

# Systemic and local production of $\alpha$ 1-antitrypsin and $\alpha$ 1-acid glycoprotein in rheumatoid arthritis in man and adjuvant arthritis in rats

GEORGIOS PANTAZIDIS<sup>1</sup>, ELISAVET KOUZIORTI<sup>1</sup>,  
SPYROS ASLANIDIS<sup>2</sup>, ZAFIRIS POLYMENIDIS<sup>3</sup>,  
LYGERI HADJIPETROU-KOUROUNAKIS<sup>1</sup> and MINAS YIANGOU<sup>1\*</sup>

<sup>1</sup> Department of Genetics, Development and Molecular Biology, School of Biology,  
Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

<sup>2</sup> 2nd Propedeutic Department of Internal Medicine, Aristotle University of Thessaloniki,  
Hippokration General Hospital, Thessaloniki, Greece

<sup>3</sup> Histocompatibility Center and Department of Immunology,  
Hippokration General Hospital, Thessaloniki, Greece

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We have studied the systemic and local production of  $\alpha$ 1-antitrypsin (AAT) and  $\alpha$ 1-acid glycoprotein (AGP) in serum and synovial fluid (SF) respectively of rheumatoid arthritis (RA) patients during both active and inactive phase of disease and this response has been compared to the respective response in adjuvant arthritic (AA) rats. We observed potential differential regulation and activity of AAT and AGP in active RA and similar data observed also in AA rats or in dorsal rat air pouch model suggesting that these animal models may be useful for further studies to understand RA pathogenesis. Elevated serum or synovial fluid AAT levels do not change in active or inactive RA while increased number of AAT positive PMN cells observed in both blood and SF of active RA patients suggesting a potential marker to estimate disease phase. Honey bee venom (HBV) inhibits AA induction and affects local and systemic AAT and AGP production. In the present study, we provide evidence from *in vivo* experiments in AA rats that AAT may suppress AA induction by exhibiting biological activity independent of inhibition of serine proteases. The potential anti-inflammatory and immunosuppressive activity of AAT and AGP in synovial fluid in combination with HBV activity is discussed.

**Key words:** Rheumatoid arthritis, adjuvant arthritis,  $\alpha$ 1-antitrypsin,  $\alpha$ 1-acid glycoprotein.

## INTRODUCTION

Rheumatoid arthritis (RA) is a multifactorial and polygenic disease that primarily affects peripheral joints, with chronic inflammation, cartilage and bone destruction, and finally, joint deformation (Feldmann *et al.*, 1996). The pathogenesis of RA is poorly understood, and the diagnosis is based on its clinical picture descriptions rather than understanding of the disease mechanisms (Aho *et al.*, 1986; Silman *et al.*, 1993). RA is believed to be the result of envi-

ronmental factors acting in combination with a genetic predisposition to the disease. Unfortunately, heterogeneity of the population hampers linkage analyses of RA in humans and thus considerable effort has been invested in establishing animal models of disease that resemble RA in humans. Adjuvant arthritis (AA) is an experimental model of autoimmunity in rats induced by the intradermal injection of Freund's complete adjuvant (FCA) and resembles rheumatoid arthritis in man (Pearson, 1956). The mechanism of AA development in rats remains unclear. Additionally, the 6 day-old air pouch in rats develops phagocytic and fibroblast-like lining cells

\* Corresponding author: tel.: +30 2310 998333, e-mail: [Yiangou@bio.auth.gr](mailto:Yiangou@bio.auth.gr)

followed by organized vasculature that acts as a mechanical barrier which retains the products of the inflammatory response and resembles the synovial tissue (Sedgwick *et al.*, 1983).

The acute phase response (APR) is the immediate set of host inflammatory reactions that counteract challenges such as tissue injury, infection, trauma or environmental stress such as heavy metal poisoning or heat shock (Kushner, 1982; Yiangou *et al.*, 1991; Baumann & Gauldie, 1994; Yiangou *et al.*, 1998). Acute phase reactants are the products of a distinct family of genes such as  $\alpha$ 1-acid glycoprotein (AGP), C reactive protein (CRP), serum amyloid A (SAA) or  $\alpha$ 1-antitrypsin (AAT) whose activation initiates an inflammatory mediator cascade that is characterized by both local vascular effects and systemic, multiorgan effects (Baumann & Gauldie, 1994). Several primary or secondary inflammatory mediators such as IL-1, IL-6, TNF and glucocorticoids, regulate APR gene induction (Cassatella, 1995). Although the acute-phase response has evolved as a short-term homeostatic mechanism its sustained activation as in cases of chronic inflammation may have negative clinical consequences. Acute phase reactants have been extensively used as markers of arthritis severity and there is considerable evidence of their potential role in development of arthritis (Arvidsson *et al.*, 1998). In previous work, we have demonstrated that AGP may be involved in the pathogenesis of adjuvant arthritis (AA) in rats (Yiangou *et al.*, 1993). Administration of purified AGP into the foot of arthritic rats accelerates the onset of AA, and increases the severity and duration of the disease (Yiangou *et al.*, 1993), thus raising the question of a possible local action of AGP on AA development in rat joints. Furthermore, honey bee venom (HBV) mediated AA remission may be the result of a local rather than a systemic anti-inflammatory or immunosuppressive effect (Hadjipetrou-Kourounakis & Yiangou, 1988) which coincides with AGP downregulation at the early stages of disease development (Yiangou *et al.*, 1993). Serum amyloid protein (SAA) that is locally produced in the synovial fluid induces metalloproteinase (MMPs) production and subsequent destruction of cartilage (Brinckerhoff *et al.*, 1989; O'Hara *et al.*, 2000) and it is widely accepted that MMPs may play critical role in the pathology of arthritis (Brinckerhoff, 1991). AAT is the most abundant serine proteinase inhibitor in human plasma and its levels increase rapidly in the circulation, as a response to inflammatory or infective stimuli (Travis *et al.*, 1988;

Potempa *et al.*, 1994; Janciauskiene, 2001; Kalshenker *et al.*, 2002). AAT is predominantly synthesised in the liver, but neutrophils, monocytes, and alveolar macrophages also express AAT in response to a variety of inflammatory mediators (Perlmutter *et al.*, 1988; Knoel *et al.*, 1998). Since AAT is a powerful inhibitor of neutrophil/derived proteases, including neutrophil elastase and proteinase 3, it is generally assumed that its physiological role is to protect host tissue from the potentially destructive proteolytic activity of these serine proteases produced during inflammation. There is conflicting evidence however, concerning the serum or synovial fluid  $\alpha$ 1-antitrypsin levels or activity and disease development (Housiau *et al.*, 1988; Ciobanu *et al.*, 1992; Lacki *et al.*, 1994, 1995; Moore *et al.*, 1999).

