture. Thick cryostat sections (20 μm) were then cut and processed for electron microscopy as described by Bianchi, Penfold & Roitt (1973). Experiments were made to select the optimum period of incubation with the peroxidase conjugates. For this, several thick sections were immersed in 0·5 ml of undiluted conjugate and incubated at room temperature for 30, 60 and 90 min respectively.

0·5 μm sections were viewed in the light microscope with and without Toluidine Blue counterstain. Araldite-embedded thin sections were examined unstained on supported grids with a JEOL 100 B and an AEL EM 6B electron microscope.

TABLE 1. Ribosome–related serology in four patients

Case	S/A	Diagnosis	Ribosomal			DNA	RNA
			Precipitin	IFL	CFT*	CFT	CFT
An	F22	SLE	+	100†	256	Negative	Negative
SL	F23	SLE	++	320	256	16‡	Negative
Rou	F67	Systemic sclerosis	++	40	64	Negative	Negative
Jo	M57	Collagenosis	Negative	80	Negative	Negative	Negative

* CFT = complement fixation test, microtitre technique.
† Numbers indicate reciprocal of maximum serum dilution reacting in test.
‡ Related to co-existing nuclear antibodies. For details compare with Homberg *et al.* (1974).

RESULTS

Effect of fixation

Fixation with glutaraldehyde, formaldehyde, acetone, ether, ethanol and methanol did not diminish the ribosomal immunofluorescence of sera Le and Rou in any of the organs tested.

Digestion with RNase and trypsin

Tissue sections treated with RNase reacted with the ribosomal antibodies, showing no decrease in the intensity of IFL. Trypsin digested the sections, breaking them into fragments which floated off on washing and made IFL testing impossible.

Precipitins

Three of the four sera tested showed positive precipitins (SL, Rou, An) against rat liver

