

## IMMUNOREGULATION BY $\alpha$ 1 ANTITRYPSIN

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**SUMMARY**  $\alpha$ 1 antitrypsin ( $\alpha$ 1 AT) deficiency is a common genetic disorder seen in about 10% of the population. It predisposes to the development of a large number of inflammatory and immunologic disorders including rheumatoid arthritis, systemic lupus erythematosus, juvenile chronic arthritis, anterior uveitis, ankylosing spondylitis, fibrosing alveolitis and emphysema. We have investigated immunologic function in subjects with severe  $\alpha$ 1 AT deficiency and demonstrated serum mediated enhancement of lymphocyte response to PHA and increased zymosan activation of mononuclear cells and neutrophils as measured by their chemiluminescence. These patients also have accelerated delayed hypersensitivity responses and increased levels of factor B, C3 and C5 but normal levels of immunoglobulin and other complement components. Such abnormalities in immunoregulation demonstrate a tendency to hyperreactivity that may contribute to disease predisposition.

### INTRODUCTION

ALPHA 1 ANTITRYPSIN ( $\alpha$ 1 AT) deficiency is a common genetic variant, being found in about 10% of the population (1). Some  $\alpha$ 1 AT phenotypes are associated with a variety of inflammatory and immunologic disorders including rheumatoid arthritis (RA) (2, 3, 4), juvenile chronic arthritis (JCA) (5), systemic lupus erythematosus (SLE) (4), anterior uveitis (4, 6), ankylosing spondylitis (AS) (3), asthma (7), fibrosing alveolitis (8), emphysema (9) and chronic liver disease (10, 11). To explore the possible mechanisms underlying this association we have investigated a variety of parameters of inflammatory and immunologic functions in patients with severe  $\alpha$ 1 AT deficiency (serum levels less than 25% of normal) compared with a control population.

### METHODS

#### a. Patient Selection

Investigations were carried out on 10 members of 5 families with severe  $\alpha$ 1 AT deficiency (PiZZ and serum levels less than 25% of normal) and a control population consisting of 10 sex matched medical students with normal  $\alpha$ 1 AT phenotypes and levels. With the exception of several patients with emphysema all were disease free at the time of study. Any medications were ceased 24 hr prior to sample collection.

#### b. Serum Protein Determination

Using standard radial immunodiffusion techniques, levels of the following serum proteins were determined: IgG, IgM, IgA, C1q, C3, C4, C5, factor B, C1 inhibitor,  $\alpha$ 1 AT,  $\alpha$ 2 macroglobulin,  $\alpha$ 1 antichymotrypsin and inter alpha trypsin inhibitor. Functional assessment of the

classic and alternative pathways of complement activation was undertaken by measuring the reciprocal of the titre of serum lysing 50% of sensitised sheep red cells (CH50) (12) and unsensitised rabbit erythrocytes (PH50) (13) respectively.

#### c. Skin Tests

*In vivo* assessment of delayed hypersensitivity response (DTH) was carried out by intradermal injection of 0.1 ml of candida (1:20 dilution Dermatophytin O Hollister Stier Spokane, USA), SKSD 5 units (Varidase, Lederle Lab. Div., St. Leonards, N.S.W., Australia), PPD 10 units (Commonwealth Serum Laboratories, Melbourne, Australia). An Arthus reaction was also induced by injection of 0.1 ml of aggregated IgG. Autologous serum was administered as a control. Aggregated IgG was prepared by making a 1:8 dilution in normal saline of a 160 mg/ml gammaglobulin preparation (Commonwealth Serum Laboratories, Melbourne, Australia) which had been heated at 63°C for 10 minutes. The induration associated with all injections was read at 1 hr, 4 hr, 8 hr and 48 hr and recorded as the mean of the largest and smallest diameters of the lesions. A result greater than 0.5 cm was considered positive.