In this study, we compared the AAT and AGP production in rheumatoid arthritis in man and in adjuvant arthritis in rats during the active and inactive phases of disease. AAT and AGP are differentially regulated in serum or synovial fluid of both active RA and AA rats. We provide evidence that AAT may exert dual immunomodulatory effect on arthritis development.

## MATERIALS AND METHODS

### *Patients*

Twenty-two patients with RA (16 females and 6 males), and 21 patients with systemic lupus erythematosus (SLE) all males were recruited from a hospital-based sample and were included in the study. All patients gave their informed consent in accordance with the Declaration of Helsinki ethical guidelines. The patients fulfilled the respective RA and SLE classification criteria of the American College of Rheumatology (Tan *et al.*, 1982; Arnett *et al.*, 1988). The RA subjects were 11 patients aged between 20 to 45 years and another 11 patients aged from 48 to 68 years while SLE subjects were 16 patients between 20 to 45 years and another 5 patients from 48 to 68 years of age. The patients were further divided in two groups: i) thirteen of the RA and 12 of the SLE subjects were included in the non medicated-active phase group, and ii) RA or SLE patients receiving medication that reduced disease activity were included in the inactive phase group. In RA patient's medication was consisted of 15 mg methotrexate per week and prednisolone  $\leq$  10 mg daily and for SLE antimalarials (200 mg twice daily) and/or prednisolone  $\leq$  10 mg daily. Twelve healthy subjects, 8 females and four males,

were evaluated as the control group; the mean age in the healthy subjects group was  $37 \pm 4.6$  years.

#### *Serum and synovial fluid samples*

Blood and synovial fluid (SF) samples were collected with the informed consent of individuals with knee effusion requiring arthrocentesis for diagnostic or therapeutic reasons. SF was collected in heparinized tubes and cells were isolated by centrifugation at 2000 rpm for 20 min. The SF cells washed repeatedly in saline and counted using a haemocytometer and processed immediately for immunocytochemistry. The number of dead cells was determined by trypan blue exclusion. Serum was obtained by the centrifugation of blood at 3000 rpm for 5 min. Both SF and serum were aliquoted and stored at  $-30^{\circ}\text{C}$  until used.

#### *Animals*

Male Fisher-344 inbred rats (130-200 g) from our colony were housed under standard laboratory conditions (12-24 h light-dark cycle) and received a diet of commercial food pellets and water *ad lib*. This study complied with the current ethical regulations on animal research of our university that is according to EEC ethical regulations and all rats used in the experiments received human care.

#### *Treatments of animals and air pouch formation*

Adjuvant arthritis (AA) induced in rats by intradermal injection of Freund's complete adjuvant (FCA) to the third distal of the tail as previously described (Hadjipetrou-Kourounakis & Yiangou, 1988). HBV (0.25 mg/Kg bw, kindly provided by Mr C. Mraz, Champlain Valley Apiaries, Middlebury, NY) administered intramuscularly day by day for 3 weeks. At the indicated time post treatment, animals were bled by cardiac puncture and the serum was collected immediately and stored at  $-30^{\circ}\text{C}$ . Synovial fluid was collected by injecting 50  $\mu\text{l}$  of saline in joints. Air pouches were created on the back of rats as described previously (Sedgwick *et al.*, 1983) by injecting on day zero 20 ml air and two times 10 ml air day by day. Rats were injected with 0.1 ml Freund's complete adjuvant (FCA) and the cells accumulated in the air pouches harvested by injecting 2 ml saline. After centrifugation, the supernatant was collected and used as air pouch exudates. The air pouch exudates cells washed twice in saline and counted using

a haemocytometer. Dead cells determined by trypan blue exclusion. The air pouch lining tissue separated from skin and processed immediately.

Air pouch PMN cells accumulated in air pouch exudates 24 hours post FCA treatment were used for passive transfer of arthritis. PMN exudates cells ( $165 \times 10^6$ ) were injected in to the right footpad in 0.1 ml saline. Onset of arthritis estimated on arbitrary scale as previously described (Hadjipetrou-Kourounakis & Yiangou, 1988).

#### *Western blot analysis*

For Western blot analysis 5  $\mu\text{l}$  human or 7.5  $\mu\text{l}$  rat serum (1/50 dilution) or 5  $\mu\text{l}$  synovial fluid (1/25 dilution) as well as rat air pouch exudates were electrophoretically separated on a 10% SDS-polyacrylamide gel as described (Laemmli, 1970). Samples containing several concentrations of purified rat AGP or human  $\alpha 1$ -Antitrypsin (Sigma, St. Louis, MO) were also included as internal control. The proteins were then electrotransferred to Westram PVDF membrane (0.45  $\mu\text{m}$  pore size) in a buffer containing 25 mM Tris-Cl, 192 mM glycine and 20% methanol. The membranes then subjected to Western analysis (Yiangou *et al.*, 1998) using specific polyclonal antibodies. Specific polyclonal antibodies against human AAT or AGP were purchased from Sigma, St. Louis, MO and for rat AAT from RDI Res. Diagn., NJ, USA. Rat AGP was detected using specific polyclonal antibodies raised against purified rat AGP (Sigma, St. Louis, MO) prepared by us in rabbits. The resulting Western bands scanned and expressed as relative amount of protein in arbitrary densitometry units.

#### *Immunocytochemistry*

Cells ( $2 \times 10^5$ ) isolated from blood, synovial fluid or rat air pouches were loaded on slides using a cyto-centrifuge, fixed for 1 min in 4% paraformaldehyde and stored in 70% ethanol at  $4^{\circ}\text{C}$ . Small pieces of air pouches lining tissue were initially fixed in 4% paraformaldehyde for 24 hours and then embedded in paraplast, sectioned at 5  $\mu\text{m}$  thickness onto gelatine-coated slides and treated as it was previously described (Yiangou *et al.*, 1998). Immunocytochemistry of air pouch cell smears or paraplast sections performed as previously described (Avramidis *et al.*, 2002).

The human AAT or AGP producing cells were detected using specific anti-human AAT (Sigma, St

Louis, MO or RDI Res. Diagn., NJ, USA) or AGP (Sigma, St Louis, MO) antibodies. As control irrelevant rabbit IgG was tested as well. After washing with PBS slides were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (Sigma) 1/1000 in PBS-BSA. The slides were washed repeatedly with PBS and thereafter incubated for 5 min in 0.5 mg/ml 3,3-diaminobenzidine-tetrahydrochloride (Sigma Chemical Co., St Louis, MO) in PBS (pH 7.4) containing 0.01%  $H_2O_2$ . Finally, the slides were counterstained with Giemsa solution and positive cells were determined microscopically. Two independent investigators performed these microscopic studies; for the final scoring two investigators achieved consensus.