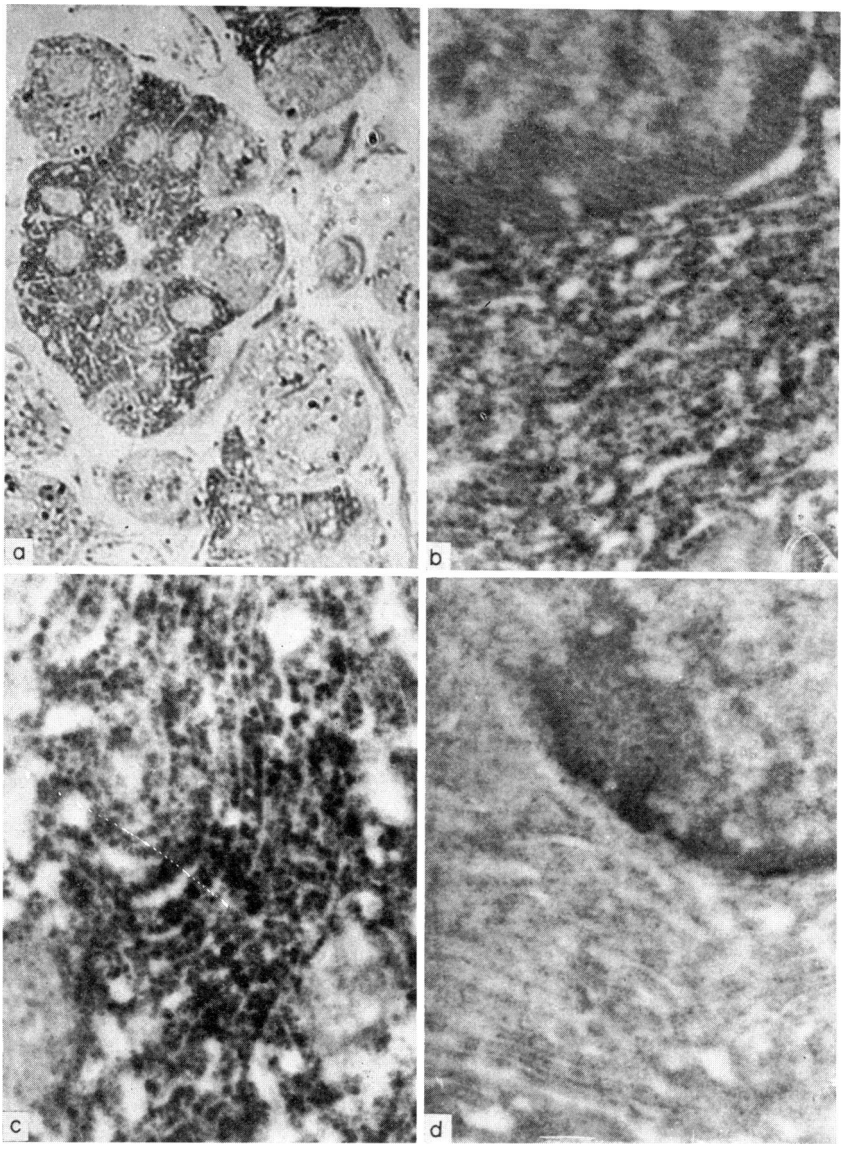

FIG. 1. Rat stomach. (a), (b) and (c) Px-Ribo IgG. (d) Px-normal IgG. (a) Light micrograph of a 0·5 μm section; no counterstain; cross-section of glands. The cytoplasm in the chief cells is positive, that in the parietal cells is negative. (Magnification $\times$ 400.) (b) Chief cell. The nucleus (upper part) is negative. Granular stain of the rough ER in the position of the ribosomes. (Magnification $\times$ 28,000.) (c) Same distribution as (b) of the stain in the cytoplasm of a chief cell. (Magnification $\times$ 32,000.) (d) Unstained ribosomes in gastric chief cell. The nucleus is to be seen in top right hand corner.

ribosomes. Two lines were obtained with each serum. One compact line near the antigen well which showed complete identity for the three sera and a second more diffuse short thick band situated nearer the serum well which did not merge with the lines of adjacent wells, although they looked similar for the three positive sera. Comparison of precipitin results with other reactions obtained with these sera are shown in Table 1.

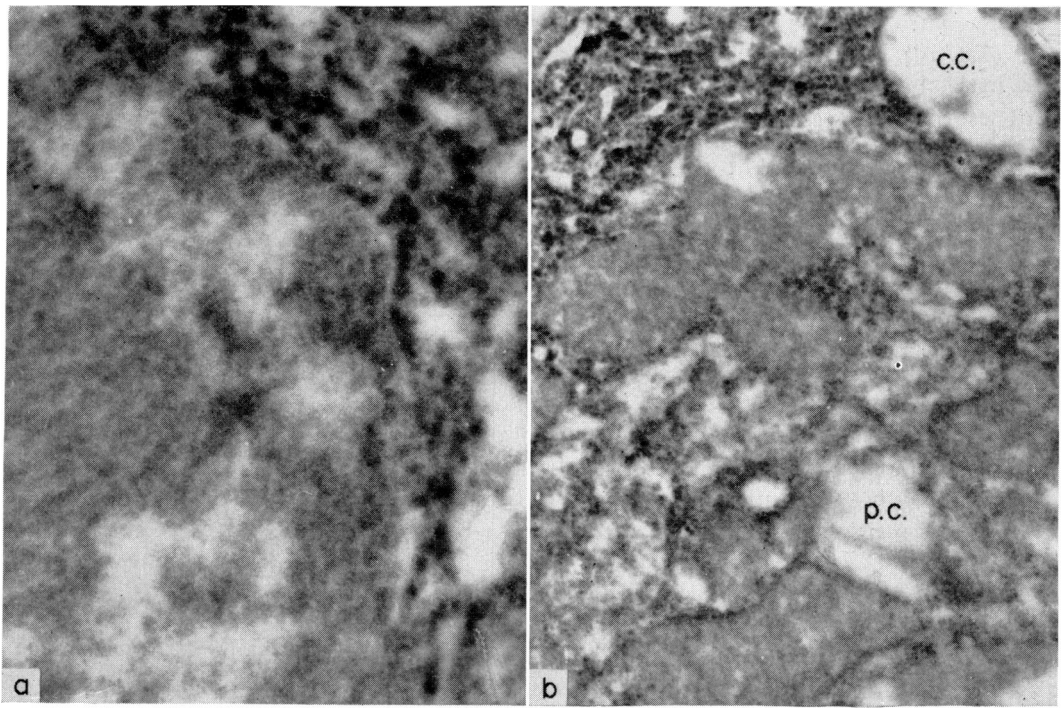

FIG. 2. Rat stomach. Px-Ribo IgG. (a) Positive ribosomes attached to the outer membrane of the nucleus. (Magnification ×49,000.) (b) c.c. = chief cell; p.c. = parietal cell. In the latter scanty positive ribosomes are seen scattered among the mitochondria. (Magnification ×20,000.)

Immunoelectronmicroscopy

Fixation in 1 and 2% formaldehyde gave the same results with respect to antigen preservation and morphology. The shorter incubation time of 30 min with Px conjugate gave the best preservation of ultrastructural morphology without significant loss of penetration into the 20 μm blocks.

At light microscopy level with 0·5 μm sections, cells in the topographical position of the chief cells showed diffuse staining of the cytoplasm (Fig. 1a). Parietal cells were negative except for occasional peroxisome granules. Cell nuclei were consistently unstained.

