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Sialidase activity and antibodies to sialidase-treated autologous erythrocytes in bronchoalveolar lavages from patients with idiopathic pulmonary fibrosis or sarcoidosis

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SUMMARY

Sialidases catalyse the hydrolysis of terminal sialic acid of the carbohydrate moiety of glycoconjugates. Sialic acids play a key role in the expression or masking of antigenic sites and in cell–cell interactions. As an example, removal of sialic acid from the human erythrocyte membrane unmasks underlying molecules such as the specific carbohydrates (Gal-GalNac) of the so-called T or Thomsen–Friedenreich cryptic antigen. A consequence of this, is the recognition of that antigen by natural serum antibodies. Since the T antigen has been shown to be present in the lung, we have investigated the possible presence of sialidase and of specific antibodies to sialidase-treated cells in bronchoalveolar lavage fluids (BALF) from patients with pulmonary sarcoidosis or idiopathic pulmonary fibrosis (IPF). By using a fluorogenic substrate (4-methyl umbelliferyl-α-D-N-acetyl sodium neuraminate), we were able to detect a sialidase activity in BALF from eight out of nine patients with IPF and from ten out of thirty-five patients with sarcoidosis. BALF from normal volunteers and serum from both patients and normal volunteers were devoid of activity. BALF sialidase has an optimum pH activity of 5·4, it is not inhibited by EDTA and has a molecular weight close to 21 kD. BALF anti-T antibodies (galactose specific) were detectable in minute amounts in only one out of the nine normal volunteers. By contrast, they were frequently present in BALF from sarcoidosis (77%) or IPF (66%) patients and sarcoidosis patients had a higher mean activity. No correlation was observed between the enzymatic and antibody activities.

Keywords sarcoidosis bronchoalveolar lavages sialidase carbohydrates autoantibodies

INTRODUCTION

Sarcoidosis and idiopathic pulmonary fibrosis (IPF) are two interstitial lung diseases of unknown cause. They are associated with numerous immunological abnormalities and both cell-mediated and humoral systemic immunity are affected (Crystal *et al.*, 1984; Thomas & Hunninghake, 1987). With the use of the bronchoalveolar lavage technique immunological abnormalities were also demonstrated locally in the lungs (Daniele *et al.*, 1985). Most of them are associated with dysfunctions of immunocompetent cells which are accompanied by, and are possibly due to, alterations of cell membrane receptors. Indeed, membrane signal molecules, as part of receptors or acceptor sites, represent the major means by which cells are able to collaborate and interact with other cells or with soluble mediators. Among these signals, membrane glycosylation represents one of the most powerful systems (Stanley, 1987). This information-bearing role of the carbohydrate moiety of membrane glycoconjugates has been shown to be of primary importance for immunocompetent cells (Hooghe & Pink, 1985; Akasaki, Jardieu & Ishizaka, 1986). Due to their location at the extremity of the carbohydrate moiety, sialic acids play a crucial role in these mechanisms (Nakano *et al.*, 1980; Schauer, 1985; Landolfi & Cook, 1986; Miller *et al.*, 1986). Sialidase-induced membrane desialylation results in profound modifications of lymphocyte functions (Frohman & Cowing, 1985), sometimes leading to tissular alterations (Kolb-Bachofen & Kolb, 1979). Autoimmune phenomena, some of them resembling those observed during sarcoidosis, have been shown in mice treated with natural antibodies to saccharides such as galactose (Nakanishi *et al.*, 1982). As this saccharide is located immediately below sialic acid residues in the carbohydrate moiety of glycoconjugates, it is unmasked after sialidase treatment. This shows that, besides being able to recognize and to react with desialylated cell surface (Springer *et al.*, 1979; Bray, Lemieux & McPherson, 1981), anti-galactose natural auto-antibodies may modify the normal cellular metabolism. As this could be the case

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for epithelial and immunocompetent cells in the lung of patients with pulmonary sarcoidosis or IPF, we looked for the possible simultaneous presence of sialidase and of autoantibodies to desialated cell surface in bronchoalveolar lavage fluids recovered from these patients.