#### Assessment of cytokines

Cytokines in human or rat serum and in rat air pouch fluids were determined by the enzyme linked immunoassay. In human samples IL-1 and TNF $\alpha$  were detected using the Cytelisa kit (CytImmune Sciences Inc., MD 20740) while rat IL-1 or IL-6 levels were

detected using the Quantikine M Murine kit (Endogen) according to manufacturer directions.

#### Statistical Analysis

Statistical analysis was done using the two-tailed Student's t-test and statistical significance was accepted with values of  $p < 0.05$ .

## RESULTS

#### Detection of AAT and AGP pool levels in serum and synovial fluid

The AAT or AGP pool levels in serum or knee synovial fluid at the active or inactive phase of RA were determined by SDS-page and Western immunoblot analysis using specific anti human AAT or AGP antibodies. These antibodies detected a single band with an apparent molecular weight of 54 kDa that corresponds to purified active AAT (Fig. 1A). Serum AAT levels in either active or inactive phase of RA were similar to healthy controls (Fig. 1A). Confirming previous studies (Houssiau *et al.*, 1988; Ciobanu

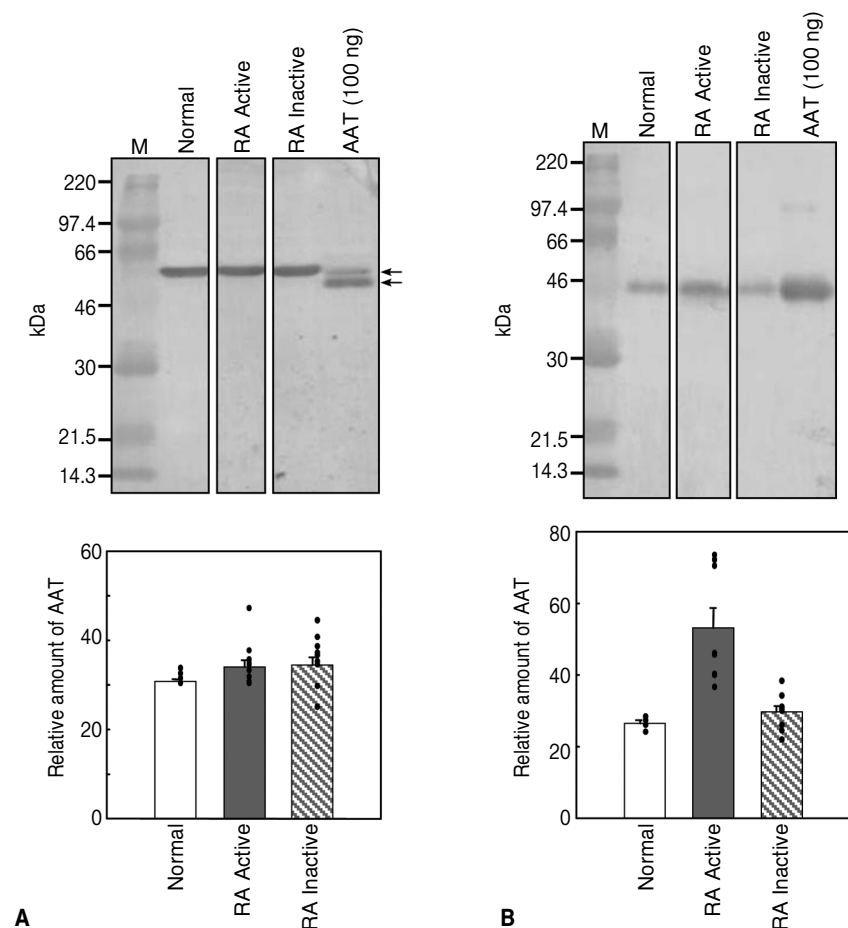


FIG. 1. Determination of AAT and AGP pool levels in the serum of RA patients during the active and inactive phase of disease. In the upper of panels representative Western analysis showing the AAT (Panel A) or AGP (Panel B). Lane M represents molecular weight markers and lanes AAT or AGP purified proteins. In panel A arrows show the active and inactive AAT present in purified AAT sample. The resulting Western immunoblotting bands were scanned and expressed in arbitrary densitometry units as relative amount of protein (lower part of panels A and B). In the histogram (lower part of panels) each bar represents the serum values mean  $\pm$  S.E.M. and each dot over bars represent the value obtained from each individual.