At ultrastructural level the chief cells showed rows of discrete stained granules in the position of rough endoplasmic reticulum with no apparent membranous structures supporting them (Fig. 1b, c). Identical rows of granules were seen in the position of the outer nuclear membrane (Fig. 2a). The other organelles were negative. In the parietal cells there were

scanty positive granules among the mitochondria representing ribosomes or rough ER (Fig. 2b). The nucleoli of both chief and parietal cells were negative. Plasma cells, lymphocytes and eosinophils were seen in the blocks examined. The plasma cells were strongly positive with the same rows of stained ribosomes as in chief cells. Lymphocytes had polysomes and many single ribosomes which were fully reactive. Eosinophils showed scanty black dots, among their large specific granules. Mucous neck cells were not examined owing to the selection which is necessary at EM level. In sections treated with Px-N conjugate and in controls for endogenous peroxidase, ribosomes were faintly visible along the rough ER (Fig. 1d). Moderate endogenous Px activity was confined to some of the lysosomes in the parietal cells.

These observations strongly suggest that the antigen is localized exclusively in the cytoplasmic ribosomes, while the membranes of the endoplasmic reticulum appear to be non-reactive.

DISCUSSION

The electron microscopic appearances of tissue treated with the peroxidase conjugate of an SLE serum showed clear-cut staining of all cytoplasmic ribosomes, including those on rough ER, and single ribosomes or polyribosomes, thus confirming the findings with immunofluorescence. Gastric chief cells showed uniform staining at light microscopy and at EM levels. Parietal cells appeared negative by fluorescence but contained reactive ribosomes which could not be seen by IFL owing to their low concentration in these cells. Other tissues also react with these antibodies as confirmed by EM in plasma cells, lymphocytes and eosinophils seen among the gastric glands, and which all combined with the peroxidase-conjugated IgG in proportion to their ribosome content. The antibody is non-species-specific and it is now confirmed that all cytoplasmic ribosomes react with it. Nucleoli were negative in all cell types examined, suggesting that their ribosomes have not yet acquired the antigen. EM appearances agreed with previous complement fixation studies where DOC-purified ribosomes proved the most highly reactive subfraction, microsomes reacted to a lesser extent and ribosomal RNA gave negative CFT results (Homberg *et al.*, 1974).

The absence of visible staining of the supporting membranes in the rough ER of chief cells was striking with the ribosomal antibodies and contrasts with the appearances seen when the liver/kidney microsomal (LKM) antibodies found in patients with liver disease (Rizzetto, Swana & Doniach, 1973) were similarly applied. LKM antibodies stained both the ribosomes and the membranes of rough ER and fixed complement with both smooth and rough membrane subfractions, but not with isolated ribosomes (Rizzetto, Bianchi & Doniach, 1974).

In this context it is of interest to mention that localization by immunoelectronmicroscopy has also been done with the mitochondrial antibodies found in primary biliary cirrhosis (Bianchi *et al.*, 1973). In all these studies it was essential to use a serum with high titre antibodies and to make a direct peroxidase conjugate of the isolated IgG fraction of each patient.

The ribosomal antigen is resistant to strong fixatives such as 0·5% glutaraldehyde and 2% formaldehyde and appears to be equally resistant to lipid solvents including acetone alcohol and ether as well as detergents such as deoxycholate. RNase digestion of the sections did not diminish the fluorescence. In previous CFT studies, there was a slight diminution

of antigen activity after RNase treatment of liver ribosomes, but this was probably due to non-specific denaturation of proteins by excess enzyme. Trypsin digestion could not be done on sections but in previous CFT studies, it was shown that this enzyme destroyed the ribosomal antigen. These studies taken together suggest that the antigen is a protein rather than a lipoprotein.

Some of the other cytoplasmic tissue autoantigens studied so far have been lipoproteins. Thus the LKM antigen mentioned earlier, and the organ-specific microsomal antigens reacting in the thyroid-gastric-adrenal group of autoimmune disorders are all sensitive to lipid solvents and detergents as well as to proteolytic enzymes (Roitt *et al.*, 1964; Baur, Roitt & Doniach, 1965; Goudie *et al.*, 1968). The mitochondrial antigen is also a lipoprotein (Berg *et al.*, 1969; Ben-Yoseph, Shapira & Doniach, 1974). Another interesting lipoprotein is the liver-specific membrane antigen (Hopf *et al.*, 1974).

The ribosomal fluorescent antibody has only been found so far in seven patients, of whom five had classical SLE and two had related collagenoses. It appears to be distinct from the ribosomal precipitins which have been described by several authors (Sturgill & Prebble, 1967; Schur, Moroz & Kunkel, 1967) and were also present in our SLE cases. However precipitins and ribosomal agglutinins can be demonstrated in up to 50% of SLE cases, while ribosomal fluorescence is found in less than 1%. Furthermore the antigen in previous studies was thought to be an RNA–protein complex, whereas the present antigen appears to be independent of ribosomal RNA. The pathogenic significance of these antibodies is unknown but nearly all the patients showing this fluorescence have had renal involvement and three died within 2–7 years of onset.

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