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α 1-Acid glycoprotein production in rat dorsal air pouch in response to inflammatory stimuli, dexamethasone and honey bee venom

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Abstract

This study shows the rapid and differential production of the 40–43 kDa and the 70–90 kDa α 1-acid glycoprotein (AGP) fucosylated glycoforms after treatment of the dorsal air pouch with bacterial lipopolysaccharide (LPS), HgCl₂ or Freund's complete adjuvant (FCA). The 40–43 kDa and the 70–90 kDa AGP production is peaked 1–3 h post-LPS treatment. We observed that the responses to LPS and FCA are similar in that both AGP isoforms are induced whereas they differ in that the FCA exhibits a 6 h lag period. The response to HgCl₂, however, exhibits the specific biphasic induction only of the 40–43 kDa AGP. The serum 40–43 kDa AGP glycoform gradually increases in response to all of the above stimulants and peaks by 24 h post-treatment. The increase of the 70–90 kDa AGP levels in the air pouch occurs in association with the accumulation of polymorphonuclear (PMN) cells while dexamethasone (DEX) increases only the 40–43 kDa AGP production in the absence of PMN accumulation. Macrophage–monocyte lineage cells forming the air pouch lining tissue may potentially be the cells that secrete the 40–43 kDa AGP while polymorphonuclear cells that infiltrate the air pouch secrete the 70–90 kDa AGP. The 40–43 kDa and 70–90 kDa AGP production induced by LPS in the air pouch precedes that of interleukin-1 (IL-1) or interleukin-6 (IL-6) while the 40–43 kDa AGP glycoform potentially increases IL-6 production by air pouch PMN exudate cells. These significant differences suggest a local pro-inflammatory role of AGP. Honeybee venom suppressed arthritis development and exhibited differential local or systemic regulation of AGP in serum vs. air pouch exudate or synovial fluid. This study with the air pouch model of facsimile synovium tissue suggests that local α 1-acid glycoprotein (AGP) production may contribute to pro-inflammatory and anti-inflammatory activities during the local acute phase response or during chronic inflammatory stress as in arthritis.

Keywords

α -Acid glycoprotein; Air pouch; Synovial fluid; Cytokines; Inflammation; Adjuvant arthritis

Introduction

Eukaryotes possess natural defence mechanisms by which restorative processes are initiated in response to several stress stimuli. Acute phase reactants (APR) are the products of a distinct family of genes that immediately respond to several chemical and physical stress factors or pathological insults such as inflammatory agents, infection, myocardial infarction, surgery, burns, neoplastic disease, heavy metal poisoning or heat shock (Kushner, 1982; Fey and Fuller, 1987; Yiangou et al., 1991, 1998, Yiangou and Papaconstantinou, 1993a). The $\alpha 1$ -acid glycoprotein (AGP) gene is a member of the family of APR genes, and its expression in the liver is activated by various chemical or physical stress stimuli such as Freund's complete adjuvant (FCA), bacterial lipopolysaccharide (LPS), heavy metals and heat shock (Kushner, 1982; Fey and Fuller, 1987; Yiangou et al., 1991, 1998). The AGP gene is also inducible by glucocorticoids (Baumann et al., 1983) and mediators of the inflammatory response such as IL-1, IL-6 and tumour necrosis factor (Fey and Fuller, 1987). Although AGP is considered to function as a natural pro-inflammatory and anti-inflammatory agent (Hochebied et al., 2003), the mechanism of these biological functions remains unclear. Several biological functions attributed to AGP (Fournier et al., 2000; Hochebied et al., 2003) include inhibition of platelet aggregation activity, polymorphonuclear neutrophil activation, chemotaxis and oxidative metabolism (Costello et al., 1979; Vasson et al., 1994). In addition, an AGP function that is unique among other acute phase response proteins suppresses IL-1 activity *in vitro* by increasing IL-1 receptor antagonist (IL-1Ra) synthesis (Bories et al., 1990).