MATERIALS AND METHODS

Study population

The study concerned 53 subjects: nine healthy volunteers and 44 out-patients, divided into three groups: 1. Pulmonary sarcoidosis group consisting of 20 men and 15 women (mean age: 33 years, range 24–45 years). They had biopsy-proved pulmonary sarcoidosis, 31 had no clinical extrathoracic symptoms. 2. Idiopathic pulmonary fibrosis group consisting of seven men and two women (mean age: 54 years, range 44–65 years). They were all smokers, the diagnosis of IPF was biopsy proved. 3. This group consisted of nine healthy volunteers, four men and five women, five of them being smokers. Their mean age was 29 years (range 20–43 years).

Bronchoalveolar lavage (BAL)

The procedure used was a simple extension of fibre optic bronchoscopy, used routinely in this institution and previously described (Lambré *et al.*, 1986). Cells were numbered and differential cell counts were done on smears stained with Wright Giemsa. A percentage was established from at least 200 cells comprising macrophages, lymphocytes and polymorphonuclear neutrophils (PMN).

BAL was centrifuged at 300 ***g*** for 15 min at 20°C and the supernatant was concentrated 25 times by ultrafiltration on an Amicon membrane (Diaflo PM10, Amicon Paris, France). Concentrated bronchoalveolar lavage fluid (BALF) was frozen at −80°C in 200 μl aliquots and assayed for sialidase and antibody activities within 1 month.

Protein assay

The protein concentration was determined using Bradford's method (Bradford, 1976) with bovine serum albumin as standard.

Sialidase assay

The sialidase activity was assayed by using a fluorogenic substrate (Potier *et al.*, 1979) sodium 4-methyl umbelliferyl neuraminate (Mu-NeuAc) (Sigma Chemicals, St Louis, MO). BALF (25 μl) were incubated in a 100 mM sodium acetate buffer at various pH for 1 to 4 h at 37°C with 250 μM substrate. The reaction was stopped with 1 ml absolute ethanol. Sialidase activity was determined after adding 100 μl 1M NaOH then measuring the fluorescence intensity of released 4-methyl-umbelliferone (4-Mu). Fluorescence emission was read at 450 nm after excitation at 365 nm on a Dynatech Microfluor Reader. Pure 4-Mu was used as standard.

Sialidase treatment of erythrocytes

Outdated human O Rh^- erythrocytes (Eh) were obtained from the 'Centre de transfusion sanguine', Hopital Henri Mondor, Créteil and treated with *Vibrio cholerae* sialidase as previously described (Lambré *et al.*, 1985). In some cases, autologous instead of homologous erythrocytes were used. Sham-treated Eh were also prepared.

For determination of sialic acid associated with membrane of normal or sialidase treated erythrocytes, stroma from 1×10^9 erythrocytes was hydrolysed in 0·1 M HCl at 80°C for 60 min and free sialic acid was assayed by the thiobarbituric acid method (Warren, 1959). More than 95% of the sialic acid associated with the erythrocyte membrane was removed following sialidase treatment.

Microhaemagglutination tests

Serial dilutions (25 μl) of BALF in a veronal buffer containing 1% bovine serum albumin (VBS-BSA) were prepared in microagglutination plates. To each dilution (in duplicate) was added 25 μl of sialidase-treated erythrocytes at a concentration of 10^8 cells per ml in VBS-BSA. The plates were shaken to suspend the cells and kept for 45 min at room temperature, then for 18 h at 4°C. The haemagglutinating titre was expressed as the reciprocal of the highest sample dilution giving definite agglutination.

Immunofluorescence study

Sialidase-treated erythrocytes (2×10^7 cells in 0·2 ml VBS-BSA) were incubated with 0·2 ml of BALF (1/5–1/50 in VBS-BSA) for 1 h at room temperature. Cells were washed three times with 3 ml VBS-BSA at 4°C then incubated with 0·2 ml fluorescein-conjugated monospecific goat antiserum anti-human IgG, IgA or IgM (Atlantic antibodies) 1/100 for a further 45 min on ice. After three final washes, as above, erythrocytes were resuspended in 0·2 ml PBS-glycerol (80/20) and examined with a Leitz orthoplan fluorescent microscope.