#### d. Lymphocyte Function

Serum from 7 of the  $\alpha$ 1 AT deficient patients and 7 controls were obtained and either used fresh or stored at -70°C. These sera were used as a 20% supplement in determining the PHA response of isolated peripheral blood mononuclear cells of all experimental subjects. The culture was carried out using a standard microculture technique (14) on quadruplicate samples at PHA (Purified PHA, Wellcome Reagents Ltd., Beckenham, England) doses of 0.1, 0.15, 0.2, 0.3 and 0.5  $\mu$ g per ml of culture. At the end of 3 days culture, the rate of DNA synthesis as measured by tritiated thymidine incorporation was determined.

#### e. Neutrophil and Monocyte Function

Neutrophil and monocyte function was assessed using the chemiluminescence technique essentially as previously described (15). In brief, mononuclear cells (MN) and neutrophils (PMN) were isolated from the peripheral blood of 8  $\alpha$ 1 AT deficient patients and 8 controls using Ficoll Hypaque density gradient centrifugation and dextran sedimentation. MN or PMN ( $1 \times 10^6$  in 1 ml of Hanks' Balanced Salt Solution)

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Table 1 Mean levels of serum proteins in  $\alpha 1$  AT deficient patients and controls

	Serum Proteins					
	IgG g/l	IgA g/l	IgM g/l	$\alpha_2$ M g/l	$\alpha_1$ anti- chymotrypsin g/l	inter $\alpha$ trypsin inhibitor g/l
Patients (mean)	10.4	1.8	1.0	2.30	0.45	0.52
Controls (mean)	10.3	1.9	1.0	2.64	0.37	0.46

from each subject were placed in vials in the presence of 50  $\mu$ l of 0.1% luminol, 2.2 ml phosphate buffered saline and 700  $\mu$ l of the appropriate serum from subject or control. These were counted repeatedly in a liquid scintillation counter in the out of coincidence mode to obtain a baseline value. One ml of opsonised zymosan (5 mg/ml) was then added to activate the cells which were then counted every 2 min for 6 sec. The peak response was obtained for each sample.

f. Statistical Analysis

All results were analysed using Wilcoxon matched pairs signed ranks test.

RESULTS

a. Serum Protein Determination (tables 1 and 2)

Serum levels of C3, factor B, C5 and PH<sub>50</sub> were all elevated in  $\alpha 1$  AT deficient subjects with respect to the control group ( $p < 0.01$ ). There was no significant difference in the level of any other serum protein.

b. Skin Tests

There was a marked difference in the time course of the DTH response (table 3), although there was no difference between the two groups in terms of the number of positive responses or their magnitude. Of the 15 positive DTH responses in  $\alpha 1$  AT deficient subjects, 10 were already positive by 8 hours. By contrast, of the 20 positive responses in the controls, only one was positive by 8 hours.

c. Lymphocyte Function

Analysis of PHA responses of peripheral blood lymphocytes indicates significant enhancement in the presence of

Table 3 Difference in the time course of positive DTH responses in  $\alpha 1$  AT deficient patients and controls

	Delayed hypersensitivity responses		
	Number positive 8 hr	Number positive 48 hr	Number negative 48 hr
Patients	10	15	15
Controls	1	20	10

$\alpha 1$  AT deficient serum at doses of 0.1, 0.15, 0.2, 0.3  $\mu$ g/ml ( $p < 0.01$ ) (fig. 1). There was no significant difference at a dose of 0.5  $\mu$ g/ml or in control cultures with no PHA added. Figure 2 documents the results of all experiments at a dose of PHA of 0.2  $\mu$ g/ml. Experiments comparing the effect of  $\alpha 1$  AT deficient and control serum on lymphocytes clearly illustrate that the enhanced PHA

