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Anti-golgi complex autoantibodies in a patient with Sjögren's syndrome and lymphoma

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SUMMARY

During routine immunofluorescence studies of the serum of a patient with Sjögren's syndrome and lymphoma we detected antibodies giving a cytoplasmic pattern which did not correspond to previously described patterns found for autoantibodies. Using different cells and tissues as substrates for indirect immunofluorescence, including rat liver, rat small bowel, rat testicle, human thyroid, guinea-pig plasma cells and cultured human fibroblasts, the cytoplasmic structure to which these autoantibodies are directed seems to be the golgi complex, a conclusion supported by histochemical studies. Furthermore, these antibodies were absorbed by isolated golgi vesicles. The autoantibodies are of IgG and IgA classes, and the antigen(s) with which they react is(are) resistant to treatment with DNAase and RNAase. None of the sera from 50 normal individuals, seven patients with Sjögren's syndrome (five of them primary and two associated with rheumatoid arthritis; none of them with lymphoma), 25 patients with mixed connective tissue disease, 10 patients with systemic lupus erythematosus and five patients with progressive systemic sclerosis, had antibodies directed against this cytoplasmic specificity, as determined by indirect immunofluorescence. This is the first time that autoantibodies directed to the golgi complex are reported. The significance of this finding awaits further descriptions in patients with a clinical picture similar to the one reported here.

INTRODUCTION

A wide variety of autoantibodies directed to antigens present in the nucleus, cytoplasm and plasma membrane of cells in different tissues have been described. A number of these antibodies have been found to be of value in the diagnosis and follow-up of some diseases in which several other immunological parameters are altered. It has been suggested that a disregulation of immune homeostasis is a most important event in the pathogenesis of diseases such as systemic lupus erythematosus (Decker, 1979), myasthenia gravis (Lisak *et al.*, 1977), autoimmune thyroid disease (Doniach & Marshal, 1977), this disregulation being presumably responsible for the appearance of the autoantibodies. Several roles have been adscribed to them in these and other clinical pictures: (i) a direct role in pathogenesis, as in anti-receptor autoimmune diseases (Doniach & Marshal, 1977; Mackay & Carnegie, 1977); (ii) as components of immune complexes, which could produce vascular inflammatory lesions through the activation of complement (Dixon, Feldman & Vazquez, 1961); (iii) some autoantibodies directed against antigens on the surface of lymphocytes could increase the disregulation of the immune system (Morimoto *et al.*, 1980); (iv) as agents capable of interfering with the function of cells into which they would penetrate and react with nuclear structures (Alarcon-Segovia, Ruiz-Arguelles & Fishbein, 1978). New roles will possibly be added to those

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mentioned above. On the other hand, some autoantibodies have been considered just as epiphenomena secondary to tissue damage (Liem *et al.*, 1979), or to injury induced by chemical or biological agents (Vergani *et al.*, 1980; Mead, Cowin & Whitehouse, 1980).

In the present paper we report the finding of autoantibodies which detect a previously undescribed cytoplasmic specificity, in a patient with Sjögren's syndrome and lymphoma. By morphological, immunofluorescent and absorption studies we have identified this structure as the golgi complex.

CASE REPORT

The patient is a 38 year old woman with a past history of recurrent parotiditis with a pathological diagnosis of 'non-specific chronic syaloadenitis'. She was admitted to our hospital because of dry mouth and eyes, fever, nausea vomiting and bilateral subcostal discomfort. Physical examination revealed a pale patient and enlarged liver and spleen. Initial laboratory data included: 5,300 WBC/μl (7% bands, 58% neutrophils, 23% lymphocytes, 12% monocytes), Hb 9 g/l, ESR (Westergreen) 130 mm/hr, MCV 66 fl, MCH 23 pg, MCHC 33·6%, serum iron 3 μmol/l, iron binding capacity 38 μmol/l with a saturation index of 8%, platelets 550,000/μl, total serum protein 85 g/l with hypoalbuminaemia and hypergammaglobulinaemia (38·2 g/l). There was a mild increase in serum AST and LDH, and moderate increases in gammaglutamyl transpeptidase and alkaline phosphatase. Chest X-ray was unrevealing. A ^{99}Tc liver and spleen scan showed a diffusely enlarged liver and a cold image in the spleen which corresponded to a nodule uptaking ^{67}Ga. The abdominal bidimensional echography showed a splenic nodule with a diameter of 6·1 cm.

Needle biopsy of the liver revealed mononuclear cell infiltrates in portal spaces, dilation of sinuses and luminal invasion by mononuclear cells. Bone marrow biopsy showed slight erythroblastic hyperplasia. Lip mucosal biopsy was suggestive of Sjögren's syndrome.