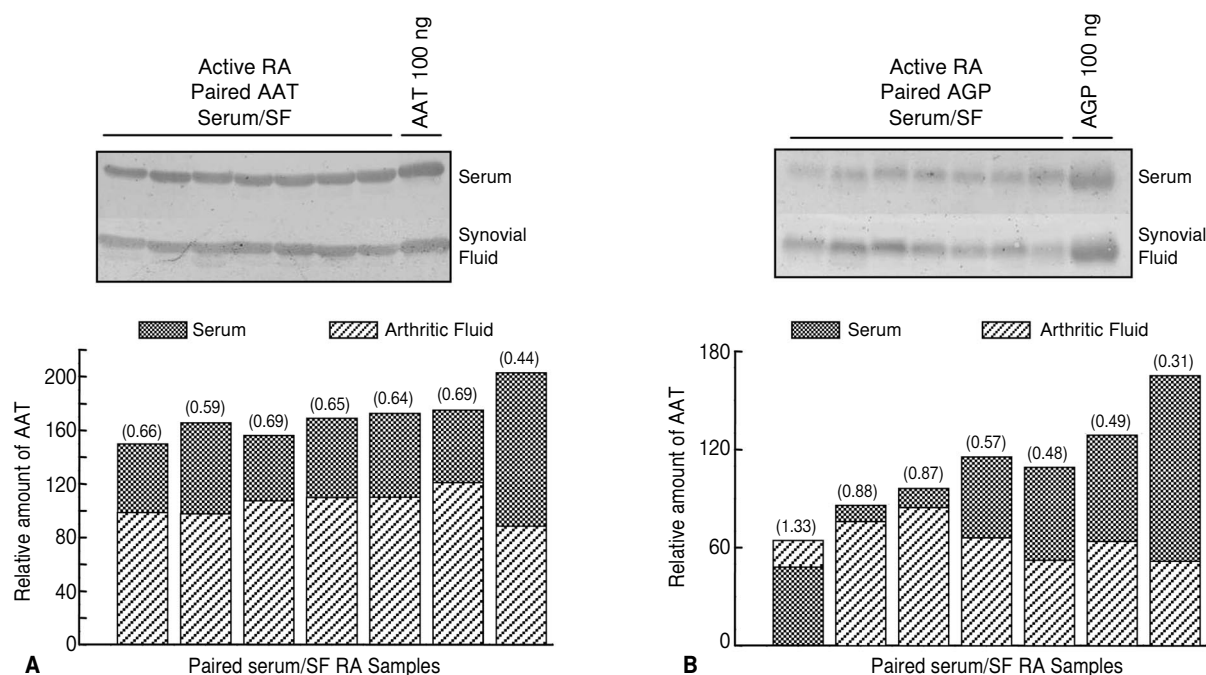


FIG. 2. Western analysis showing paired AAT (panel A) and AGP (panel B) pool levels in the serum and synovial fluid of RA patients during the active phase of disease. In the upper part of panels representative SDS-page and immunoblotting using specific antibodies raised against human AAT or AGP. Serum and synovial fluid samples were subjected to electrophoresis in separate gels. The resulting Western immunoblotting bands were scanned and each value normalized to internal control samples of purified AAT or AGP. The histogram (lower part of panels) shows the paired serum/synovial fluid AAT and AGP levels and numbers over bars is the serum/synovial fluid ratio.

*et al.*, 1992) AGP protein pool levels in the same samples were found increased in serum of active RA and reduced in inactive RA ( $p < 0.05$ , Fig. 1B). Moreover, analysis of AAT or pool levels in paired sera and SF samples from patients with active RA, revealed that there is correlation between serum and SF AAT in all samples tested and no correlation for AGP in 5 out of 7 samples tested (Fig. 2A-B). The above data suggested significant differences between systemic *versus* local production of AGP or AAT.

#### Detection of AAT and AGP positive cells in blood and synovial fluid

AAT as well as AGP are synthesized predominantly in the liver, but also in extrahepatic tissues and cells, including neutrophils, monocytes and macrophages (Boskovic & Twining, 1998; Fournier *et al.*, 2000). To investigate the extrahepatic AAT production in RA patients we performed immunocytochemistry on cell smears prepared from blood or SF from RA patients to detect intracellular AAT. The immunocytochem-

istry analysis revealed increased number of AAT positive cells as well as AGP positive cells in both active RA and active SLE patients (Fig. 3A-D). This fact suggests significant differences between serum or SF AAT extracellular levels *versus* AAT intracellular levels during active phase of disease. Furthermore, during inactive RA or SLE the number of AGP or AAT positive cells in blood remains constant (Fig. 3A-B) suggesting that additional factors or modulation of AAT or AGP activity are involved directly or indirectly in disease development. In paired blood and SF samples AAT or AGP positive PMN cells were significantly higher in SF than in blood (Fig. 3C-D). The morphology of AAT or AGP positive cells isolated from either serum or SF of active RA is similar and belongs to the neutrophils as well as macrophage-monocyte lineage (Fig. 3E-G). The above data show significant differences between systemic *versus* local AAT and AGP production in RA suggesting potential local role of AAT or AGP on the development of the disease.