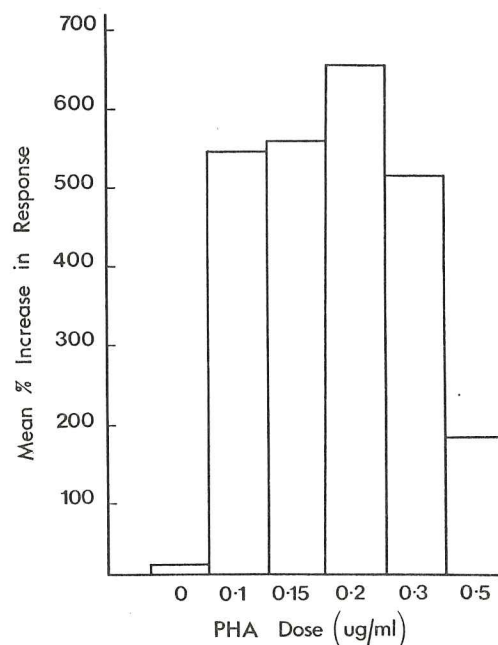


FIG. 1. Mean percentage increase in PHA response of lymphocytes cultured with  $\alpha 1$  AT deficient sera compared to control sera.

$$\left[ \frac{\text{response in patient} - \text{control}}{\text{control}} \times \frac{100}{1} \right]$$

Increases are significant at PHA doses of 0.1, 0.15, 0.2 and 0.3  $\mu$ g/ml.

Table 2 Mean levels of complement components in  $\alpha 1$  AT deficient patients and controls

	Complement components							
	C1q % Normal	C3* g/l	B* % Normal	C4 g/l	C5* % Normal	CH50 Units	PH50* Units	C <sub>1</sub> INH g/l
Patients (mean)	86.1	1.32	84.6	0.42	112.3	325.9	22.9	0.33
Controls (mean)	90.4	1.08	65.6	0.35	84.2	269.6	17.7	0.29

\* Differences between groups significant with  $p < 0.02$ .

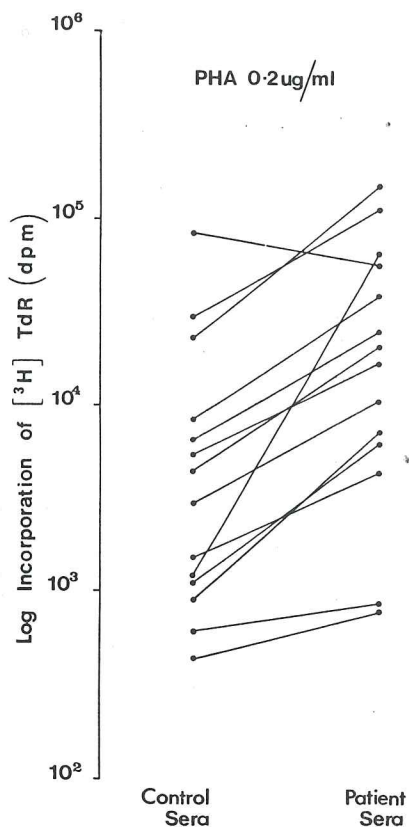


FIG. 2. Individual results of PHA responses in  $\alpha 1$  AT deficient sera compared with control sera.

responsiveness in  $\alpha 1$  AT deficient subjects is serum mediated.

#### d. Neutrophil and Monocyte Function

In the 8 patients and controls studied there was enhanced activation of both MN and PMN in the presence

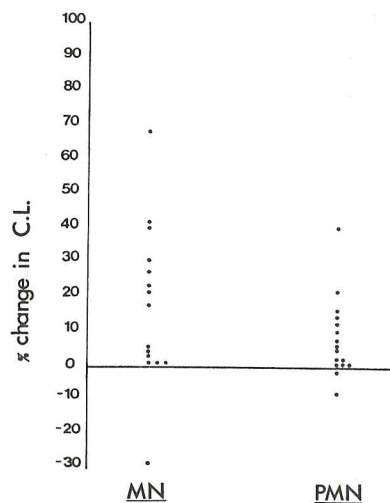


FIG. 3. Percentage change in chemiluminescence response of monocytes and neutrophils activated in  $\alpha 1$  AT deficient sera compared to control sera.

$$\left[ \frac{\text{response in patients} - \text{control}}{\text{control}} \times \frac{100}{1} \right]$$

of  $\alpha 1$  AT deficient serum ( $p < 0.01$ ) (fig. 3) with mean percentage increases of 21% and 8% respectively. In spite of the wide scatter of results, Figure 3, which examines the percentage change in CL, shows that in only one instance for MN and two for PMN was there a decreased CL response in the presence of  $\alpha 1$  AT deficient serum.