One month after admission the patient underwent splenectomy. A compact central area of 9 cm diameter was seen at macroscopic examination, which was histologically diagnosed as poorly differentiated histiocytic lymphoma. Cytochemical studies of the tumour cells were positive for alkaline phosphatase and acid phosphatase stainings. Diagnoses on discharge were Sjögren's syndrome and histiocytic lymphoma, stage II_s and treatment was established with a CHOP schedule.

Immunological findings before splenectomy were: IgG 39·3 g/l, IgA 5·8 g/l, IgM 1·5 g/l; total haemolytic complement, C1q, C4 and C3 were within normal limits. Circulating immune complexes (CIC) were 2·0 g/l (normal $<1·7$). Anti-thyroglobulin and anti-2nd component of colloid antibodies were weakly positive. Smooth muscle antibodies were positive at a titre of 1/20, and anti-nuclear antibodies were positive at a titre of 1/160 with a speckled pattern. Anti-microsomal, anti-epithelial, anti-skeletal muscle, anti-parietal gastric cell, anti-salivary duct cell, anti-dsDNA and anti-ssDNA antibodies were negative. Anti-ENA antibodies were negative by haemagglutination but a precipitation line was detected by immunodiffusion. By immunodiffusion the ANA were shown to be anti-SS-B. Waaler–Rose test for rheumatoid factor was positive with a titre of 1/128. Surface markers for peripheral blood lymphocytes: active E rosettes 50% (364/μl), E rosettes 78% (599/μl), EAC3b rosettes 8% (61/μl), and surface Ig positive cells 10% (75/μl).

After splenectomy the findings were: IgG 47 g/l, IgA 5·8 g/l, IgM 1·4 g/l, CIC 2·7 g/l. Results of autoantibody tests were the same as before splenectomy except for an anti-nuclear antibody titre of 1/640, with a speckled pattern. Surface marker in non-tumoural spleen revealed: active E rosettes 48%, E rosettes 49%, EAC3b rosettes 33%, and sIg-positive cells 40%. None of these surface markers were present on tumoural spleen cells. Sixty-five percent of cells from the tumoural mass had intracytoplasmic μ and κ chains, as detected by direct immunofluorescence.

MATERIALS AND METHODS

Indirect immunofluorescence (IIF). IIF was performed on 4 μm frozen sections of rat liver, rat small bowel, human thyroid, imprints of rat testicle, rat liver, guinea-pig lymph nodes and on slide

cultured human fibroblasts. Preparations were fixed in acetone for 3 min before incubation with antisera. Human fibroblasts were fixed with methanol–acetic acid at −20°C for 5 min. Commercial FITC-conjugated rabbit anti-human gammaglobulin (Dako) and rabbit anti-human IgG, IgA and IgM (Behringwerke) were used at 1/30 dilutions in phosphate-buffered saline (PBS). The preparations were seen under a Leitz Dialuz 20 fluorescence microscope.

Enzymatic treatments. Acetone-fixed tissue sections and imprints were treated with DNAase and RNAase (Sigma) at a concentration of 4 mg/100 ml in saline solution at pH 7, containing 0·4 mM magnesium chloride, at 37°C for 60 min (Sharp *et al.*, 1972). After washing with PBS the sections were processed as above for indirect immunofluorescence.

Cytochemical studies. Haematoxylin-eosin, acid phosphatase and methenamide silver stainings were performed according to standard techniques. In some silver stain preparations a haematoxylin-eosin counterstain was used.

Electron microscopy. Rat testicle was fixed in Zamboni's fluid and processed for a standard osmium tetraoxide staining.

Crude antigen extract. Rat testicle was teased in Hank's balanced salt solution (HBSS) and centrifuged at 500 ***g*** for 15 min at room temperature. After removing the supernatant, the pellet was resuspended in 0·1 mM phenylmethylsulphonyl fluoride (PMSF, Sigma) in 0·15 M NaCl, ultrasonicated six-fold for 15 sec (Ultrasound Heat System, setting at 4), and extracted overnight at 4°C with continuous stirring.

Immunodiffusion. Crude rat testicle extract was used for immunodiffusion in 0·5% agarose plates. In some experiments 0·5 ml of the crude extract was incubated with 0·3 mg of RNAase for 1 hr at 37°C (enzyme to substrate ratio = 1:5, w/v).