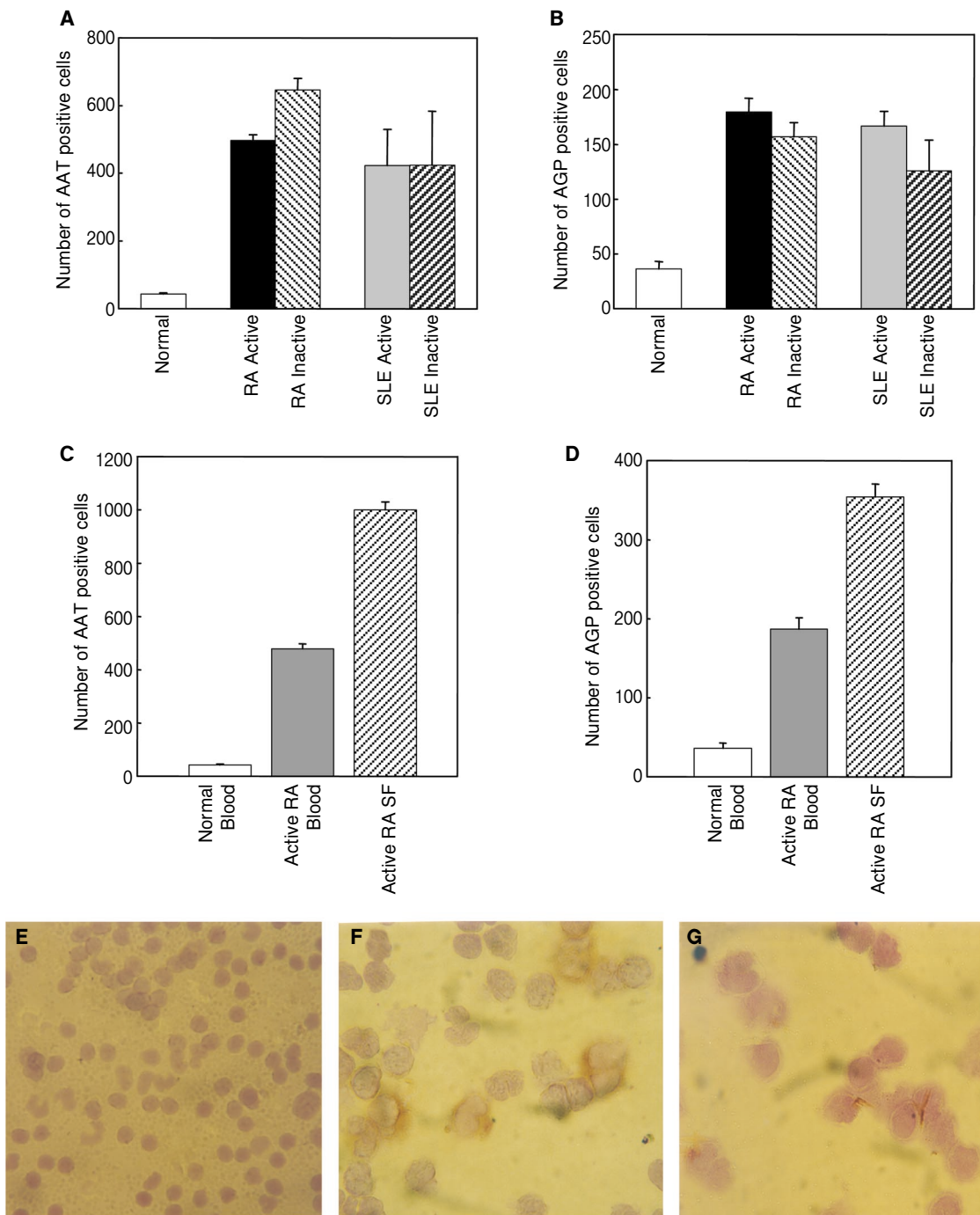


FIG. 3. Determination of intracellular AAT or AGP in cells isolated from blood or synovial fluid. AAT or AGP positive cells were determined by immunocytochemistry on cell smears prepared from blood or synovial fluid. Panel A-B: Number of AAT or AGP positive cells in the blood of normal or RA and SLE patients during either active or inactive phase of disease. Panel C-D: The histograms shows the number of AAT or AGP positive cells in paired samples prepared from blood and synovial fluid isolated from RA patients during the active phase of disease. Bars represent mean  $\pm$  S.E.M. Panels E-G: The photographs show the morphology of AAT or AGP positive cells in the blood of normal (panel E) or active RA (panel F) and synovial fluid of active RA (panel G) individuals.

TABLE 1. Determination of IL-1 $\beta$  and TNF $\alpha$  levels in serum and synovial fluid of RA patients during the active and inactive phase of disease

	Serum (ng/ml)			Synovial Fluid (ng/ml)		
	Normal	RA Active	RA Inactive	Normal	RA Active	RA Inactive
IL-1 $\beta$	1.040 $\pm$ 0.42	33.77 $\pm$ 11.12*	46.21 $\pm$ 14.02*	ND	117.10 $\pm$ 44.09	ND
TNF $\alpha$	0.997 $\pm$ 0.5	13.91 $\pm$ 0.93*	7.25 $\pm$ 1.4*	ND	113.27 $\pm$ 39.16	ND

ND: Non determined,

\* two-tailed Student *t*-test  $p < 0.05$

*Detection of cytokine levels in serum and SF*

The cytokines IL-1 $\beta$  and TNF $\alpha$  are increased significantly in SF and serum in active RA (Table 1) confirming previous studies (Duff, 1993). However, during inactive phase RA cytokine levels do not decrease. This could at least in part be attributed to the fact that the medications used for the treatment of arthritis affects factors that participate in arthritis development but not be involved in cytokine regulation.

*Detection of AAT pool levels in serum and synovial fluid of normal and arthritic rats – effect of HBV treatment*

Since SF samples from healthy control or inactive RA groups were not included in the above study, we expanded our experiments using the animal model of adjuvant arthritis. Honey bee venom was also used in our experiments to suppress arthritis development. As well as with human normal serum, elevated AAT levels also detected in normal rat serum (Fig. 4A-B). In arthritic rat serum, AAT pool levels do not change

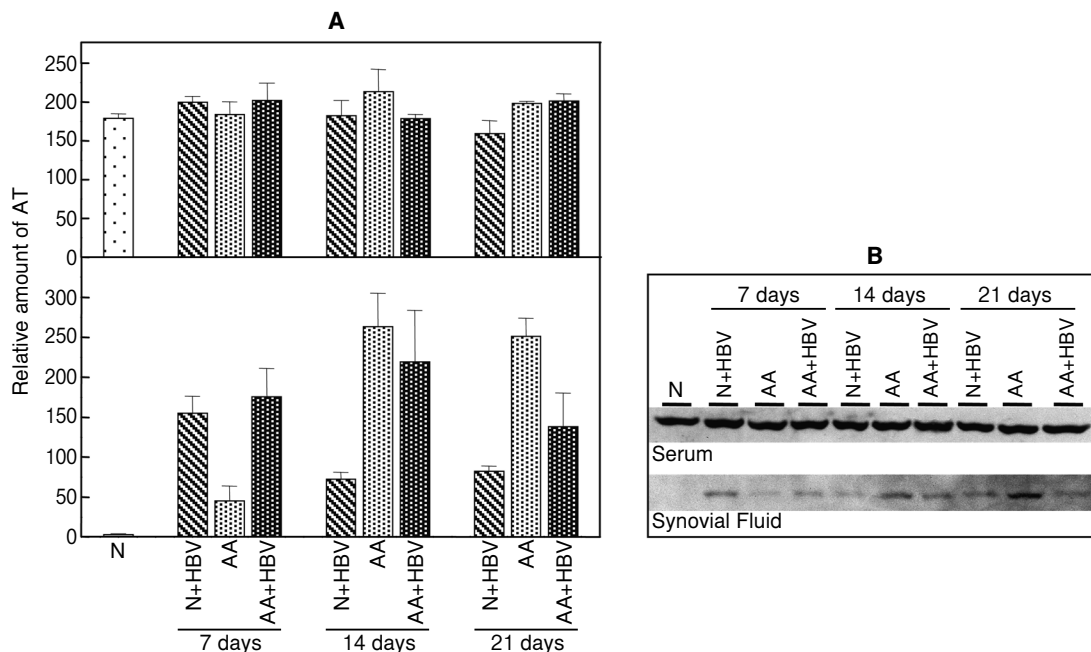
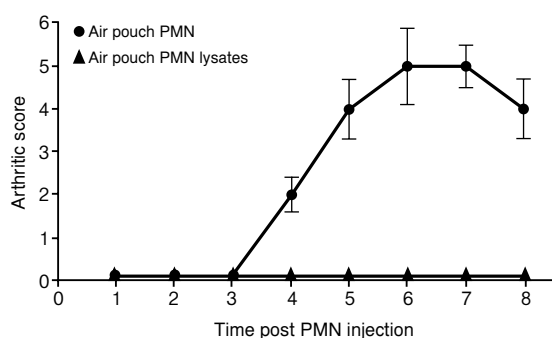


FIG. 4. Effect of honey bee venom on AAT pool levels in serum and synovial fluid isolated from normal and adjuvant arthritic rats. HBV (0.5 mg/Kg bw) was administered intramuscularly day by day for 3 weeks and at the indicated time post interval serum and synovial fluid subjected to SDS-page and immunoblotting. Panel A: The histogram is one out of three independent experiments and shows the relative amount of AAT in serum and synovial fluid of normal and AA rats treated or not treated with HBV. Each bar represents the mean  $\pm$  S.E.M of 5 rats. Panel B: Representative SDS-page and immunoblotting of serum and synovial fluid samples presented in panel A.

through the development of disease or when suppression of arthritis occurred by HBV administration (Fig. 4A-B). In normal rat SF, AAT pool levels are not detectable. However, in SF of AA rats AAT pool levels are increased with disease progression suggesting that AAT may be involved in the mechanism of AA development. Moreover, HBV mediates suppression of AA development and regulates differentially AAT production in serum and SF. Serum AAT pool levels are not affected by HBV treatment in either normal or AA rats while are increased in SF of AA rats at the early stages of disease development and decreased at the late stages of disease. Furthermore, HBV administration in normal or AA rats results in differential regulation of AGP production in serum versus SF (Vasiliadou K. personal communication). The above data show that the profile of serum AAT pool levels in RA and AA in rats is similar suggesting that this may also occur in SF. Local production of AAT in SF of AA rats may have a dual and distinct immunomodulatory effect when is expressed at the early stages or at the late stages of disease development.