Carbohydrate inhibition studies

The effect of various saccharides, D(+)-galactose, D(+)-mannose, D(+)-glucose and lactose (4-0-β-D-galactopyranosyl-α-D-glucose) (Sigma Chemicals, St Louis, MO) on the BALF induced agglutination of sialidase-treated erythrocytes was examined. Stock solutions (0·5 M) were prepared in VBS-BSA and stored for no longer than 2 weeks at −20°C. Once diluted they were used immediately.

Statistical analysis

The mean values were expressed with their respective standard deviation. The difference between two groups was established by using the analysis of variance (*F*-test).

RESULTS

Volumes of retrieved bronchoalveolar lavages

The mean retrieved volume of BAL was, respectively, 110 ± 15, 90 ± 20 and 137 ± 15 ml for the sarcoidosis, IPF and normal group. There was no significant difference in the volume of return between smoking and non-smoking normal volunteers.

Analysis of the cellular population of BAL

As expected, with regard to the normal group, BAL cellular populations were significantly increased in the two groups of patients. As usual, the sarcoidosis group was characterized by an increased number of lymphocytes, and the IPF group by an increased number of polymorphonuclear neutrophils (Table 1).

Table 1. Cellular population in bronchoalveolar lavage from normal healthy volunteers, or patients with idiopathic pulmonary fibrosis (IPF), or sarcoidosis (Sarc)

	Normal 18 ± 10*	IPF 65 ± 30*	Sarc 45 ± 18*
% Macrophages	$89{\cdot}5 \pm 5$	$78{\cdot}2 \pm 13$	$53{\cdot}8 \pm 13$
% Neutrophils	$0{\cdot}5 \pm 0{\cdot}2$	$18{\cdot}8 \pm 10$	$1{\cdot}2 \pm 0{\cdot}8$
% Lymphocytes	$9{\cdot}8 \pm 4$	3 ± 2	45 ± 12

* Cell number ($\times 10^4$)/ml BAL.

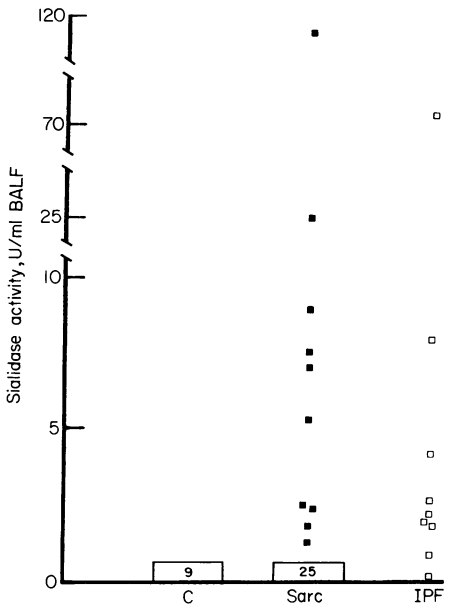

Fig. 1. Sialidase activity in bronchoalveolar lavage fluids (BALF) from control healthy volunteers (C), and patients with sarcoidosis (Sarc) or idiopathic pulmonary fibrosis (IPF). The activity is expressed as enzymatic units per ml of BALF.

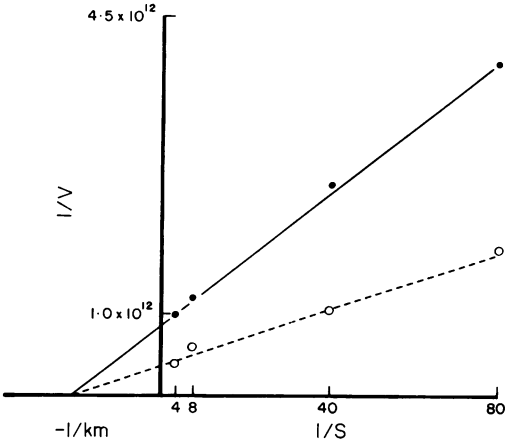

Fig. 2. Determination of the apparent *Km* (Lineweaver and Burk plot) of BALF sialidase from patients with sarcoidosis (●) or IPF (○). 1/V $(\text{mole/min})^{-1}$; 1/S $(10^3 \text{ mole})^{-1}$.