Isolation of golgi vesicles. Golgi vesicles were isolated from rat testicle, according to the following procedure. Rat testicles are freed of albuginea and grossly visible blood vessels, and are teased in cold PBS. The cell suspension is centrifuged at 200 ***g*** for 10 min, and the resulting pellet is resuspended in 0·1 mM PMSF in PBS, homogenized with a manual Potter homogenizer, filtered through a cotton gauze, and centrifuged over a discontinuous sucrose density gradient (20–60%) at 900 ***g*** for 30 min. Golgi vesicles are enriched in the 30–40% fraction of the gradient, as controlled by morphology and the enzymatic marker thiamine pyrophosphatase, together with contaminating rough endoplasmic reticulum and spermatozoid tails. The golgi enriched fraction is resuspended in PBS and centrifuged at 200 ***g*** for 10 min, and the supernatant containing the vesicles is centrifuged at 1300 ***g*** for 20 min. The resulting pellet was used as isolated golgi vesicles for absorption studies (see below).

Absorption tests. One volume of a 1:10 dilution of the serum in PBS was mixed with 5 vol of the isolated golgi vesicle suspension for 1 hr at room temperature and overnight at 4°C. The mixture was then centrifuged at 1900 ***g*** for 20 min at 4°C and the supernatant was used for indirect immunofluorescence as described above.

Other immunological methods. IgG, IgA, IgM, C1q, C4 and C3 were determined by radial immunodiffusion. Haemolytic complement activity was measured as described by Mayer (1961). CIC were detected by 3·5% PEG precipitation and reading at 280 nm (Digeon *et al.*, 1977). Anti-ds- and ss-DNA antibodies were determined by a Farr assay using a commercial kit obtained from Amersham. Anti-ENA antibodies were detected by passive haemagglutination using calf thymus extract, as described by Sharp *et al.* (1972). Immunodiffusion studies for anti-ENA were done using the same extract in 0·5% agarose plates. Reference sera for specificity of several antinuclear antibodies were kindly provided by Dr E. M. Tan, of the University of Colorado School of Medicine, Denver, Colorado.

Active E rosettes (Wybran & Fudenberg, 1973) and EAC3b rosettes (Jondal, Holm & Wigzell, 1972), were done according to standard methods. E rosettes were performed after neuraminidase treatment of sheep red blood cells (Weiner, Bianco & Nussenzweig, 1973). Surface and cytoplasmic immunoglobulins were detected by direct immunofluorescence using FITC-labelled goat anti-human γ globulin (Hyland), and rabbit anti-human κ and λ chains (Behringwerke).

RESULTS

Indirect immunofluorescence studies

During routine studies for the detection of antinuclear antibodies in the serum of our patient using rat liver as a substrate we observed the staining of a cytoplasmic structure in a pattern not previously described for autoantibodies. In rat liver frozen sections and imprints we found a speckled cytoplasmic staining which partially surrounded the nucleus (Fig. 1); this pattern was found in all cells. When we used rat small bowel sections we could see a fluorescent structure located between the nucleus and the apical pole of the cells (Fig. 2), an image very similar to what was observed with human thyroid frozen sections.

IIF on rat testicle imprints yielded staining of a structure which in spermatids had a round shape with a paranuclear localization, and which at higher dilutions of the serum showed a hollow image (Fig. 3). Plasma cells from hyperimmunized guinea-pig lymph nodes also showed a fluorescent image located close to the nucleus. The pattern seen when using methanol–acetic acid fixed cultured human fibroblasts was clearly suggestive of the pattern expected for the golgi complex (Fig. 4).

This fluorescence was detected up to a titre of 1:1,280. None of the sera from seven other patients with Sjögren's syndrome (five of them primary and two associated with rheumatoid arthritis; none of them with lymphoma), 10 patients with systemic lupus erythematosus, 25 patients with mixed connective tissue disease and five with progressive systemic slerosis, gave such an image when using different substrates.

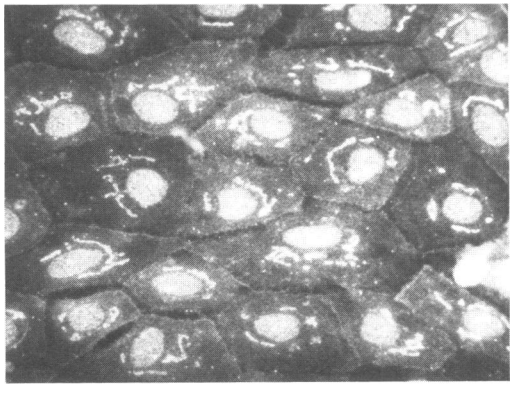

Fig. 1. Indirect immunofluorescence on rat liver imprint (× 300). A speckled fluorescence staining can be seen in the cytoplasm, partially surrounding the nucleus. A speckled nuclear pattern is also evident.