## DISCUSSION

These results suggest that  $\alpha 1$  AT is able to influence the activation of both lymphocytes and phagocytic cells. Several properties of  $\alpha 1$  AT indicate that it may be an important regulatory protein. It is an inhibitor of serine proteases with activity against a wide range of enzymes including leukocyte neutral proteases, elastase, collagenase, trypsin, thrombin and kallikrein (16). It is present in the highest molar concentration of any protease inhibitors and this coupled with its low molecular weight of 55,000 allows it to achieve high concentration in the extravascular compartment. Additionally, unlike  $\alpha 2$  macroglobulin ( $\alpha 2$  M) the other major protease inhibitor, it is an acute phase reactant.

### a. Serum Proteins

With the exception of some complement components, there was no difference in serum proteins between the  $\alpha 1$  AT deficient patients and controls. There was no compensatory rise in the other protease inhibitors measured. Proteins of the alternative pathway and the attack sequence, C3, factor B and C5, were elevated in the patients and this was paralleled by an increase in the PH50. By contrast, proteins of the classical pathway C1q and C4 were similar in the two groups, as was the CH50. Only one previous study has investigated C3, C4 and CH50 in  $\alpha 1$  AT deficient patients and found no difference (17). These patients however all had severe liver disease, known to greatly influence the metabolism of many proteins. The cause of this difference is uncertain but may be due to a compensatory increase in synthesis of C3, another acute phase protein. As will be discussed later,  $\alpha 1$  AT is also able to block the binding of C3 to its receptor on phagocytic cells and lymphocytes. This interaction is due to the carbohydrate moiety of  $\alpha 1$  AT. Changes in such interaction of C3 with its receptor in  $\alpha 1$  AT deficiency may be able to influence C3 serum levels.

### b. Skin Tests

The majority of  $\alpha 1$  AT deficient patients manifested positive DTH responses by 8 hours whereas this was seen in only one of the controls. Although there is no histological verification of the characteristics of this response, it is not simply a non specific reaction associated with intradermal injection as the 8 hour response only occurred in those patients with a positive DTH at 48 hours. Additionally, there was no positive reaction

associated with injection of autologous serum. The lack of difference in the magnitude of positive responses in the two groups may well reflect the failure of skin tests as a quantitative index of inflammation.

#### c. Lymphocyte Function

T-lymphocytes are important regulatory cells and abnormalities in their function are thought to underlie a large number of immunologic disorders. Using the PHA response, a widely accepted index of T cell function, we have demonstrated that deficiency of  $\alpha 1$  AT is associated with enhanced activation. This was observed at low and intermediate doses of PHA but not at high doses. Absence of  $\alpha 1$  AT would be expected to increase the rate of cell activation and the number of cells activated with submaximal stimulation. With maximal stimulation as seen with high doses of PHA, all potentially responding cells are activated and no further effect due to the absence of  $\alpha 1$  AT is likely. *In vivo*, its properties as an acute phase reactant would help it to limit lymphocyte activation in inflammatory disease. Conversely, decrease in  $\alpha 1$  AT levels and loss of acute phase reactant properties as is seen in some genetically determined types of  $\alpha 1$  AT could be expected to lead to exaggerated lymphocyte responses and possible abnormalities in immunoregulation.