#### Treatment of air pouches with FCA

The number of cells isolated from normal or AA rat synovial cavity is very low and thus we were not able to determine the number of AAT positive synoviocytes. To examine whether AAT induction and accumulation in SF of arthritic rats is a phenomenon accompanied arthritis development or is due to systemic activation of AAT by FCA we used the air pouch model that resembles synovium cavity. FCA administered in air pouch induces the onset of mild arthritis by 14-16 days post treatment (data not shown). Moreover, passive arthritis observed in normal rats injected in the footpad with PMN cells isolated from air pouches 24 hours post FCA treatment (Fig. 5). Lysates of air pouch PMN cells do not induce arthritis suggesting that inflammatory cells that accumulate in the air pouch membrane in response to FCA are involved in the mechanism of AA development. In addition, these data indicate similarities on AA development between synovial cavity and air pouch cavity.

FIG. 5. Passive transfer of arthritis to normal recipients using PMN cells isolated from air pouches 24 hours post FCA treatment. After harvesting from air pouch were seated on ice for 15 minutes to remove cell debris and FCA. After repeated washes with saline were counted and  $1.65 \times 10^8$  PMN cells were injected to the right hind footpad in 0,1 ml saline. Cell lysates prepared by repeated freezing-thawing cycles and heating at  $65^\circ\text{C}$  for 30 min were also included as control. Arthritic score was evaluated on arbitrary scale counting inflammations developed on limbs.

#### Detection of AAT and AGP pool levels in FCA treated air pouches

SDS-page and Western immunoblotting revealed that AAT pool levels as well as AGP pool levels in exudates isolated from FCA treated air pouches increased significantly by 7-10 days post FCA treatment (Fig. 6A-B). The time course of AAT production in FCA treated air pouch is similar with that observed in synovial fluid at the early stages of AA induction in rats (Fig. 4).

#### Effect of FCA on PMN accumulation, AAT, AGP, $IL-1\beta$ and $IL-6$ production in air pouches

FCA injection in air pouches increased the number of polymorphonuclear cell (PMN) that infiltrate the air pouch membrane and increased the volume of air pouch exudates (Table 2). The number of AAT or AGP positive PMN cells in exudates isolated from FCA treated air pouches is significantly increased (Table 2) following increased AAT or AGP pool levels in air pouch exudates. The AAT or AGP positive



TABLE 2. Effect of FCA on several inflammatory parameters in dorsal rat air pouch.

	Exudate Volume	Cell Number ( $\times 10^6$ )	AAT positive cells	AGP positive cells	IL-1 $\beta$ pg/ml	IL-6 pg/ml
<b>Normal</b>	0.2 $\pm$ 0.1	1.4 $\pm$ 0.7	21 $\pm$ 3	61 $\pm$ 2.9	13.49 $\pm$ 1.3	90.36 $\pm$ 1.2
<b>24 hours</b>	4.2 $\pm$ 0.7	9 $\pm$ 0.5	51 $\pm$ 4	152 $\pm$ 8.7	1600.87 $\pm$ 98.9	877.03 $\pm$ 76.2
<b>7 days</b>	10.7 $\pm$ 1.6	21 $\pm$ 1.7	167 $\pm$ 18	1203 $\pm$ 104.8	754.90 $\pm$ 22.4	2455.80 $\pm$ 52.3
<b>10 days</b>	15.1 $\pm$ 1.2	37 $\pm$ 2.3	332 $\pm$ 22	967 $\pm$ 85.3	875.20 $\pm$ 37.8	1927.76 $\pm$ 42.6

Each value differs from normal values with statistical significance of at least  $p < 0.05$

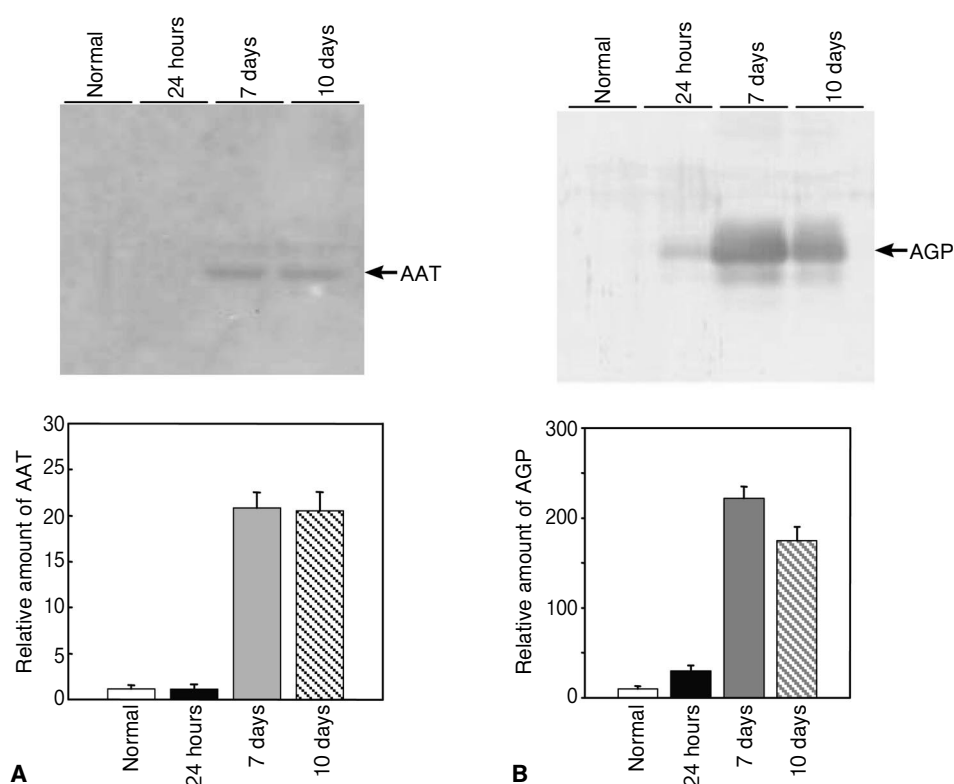


FIG. 6. Effect of FCA on AAT and AGP pool levels in air pouch exudates. Panel A-B: The upper part of panels present the Western analysis showing the AAT (panel A) and AGP (Panel B) pool levels in air pouch exudates several time post FCA treatment. The histograms in lower part of panels show the relative amount of AAT or AGP in air pouches. Each bar represents the mean  $\pm$  S.E.M. of 5 rats.