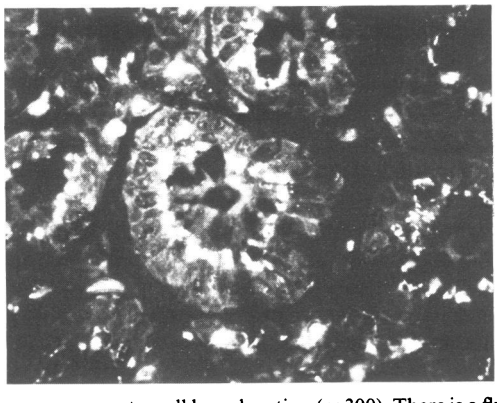

Fig. 2. Indirect immunofluorescence on rat small bowel section (× 300). There is a fluorescent staining structure located in the apical pole of the cells.

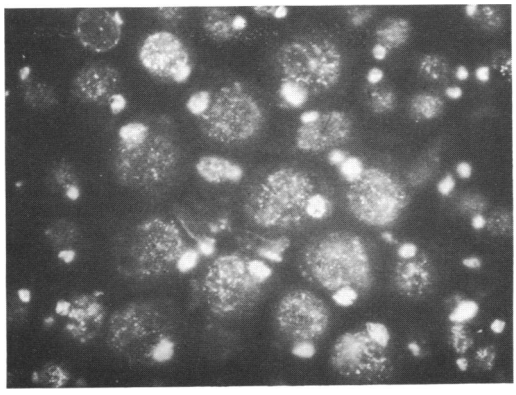

Fig. 3. Indirect immunofluorescence on rat testicle imprints (× 300). A fluorescent staining is seen at one pole of the cytoplasm of early spermatids, located close to the nucleus.

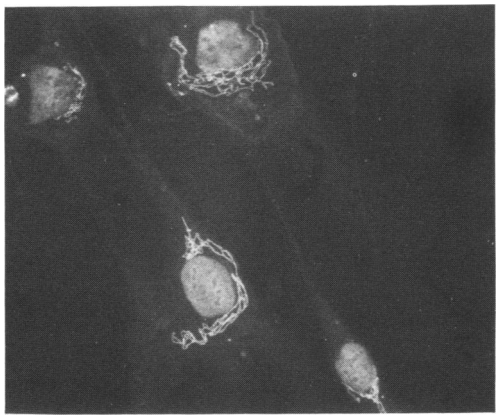

Fig. 4. Indirect immunofluorescence on slide-cultured human fibroblasts (× 300). The pattern is strongly suggestive of golgi apparatus vesicles

Using anti-class specific antisera we could detect this cytoplasmic image with anti-IgG and anti-IgA but not anti-IgM. After treatment with pepsin of the IgG fractionated from the serum of our patient (Stanworth & Turner, 1978), the resulting $F(ab')_2$ fragments gave the same results as the whole serum, thus confirming that the attachment of the antibody was not dependent on the Fc fragment.

Enzymatic treatments

After treatment of tissue preparations with RNAase we were no longer able to detect the nuclear fluorescence, while the cytoplasmic staining was left unaltered. Neither the nuclear nor the cytoplasmic antigen(s) were affected by treatment with DNAase.

Cytochemical studies

Haematoxylin-eosin stain is known to exclude the golgi complex from staining. In the preparations used in this study the area excluded from H-E staining corresponded to the cytoplasmic structure showing positive fluorescence as described above. We next used the acid phosphatase staining, which can detect several intracytoplasmic vesicular structures, among which the golgi complex. All tissues showed a pattern which corresponded to the one seen with indirect immunofluorescence. The third stain used was the methenamide silver technique, which allows detection of most

membranous cytoplasmic structures. By this method we observed a cytoplasmic organella equivalent in location and morphology to what could be seen by indirect immunofluorescence (Fig. 5).

Electron microscopy

Electron microscopy was performed only on rat testicle since we found that this was one of the tissues which most clearly showed the vesicular nature of the cytoplasmic structure to which these antibodies were detected. As can be seen in Fig. 6, the golgi complex in an early spermatid is located precisely where the stain was seen by indirect immunofluorescence in the same tissue.

Absorption tests

The patient's serum was absorbed with isolated golgi vesicles, as explained in the methods section, and used for indirect immunofluorescence on different substrates. After absorption the intracytoplasmic pattern described above dissapeared completely, while the nuclear pattern remained unchanged.