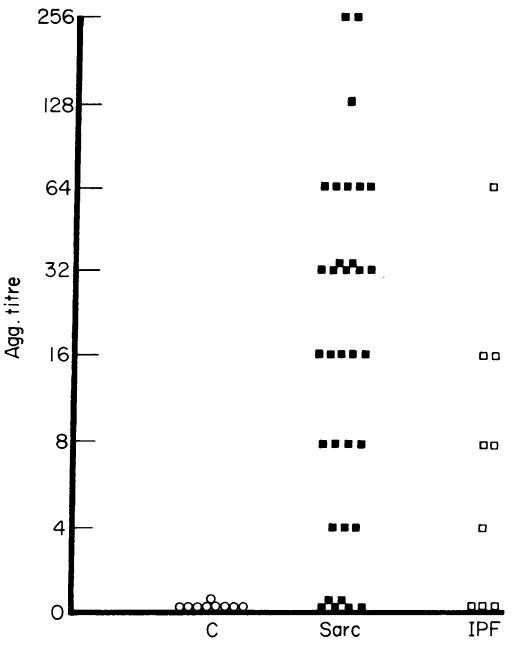

Fig. 3. Agglutinating titre of BALF from healthy controls ($n = 9$) or from sarcoidosis ($n = 35$) and IPF patients ($n = 9$).

Total protein determination in BALF

BALF from healthy volunteers contained $1{\cdot}65 \pm 0{\cdot}69$ mg/ml of protein, whereas BALF from the sarcoidosis and IPF groups contained $2{\cdot}95 \pm 0{\cdot}92$ and $5{\cdot}75 \pm 3{\cdot}12$ mg/ml respectively.

Detection of sialidase activity in bronchoalveolar lavages

No sialidase activity was detected in BALF from normal controls (Fig. 1), but in eight out of nine (90%) BALF from the IPF group, a sialidase activity was detected. An activity was also found in ten out of the 35 BALF samples (29%) from patients with pulmonary sarcoidosis (Fig. 1). The difference between duplicate determinations never exceeded 10%. There was no correlation between the BALF-sialidase activity and the number of polymorphonuclear neutrophils (PMN), macrophages, or lymphocytes in the respective BAL samples.

The enzymatic activity was destroyed by heating BALF for 15 min at 60°C; it had an optimum pH activity of 5·4. The activity was neither inhibited by ethylene-diamine-tetra-acetate (EDTA) 10^{-3} M, nor stimulated by increasing the Ca^{2+} concentration in the assay mixture. However, the activity was inhibited by a specific inhibitor of sialidases: 2-3-dehydro-2-deoxy-N-acetyl neuraminic acid, at a concentration of 10^{-4} M. The apparent Km was close to $4{\cdot}2\ 10^{-5}$ M (Fig. 2) in BALF from either IPF or sarcoidosis patients.

Gel filtration of BALF on a G-75 Sephadex gel evidenced a homogeneous and single peak having a sialidase activity. It corresponded to a molecular weight of 21 kD.

We were unable to detect any sialidase activity in the serum from either normal controls, IPF or sarcoidosis patients.

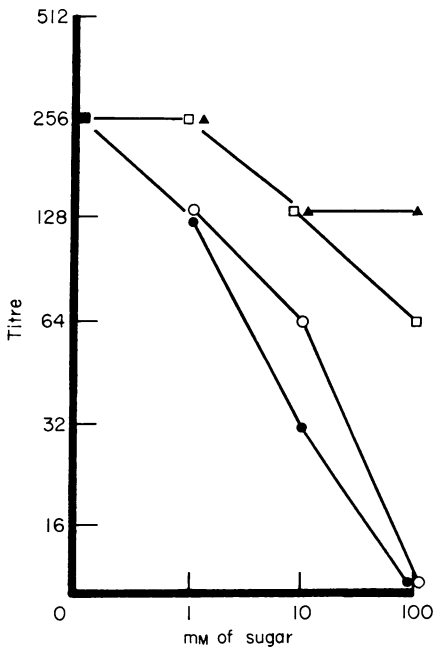

Fig. 4. Carbohydrate inhibition study of the capacity of BALF from a sarcoidosis patient to agglutinate autologous sialidase-treated erythrocytes. (■) control buffer; (▲) glucose; (□) mannose; (○) lactose; (●) galactose.