cells morphologically belong to monocyte lineage and polymorphonuclear neutrophils (Fig. 7A-B). Furthermore, IL-1 $\beta$  and IL-6 levels in air pouch exudates are increased early post FCA treatment suggesting that these proinflammatory cytokines contribute to initiation and maintenance of inflammatory response. Since serum AAT levels are not affected by FCA treatment the above data indicate that the source of AAT detected in FCA treated air

pouches or synovial fluid of AA rats are due to PMN cells that infiltrate the air pouch lining tissue or the synovial membrane.

#### *Effect of FCA on air pouch lining tissue*

It has been shown that air pouch membrane resembles synovial membrane (Sedwick *et al.*, 1983). Immunocytochemistry on paraffin sections of air pouch membrane using specific anti-AAT or anti-AGP antibodies

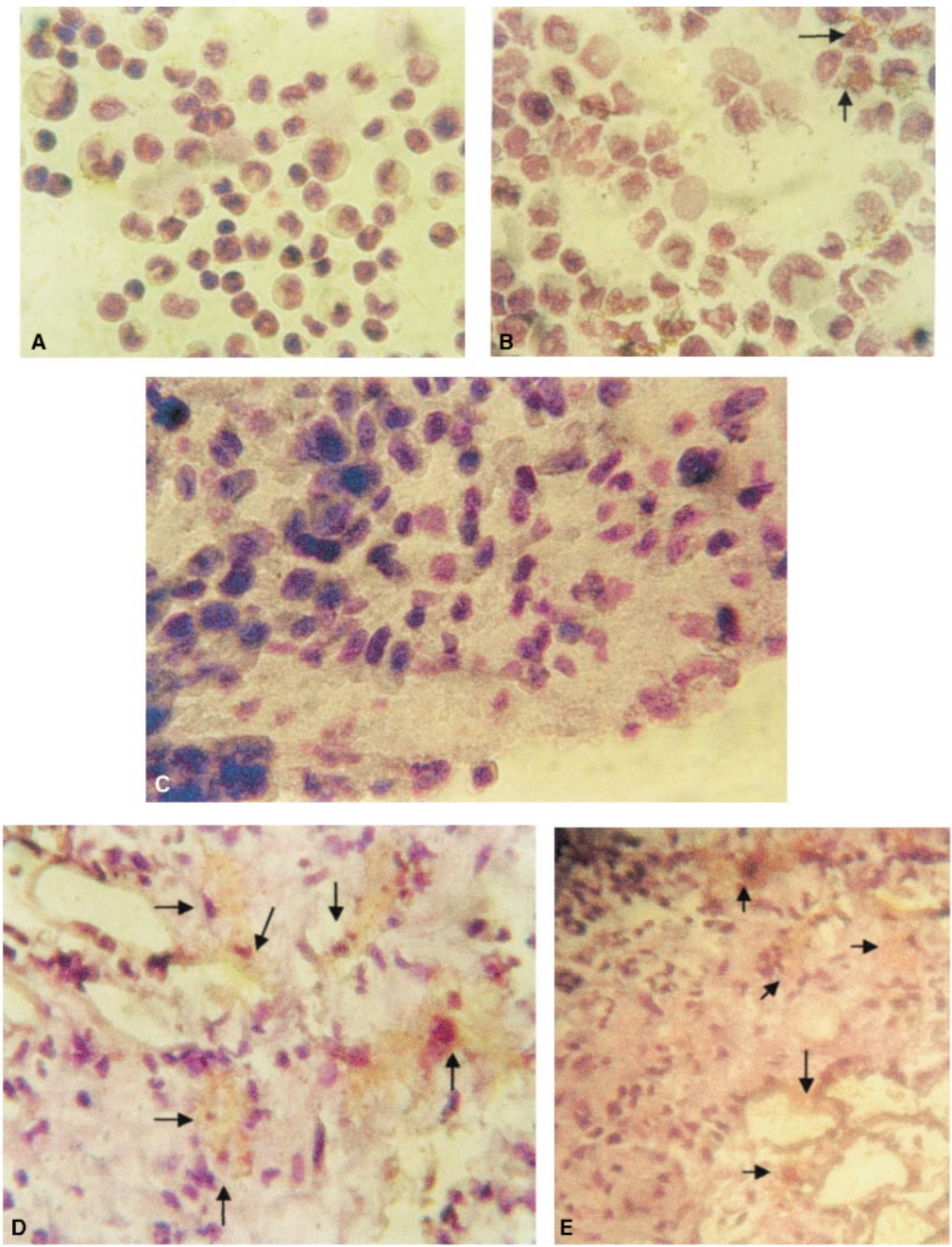


FIG. 7. Immunocytochemistry showing the morphology of AAT positive cells in air pouches exudates and in air pouch lining tissue. Panel A-B: AAT positive cells isolated from the air pouch exudates of normal (panel A) or FCA treated (panel B) rats. Panel C: Immunocytochemistry on air pouch lining tissue isolated from non-treated air pouches. Panel D-E: Immunocytochemistry showing clusters of AAT positive cells in FCA treated air pouch lining tissue (panel D). Notice the destruction of the tissue in comparison with non treated air pouch lining tissue (panel C). In panel E endothelial cells surrounding vessels positive with AAT immunostaining.

ies revealed clusters of AAT or AGP positive cells in FCA treated air pouches (Fig. 7). Furthermore, FCA treatment results in gradual destruction of the air pouch membrane (Fig. 7D). Morphologically the AAT or AGP positive cells belong to the macrophage-monocyte lineage cells. In addition, AAT or AGP

positive endothelial cells surrounding vessels were observed in specimens from air pouch membrane 7 or 10 days post FCA treatment suggesting production of AAT or AGP by endothelial cells or increased AAT or AGP permeability.