Immunodiffusion with crude antigen extract

When crude antigen extract from rat testicle was allowed to diffuse against the patient's serum, two precipitation lines were obtained. After treatment of the extract with RNAase only one

Fig. 5. Methenamide silver stain of a rat testicle imprint (× 300). In the paranuclear area corresponding to the one seen in Fig. 3, a structure can be seen which stains for silver and which corresponds to the golgi complex.

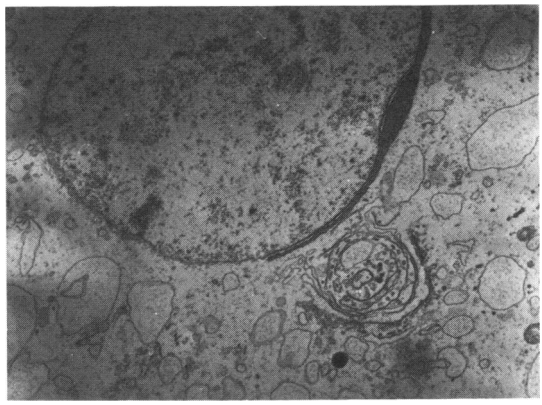

Fig. 6. Electron micrograph of a spermatid from rat testicle (× 300). The golgi complex can be seen close to the nucleus, in a location which corresponds to the fluorescence seen in Fig. 3, and to the silver staining structure of Fig. 4.

precipitation line remained. Since the nuclear antigen to which this patient had antibodies are RNAase sensitive, the remaining precipitation line could correspond to the cytoplasmic structure seen by indirect immunofluorescence.

DISCUSSION

All the evidence presented above shows that we have found autoantibodies directed to antigenic determinants located in the golgi complex. The pattern observed differs from all previously described patterns, this being to our knowledge the first time that autoantibodies with such a specificity are reported.

The antigen(s) involved in the reaction has been partially characterized as being RNAase and DNAase resistant. This feature distinguishes the specificity being detected from other previously described cytoplasmic antigenic systems to which autoantibodies are found (Niyawaki *et al.*, 1978; Mattioli & Reichlin, 1974).

Several immunological abnormalities were found in our patient, such as high γ globulin levels, CIC and presence of antinuclear antibodies and rheumatoid factor in serum. Sjögren's syndrome is related to autoimmune aggression to salivary glands (Moutsopoulos *et al.*, 1980), and it is frequently associated with diseases with autoimmune basis, especially systemic lupus erythematosus and rheumatoid arthritis (Moutsopoulos *et al.*, 1980). Since our patient did not have features of either of these diseases, the diagnosis was of 'primary' Sjögren's syndrome.

In addition, a histiocytic lymphoma was found in our patient. The high incidence of non-Hodgkin's lymphomas in patients with Sjögren's syndrome is well known (Kassan *et al.*, 1978), and it has been suggested that autoimmune disorders and chronic immune stimulation can predispose to lymphoid malignancies (Weisenburger *et al.*, 1977). Early reports linking Sjögren's syndrome to lymphoma showed a predominance of histiocytic lymphomas (Talal & Bunim, 1964), but recent studies using surface markers strongly support the B cell origin of most 'histiocyte-appearing' cells in this disease (Zulman, Jaffe & Talal, 1978). This seems to be the case in our patient, with the finding of μ and κ chains in the cytoplasm of the neoplastic cells from the splenic tumoural mass. Monoclonal serum proteins can be associated with this type of neoplastic proliferation, most frequently being μ and κ chains (Zulman *et al.*, 1978), although this was not the case in our patients. Indirect evidence suggests that the tumour cells were probably not responsible for the production of the autoantibody directed to the golgi complex found in the patient's serum, since the autoantibodies were found to be of IgG and IgA classes but not IgM.

We do not know whether the presence of this autoantibody might interfere with the function of the golgi complex. If so, it would be very interesting to find out whether this antibody can be active within the cells producing them, that is the plasma cells. This situation would be similar to what has been found in rheumatoid factor-producing plasma cells, where antibody reacts with antigen before being secreted (Munthe & Natvig, 1972). In any case, these antibodies can represent a very interesting tool in the study of the function and structure of the golgi vesicles.

Very recently, Louvard, Reggio & Warren (1982) have prepared a heteroantiserum that seems to detect antigens restricted to golgi vesicles with a molecular weight of 135,000 daltons, and Lin & Queally (1982) have produced a monoclonal antibody which reacts with antigens of a molecular weight of 110,000 daltons also located in the golgi apparatus. Further studies are now in progress in our laboratory for the definitive characterization of the antigen(s) involved in the specificity described here.

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