Agglutination of sialidase-treated erythrocytes by BALF

BALF from healthy volunteers did not agglutinate sialidase-treated erythrocytes, with the exception of a slight non-titerable activity observed in a sample obtained from only one smoker. In six out of nine cases of IPF, BALF did agglutinate sialidase-treated erythrocytes (mean 12 ± 20) (Fig. 3). BALF recovered from patients with sarcoidosis had a higher agglutinating capacity (mean 44 ± 100) but with a wide range of activity, eight out of 35 had no agglutinating activity (Fig. 3). This may be due to the heterogeneity of this group of patients with respect to the stage and/or progression of the disease.

The amount of erythrocyte membrane-associated sialic acid was identical in the different groups (124 ± 11 nmoles/1×10^9 cells).

In order to determine whether the agglutinating capacity of BALF from patients was due to the presence of autoantibodies having an anti-T like specificity, we have examined the effect of various saccharides. On the one hand, β-D-galactose, and lactose which are related to the T-antigen, and on the other hand, mannose and glucose which are not directly related to this antigen in humans. Only the two T-related saccharides could inhibit significantly the capacity of BALF to agglutinate sialidase-treated erythrocytes (Fig. 4).

By using immunofluorescence light microscopy both IgG and IgM immunoglobulins were demonstrated on sialidase-treated erythrocytes, following incubation with BALF that had agglutinating capacity. IgM was the predominant antibody class. Similar results were constantly observed when agglutination or fluorescence techniques were carried out using autologous instead of homologous (O Rh^-) erythrocytes.

Relationship between the presence of autoantibodies and of sialidase activities in BALF

No correlation was found between the presence of antibodies to sialidase-treated erythrocytes and sialidase activity in BALF from IPF patients. In patients with sarcoidosis, several sub-groups can be distinguished. In one sub-group the patients had neither BALF sialidase activity nor antibody activity (25% of the sarcoidosis patients without BALF sialidase activity). In a second sub-group the patients had both sialidase and antibodies (80% of the cases having BALF sialidase activity), but the enzymatic and antibody activities were not statistically correlated. Finally, a few sarcoidosis patients had either sialidase or antibody activity.

DISCUSSION

A BALF sialidase activity could be detected in 90% (eight out of nine cases) of the IPF patients, whereas it could be found in only 29% (10 out of 35 cases) of the sarcoidosis patients. BALF from normal volunteers had no sialidase activity. As assessed by similar biochemical properties, the sialidase activity that was detected in the two groups of patients seemed to correspond to the same enzyme. The visible heterogeneity of the sarcoidosis group was related to the different stages of each patient's disease. Furthermore, besides the stage of the disease, its progression at the time of the lavage has to be taken into consideration. Unfortunately, it is difficult to reliably assess this parameter. So far, different biological markers have been proposed: angiotensin converting enzyme activity in serum or BALF, and the number of lymphocytes in the lavages (Thomas & Hunninghake, 1987). Presently, no definite consensus has been reached on this point. It should be pointed out that in our study, no relationship has been found between the BALF sialidase activity and the number of either lymphocytes, alveolar macrophages or PMN in BALF.