## DISCUSSION

We have studied the systemic and local production of AAT and AGP in serum and synovial fluid respectively of RA patients during both active and inactive phase of disease and this response compared to the respective response in AA rats. We observed potential differential regulation and activity of AAT and AGP in active RA and similar data observed also in AA rats. In the present study, we provide evidence from *in vivo* experiments in AA rats that AAT may suppress AA induction by exhibiting biological activity independent of inhibition of serine proteases.

The data in this study concerning human RA, although performed on relatively small sample sizes, some marked differences between systemic versus local AAT or AGP production and/or activity were observed. Intracellular AAT levels in both blood and SF PMN cells during active phase of RA are increased, despite the fact that AAT pool levels in serum and SF do not change suggesting increased AAT production or activity. The AAT-metalloproteinase complexes may activate monocytes or neutrophils to secrete cytokines and indeed increased intracellular AAT levels are associated with increased IL-1 $\beta$  and TNF $\alpha$  (Table 1). Furthermore, it has been suggested that the intracellular regulation of the protease-antiprotease balance may also play a role in cellular migration (Paakko *et al.*, 1996). In the air pouch model, increased number of AAT or AGP positive cells are detected early post FCA administration suggesting that increased AAT or AGP intracellular levels may be an early event in AA development. It has been shown that upon activation PMN cells rapidly secrete stored AAT or AGP (Paakko *et al.*, 1996; Polland *et al.*, 2005). Based on the above we suggest that increased intracellular AAT or AGP in blood may serve as a marker to predict subsequent chronic inflammatory responses in peripheral organs and tissues such as synovium cavity or lungs.

In acute and chronic inflammatory processes, anti-proteases such as AAT are thought to play a major role in minimizing the harmful effects of extracellular protease activity in inflamed tissue (Churg *et al.*, 2003). HBV treatment of AA rats results in increased AAT pool levels in synovium cavity and subsequent remission of arthritis. The increase of synovial fluid AAT pool levels it is not the result of inflammatory response in synovial tissue since observed before the onset of arthritis. In addition, the

same results were also observed in SF of HBV treated normal rats as early as 7 days post treatment and in the absence of any inflammation. HBV administration or bee stings suppressed AA development only when administered before the onset of arthritis (Hadjipetrou-Kourounakis & Yiangou, 1988) occurred by day 9 post FCA treatment suggesting that HBV exerts its immunosuppressive effects on the mechanism of AA induction at the early stages. Based on the above we propose that locally produced AAT in synovium is involved in the mechanism by which HBV mediates AA suppression and that this activity is independent of its antiproteolytic action. Our hypothesis is further supported by recent studies showing that native AAT inhibits IL-1 $\beta$  and TNF $\alpha$  production by human monocytes activated *in vitro* by LPS while it increases the anti-inflammatory IL-10 cytokine (Janciauskiene *et al.*, 2004). The latter is further supported by our data showing that the appearance of AAT by 7 days post FCA treatment is accompanied by reduction of IL-1 $\beta$  (Table 2). Despite the fact that AAT production in the AA rat's synovium associates with arthritis progression and severity, AAT fails to ameliorate the consequences of inflammation, suggesting that AAT may exist in inactivated form. Neutrophil elastase is thought to be involved in the cartilage destruction occurring in rheumatoid arthritis despite the local presence of  $\alpha$ -proteinase inhibitor while IgA-AAT complex tightly inhibits neutrophil elastase (Scott *et al.*, 1998). Oxidation of AAT results in inhibition of IgA-AAT complex formation with no inhibition of elastase activity (Scott *et al.*, 1998). HBV was found to possess considerable anti-oxidant and hydroxyl radical scavenging activity (Rekka *et al.*, 1990) suggesting that part of the HBV mediated AA suppression at the late stages of disease development may be due to inhibition of AAT oxidation and subsequent reduction of metalloproteinase activity.

The immunomodulatory or immunosuppressive activity of AGP has been recently reviewed (Fournier *et al.*, 2000; Hochepped *et al.*, 2003) and some studies show that the immunomodulatory activity of AGP depends on its glycosylation (Costello *et al.*, 1979; Fournier *et al.*, 2000). In our study, AGP was increased in both serum and synovial fluid of active RA (Fig. 1, Fig. 2) as well as in AA rats (data not shown). Furthermore, HBV regulates local or systemic AGP production differentially and exhibits both immunomodulatory and immunosuppressive effects on AA development (Vasiliadou K personal

communication). Additionally, both AGP positive cells and AGP protein pool levels in air pouches follows that of AAT. Taken together our data indicate that also AGP has dual immunomodulatory activity when is locally or systemically produced. Thus, is possible that several acute phase proteins to participate in different phases of RA induction in man or AA in rats.

Our study concerning AAT production and activity in human RA correlates only with the late stages of disease development of AA in rats and thus the clarification of the involvement of AAT and/or AGP in the mechanism of RA in man must await the determination of specific markers in asymptomatic individuals. However, Nielen *et al.* (2004) has shown in a study that lasted 15 years that elevated CRP levels increased significantly 2-5 years before the onset of RA (Nielsen *et al.*, 2004).

The mechanisms regulating the recruitment of leukocytes in to the joint in inflammatory arthritis models are not fully understood. Our immunocytochemistry analysis revealed that endothelial cells surrounding air pouch vessels were immunostained for AAT and AGP. Fucosylated structures of AGP are suitable ligands for E-selectin affecting vascular permeability (Simon & Goldsmith, 2002) and pulmonary vascular endothelial cell binding of AAT derived from plasma has been identified (Aldonyte *et al.*, 2004). However, further research appears to be necessary to clarify the modulatory effects of AAT, AGP or HBV in rat arthritic joints. Inflammation initiated in air pouch or synovial fluid share similarities and since the air pouch offers more cells, tissue as well as exudates instead of joints may used for further studies in order to understand the inflammatory responses in arthritic rat joints.

FCA administration results in the destruction of the air pouch lining tissue, suggesting increased proteolytic activity similar to that one in the arthritic synovium. Glycosylation of AGP correlates with the increased collagen synthesis as well as with the destruction of articular cartilage (Haston *et al.*, 2002). Moreover, synovial fluid AGP versus normal serum AGP exhibits diminished inhibition of collagenase-3 activity versus normal serum AGP (Haston *et al.*, 2003). All of the above in combination with the reduced AAT activity in the air pouch may be due to air pouch lining tissue destruction.

In a disease so complex as RA in man or AA in rats and with AAT and AGP to possess, such a plethora of activities it is unreasonable to expect a single

mechanism of action. However, the data presented in this study suggest that local AAT production at the early stages of arthritis development leads to inhibition of disease while oxidation of AAT after the onset of arthritis results in its inactivation with subsequent increased proteolytic activity and destruction of cartilage.

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