The pro-inflammatory or anti-inflammatory role of AGP is linked to its ability to increase LPS-mediated cytokine production by monocytes–macrophages (Bories et al., 1990) *in vitro* suggesting that it may play an important role in the regulation of the immune response. Alterations of AGP glycoforms have been demonstrated in patients with inflammation (Brinkman-van der Linden et al., 1988) and rheumatoid arthritis (Elliott et al., 1997); however, their pathophysiological significance remains unclear. In addition, fucosylation of IgG heavy chains (Gornik et al., 1999; Nandakumar et al., 2007) and N-glycan microheterogeneities in AGP (Mackiewicz et al., 1987) have also been reported in rheumatoid arthritis patients, although the role of these post-translational modifications in arthritis development is not known.

The *in vivo* and *in vitro* expression of the AGP gene in response to LPS, prostaglandin-E₂ (PGE₂) or dexamethasone (DEX) occurs in several tissues and cells, such as lung (Crestani et al., 1998), kidney (Kalmovarin et al., 1991), rat intestinal epithelial cells (Boudreau et al., 1998), alveolar type II epithelial cells (Crestani et al., 1998) and alveolar macrophages (Fournier et al., 1999). AGP is also produced in infarcted myocardium (Poland et al., 2005) by infiltrating polymorphonuclear (PMN) cells suggesting an endogenous feedback inhibitory response to excessive inflammation. Thus the localized expression of AGP, at the site of the initial acute phase response, suggests that it may play a protective role against the deleterious effects of inflammation.

We previously demonstrated that in rats, AGP accelerates the onset of adjuvant arthritis (AA) and increases the severity and duration of the disease and that honeybee venom (HBV) completely suppressed AA development in rats while reducing liver AGP mRNA levels at the early stages of disease development (Hadjipetrou-Kourounakis and Yiangou 1984, 1988; Yiangou et al., 1993b). Our observations provide us with the biological system to study the mechanisms of the local action of AGP on AA development in rat joints and of the protective action of HBV. For example, 6-day-old rat dorsal air pouch develops a phagocytic and fibroblast-like cellular lining followed by organized vasculature, which acts as a mechanical barrier that retains the products of the inflammatory response (Sedgwick et al., 1983; Isaji and Naito, 1992). Importantly, the air pouch resembles the synovium tissue (Sedgwick et al., 1983) behaving as a facsimile synovium, while FCA activated air pouch PMN cells induce

mild arthritis in normal recipients (Pantazidis et al., 2005). Thus, the rat dorsal air pouch provides an excellent model to study localized effects of AGP and HBV on the inflammatory response in an *in vivo* and *in vitro* system that mimics AA. In these studies we have focused upon the effects of such inflammatory agents as LPS, FCA and HgCl₂ on AGP production in air pouches, identify those cells that produce AGP in response to these reagents, and the effects of HBV on the inflammatory response of the air pouch.

Materials and methods

Animals

Male Fisher-344 rats (130–180 g) were inbred from our colony, housed under standard laboratory conditions (12 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, which are in accordance with Greek national legislation and with EC ethical regulations. All groups included in the present study consisted of five animals (unless otherwise indicated) and each experiment was repeated at least two times.

Air pouch formation and treatments of animals

Air pouches were created on the back of rats as described previously (Sedgwick et al., 1983) by subcutaneous injection 20 ml sterile air, respectively. The formation of equal size of air pouch in each animal was achieved by refilling the 3- and 5-day-old air pouches with the appropriate volume of air and confirmed using a vernier. Subsequently, LPS [3 mg/kg body weight (BW), Sigma, St. Louis, MO] or HgCl₂ (10 mg/kg BW) or DEX (10⁻⁶ M 25 µg/kg BW, Sigma, St. Louis, MO) or both LPS and DEX (at the aforementioned doses) were injected in the air pouches after being dissolved or suspended in 2 ml saline. Air pouches were also injected with 0.1 ml Freund's complete adjuvant (FCA) containing 0.6 mg heat inactivated *Mycobacterium butyricum* (DIFCO, Detroit, MI). At the indicated time post-treatment, blood was collected by cardiac puncture with or without heparin. The serum was immediately stored at -30 °C while the air pouch exudate and the blood or air pouch exudate PMN cells were collected as described below.