The mechanism of action of  $\alpha 1$  AT is probably via inhibition of membrane bound serine proteases, known to be involved in the activation of lymphocytes. Proteases themselves may also be mitogenic to lymphocytes (18, 19) and  $\alpha 1$  AT may act by inhibiting the activity of these extracellular proteases that are present in abundance in serum and tissues at sites of inflammation. Bata *et al.* (20), have demonstrated that  $\alpha 1$  AT could inhibit the PHA response of lymphocytes in a serum free medium. Arora *et al.* (21) have also shown inhibition of the plaque forming cell response of mice after immunisation with SRBC if they were pretreated with intravenous  $\alpha 1$  AT. However, Vischer (22) has been unable to demonstrate inhibition of mitogen responses of mouse spleen cells by either  $\alpha 1$  AT or  $\alpha 2$  macroglobulin. He did however, observe inhibition by soya bean trypsin inhibitor (SBTI) and trasylol. Vischer's paper contains little methodological details but this discrepancy might be explained on the basis of species differences, inadequate dosage of  $\alpha 1$  AT or failure to use similar suboptimal doses of PHA to ourselves.  $\alpha 1$  AT has been detected on the surface of Con A stimulated human lymphocytes but not unstimulated cells (23), suggesting its attachment to membrane bound enzymes and further implicating it in activation of T cells.

#### d. MN and PMN Function

Neutrophils and macrophages are the major mediators of tissue injury. Additionally, macrophages have an important role in antigen processing and presentation to T cells. Enhanced activation of these cells such as we have demonstrated in the presence of  $\alpha 1$  AT deficient serum

could well result in increased injury in inflammatory diseases. Because of its low molecular weight  $\alpha 1$  AT can easily enter tissue spaces and this coupled with its acute phase reactant properties suggests it may be one of the important proteins regulating phagocytic cell function at sites of inflammation.

The mechanism of regulation of MN and PMN function by  $\alpha 1$  AT is probably on the basis of interaction with a membrane bound serine esterase. Whilst the molecular events involved in the initial activation of phagocytic cells are not fully elucidated, several authors have suggested serine proteases are involved. They have demonstrated that synthetic serine protease inhibitors such as soya bean trypsin inhibitor (SBTI), 2 phenylethylchloromethyl ketone (TPCK) and aprotinin are able to inhibit superoxide production by PMN and MN stimulated by a variety of different agents including wheat germ agglutinin, phorbol myristate acetate and N formylmethionyl leucine phenylalanine (24, 25, 26, 27). The fact that macromolecular inhibitors such as SBTI and aprotinin were also effective suggested that this enzyme was located at the cell surface. Under physiological conditions phagocytosis frequently is preceded by activation of MN and PMN. Musson *et al.* have demonstrated that at least 2 serine esterases are involved in phagocytosis: one present in an active form on the cell surface and a second which is activated once phagocytosis commences (28).

$\alpha 1$  AT is a serine protease inhibitor present in very high molar concentrations in serum and has inhibitory activity against a remarkably wide range of enzymes. We have demonstrated that it influences the activation of PMN and MN and enhanced activation occurs in its absence in  $\alpha 1$  AT deficiency. The mechanism of inhibition of PMN and MN activation by  $\alpha 1$  AT is probably through inactivation of membrane bound serine esterases. There is also evidence that  $\alpha 1$  AT may influence the function of phagocytic cells by other mechanisms. Dierich *et al.* have demonstrated that  $\alpha 1$  AT, via its carbohydrate portion, is able to interact with C3 and partially inhibits ingestion of C3 coated particles (29, 30). Therefore it may compete with C3 receptors on cells for the opsonised particles.

Patients with  $\alpha 1$  AT deficiency are predisposed to the development of a number of immunologic and inflammatory disorders such as RA, SLE and uveitis. These disorders have been associated with mild deficiency and phenotypes such as PiMS and PiMZ. The documented association of patients with severely deficient phenotypes (such as the ones we have used in this study) has been only with emphysema and chronic liver disease. The assumption has been made that this lack of documented association simply reflects the very low incidence of such phenotypes in the community. We have investigated patients with the most severe form of  $\alpha 1$  AT deficiency to increase the likelihood of detecting less obvious abnor-

malities. In these subjects enhanced mitogenic response of lymphocytes to PHA, increased MN and PMN activation, accelerated DTH skin test responsiveness and raised complement levels have been demonstrated. These effects may be directly due to lack of  $\alpha 1$  AT, secondary changes in other proteins or even other genetic factors linked to  $\alpha 1$  AT. This study does however suggest that these patients have abnormalities in immunoregulation with tendency to hyperresponsiveness, which may contribute to their disease predispositions.

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