BALF sialidase can be of either exogenous or endogenous origin. Indeed, numerous microorganisms possess a sialidase. Orthomyxoviruses, *Streptococcus pneumoniae* and *Pseudomonas* (Schauer, 1983) are some of the best known. However, some observations do not favour the hypothesis of an exogenous origin for the BALF activity. For instance, it is not inhibited by EDTA and it is not calcium-dependent, whereas many, although not all, microbial sialidases are. Furthermore, we had the opportunity to assay the sialidase activity in sequential lavages at 1 year intervals from the same patients ($n=3$). In every case the activity not only persisted but it was always increased. If such a sustained activity was the consequence of microbial infection, it would be surprising if it was not accompanied by clinical symptoms. An endogenous origin for the sialidase activity was suggested by its optimum pH activity and by its calcium independency. Among the cells which contain a sialidase activity are: PMN, lymphocytes, fibroblasts (Schauer, 1983), and alveolar macrophages (Pilatte, Bignon & Lambré, 1987). Since most of these cells are present and activated in the lung of patients with either IPF or sarcoidosis, one can think that a part of their sialidase activity may be released in the alveolar spaces. This may occur following cell lysis or lysosomal response to stimulation of these cells by immune complexes or by C5a. In this context, we have demonstrated the presence of activated complement in BALF from patients with IPF or sarcoidosis (Lambré *et al.*, 1986).

Whatever its origin, the presence of a sialidase in BALF may have significant pathological consequences. For instance, it is known that cell membrane desialylation, even in the absence of antibodies, leads to an activation of the alternative complement pathway (Lambré *et al.*, 1982; Gutierrez, Martin & Brown, 1987). Furthermore sialidase-induced membrane desialylation leads to unmasking of carbohydrates such as the so-called T-antigen (Thomsen–Friedenreich) (Springer *et al.*, 1979), which have antigenic properties. After treatment of histological sections with sialidase, the T-antigen was detectable on the human (Farragina *et al.*, 1986) and bovine (Vierbuchen *et al.*, 1986) bronchoalveolar epithelium. Moreover, it is also spontaneously present on the membrane of suppressor cells in the mouse (Nakano *et al.*, 1980). In this context, the presence of antibodies to the T-antigen in BALF could be relevant to the immunopathological changes that are observed during the progress of these pulmonary diseases.

In our study we found antibodies with such an anti-T antigen specificity in BALF from both IPF and sarcoidosis patients. The galactose-specific nature of these antibodies has been assessed by carbohydrate inhibition experiments. All but one BALF from normal healthy volunteers were negative and a very weak activity was detectable in the BALF from one volunteer who had a heavy smoking habit. This could be due to a passive alveolar transudation of serum proteins, among which are natural anti-T antibodies. Anti-T activity was more frequently found in BALF from sarcoidosis patients. Furthermore, the mean agglutinating activity was higher in this group. The absence of correlation between the level of anti-T antibodies and of sialidase activities could be explained by adsorption of anti-T antibodies on cell surfaces that are expressing the T specificity. This has been documented in the case of breast cancers (Springer *et al.*, 1979). Since they can be either synthesized locally or simply reflect an increased permeability to serum proteins of the capillary alveolar membrane, the origin of these BALF anti-T antibodies remains to be established.

The possible roles of anti-T antibodies in the pathogeny of pulmonary interstitial disorders are numerous. These antibodies can interact with cell membranes expressing the T antigen, and lead to pathological changes or alterations of cellular metabolisms. In this context it has been shown that mouse suppressor T lymphocytes express the T-antigen, just like mouse (Nakano *et al.*, 1980) and human (Berrih *et al.*, 1982) thymocytes do. Furthermore, natural thymocytotoxic autoantibodies can recognize the cells expressing membrane galactose residues, among which are NK and suppressor cells, and subsequently modify their metabolism. This is believed to be one of the reasons why autoimmune phenomena, such as haemolytic anaemia, lymphoid proliferation and production of anti-erythrocytes and anti-nucleic acid antibodies, are found in New-Zealand Black mice (Nakanishi *et al.*, 1982). Similarly, the anti-T antibodies we describe in this report as occurring during sarcoidosis or IPF could be responsible for the immunological abnormalities that are associated with these diseases.

The precise roles of sialidase activity and of anti-carbohydrate antibodies in interstitial lung disorders remain to be elucidated. We assume that with respect to their specific and/or synergistic pathological implications, these activities should be at least partly responsible for the persistence and/or progression of these diseases.

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