Adjuvant arthritis (AA) was induced in rats as described previously (Yiangou et al., 1993b). Crystalline HBV (kindly provided by Mr. C. Mraz, Champlain Valley Apiaries, Middlebury, NY) was dissolved in saline and injected (0.5 mg/kg BW) intramuscularly or in the air pouch every other day (three doses) in normal or arthritic rats. At the indicated time post-treatment, serum and air pouch exudate were collected as described above or below, respectively. Synovial fluid was collected from the knee joint of rats after injection of 50 µl PBS using a Hamilton syringe.

Isolation of air pouch exudates, PMN cells and lining tissue

The exudates and the PMN cells accumulated in the air pouches were harvested by injecting 2 ml saline and recollecting all contents. After centrifugation, the supernatant was collected and hence referred to as the "air pouch exudate." The cell pellet containing the air pouch exudate PMN cells was washed twice in saline and immediately used for immunocytochemistry or for lysis. PMN cells were counted using a haemocytometer while cell viability was determined by trypan blue exclusion.

PMN lysates were prepared as described previously (Poland et al., 2005). Blood and air pouch exudate PMN cells were suspended in 0.5 ml 50 mM Tris-HCl (pH 8) supplemented with 1% (w/v) Triton X-100 and a protease inhibitor mixture containing 1 µg/ml each of antipain, chymostatin, leupeptin and pepstatin. The samples were freeze-thawed three times, followed by centrifugation at 14,000 g for 30 min at 4 °C. Lysate fractions were either stored at -30 °C

until further use or immediately treated for 24 h with 1000 U of N-glycosidase F (PNGase, Sigma, St. Louis, MO) to release N-linked glycans according to the manufacturer's instructions.

The air pouch lining tissue was separated from the skin and was either processed immediately for immunocytochemistry or homogenized in a buffer containing 50 mM Tris-HCl (pH 8) supplemented with 1% (w/v) Triton X-100 and a protease inhibitor mixture and treated as previously described for PMN cell lysates. Protein concentrations were determined by the method of Bradford (1976).

AGP detection and molecular weight analysis

Air pouch exudates were further concentrated using Amicon-Centricon microconcentrators, and AGP concentrations were then determined by single radial immunodiffusion as previously described (Arnold and Meyerson, 1990), using polyclonal rabbit anti-rat AGP for detection [specific polyclonal antibodies raised against rat AGP (Sigma, St. Louis, MO) were prepared in rabbits].

The apparent molecular weight of different AGP forms determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Pantazidis et al., 2005). Briefly, air pouch exudate (7.5 μ l), serum (7.5 μ l of 1/50 dilution), synovial fluid (7.5 μ l), lysates prepared from PMN cells (20 μ g) or from air pouch lining tissue (20 μ g) were electrophoretically separated on a 10% SDS-polyacrylamide gel. Samples containing various concentrations of purified rat AGP (Sigma, St. Louis, MO) were also included as an internal control. The proteins were then electrotransferred to a Westram PVDF membrane (0.45 μ m pore size) in a buffer containing 25 mM Tris-HCl, 192 mM glycine and 20% methanol. Membranes were then subjected to Western blotanalysis (Yiangou et al., 1998) using as primary antibodies specific polyclonal antibodies raised against rat AGP. The resulting Western blotanalysis bands in each sample scanned, and AGP concentration was determined by reciprocal analysis.

Immunocytochemistry

PMN cells (2×10^5) isolated from air pouch exudates were loaded on slides using a cytocentrifuge, fixed for 1 min in 4% paraformaldehyde and stored in 70% ethanol at 4 °C. Small pieces of air pouch lining tissue were initially fixed in 4% paraformaldehyde for 24 h and then embedded in paraplast, sectioned at 5 μ m thickness onto gelatincoated slides and treated as previously described (Yiangou et al., 1998). Immunocytochemistry of air pouch exudate cells or paraplast sections was performed as previously described (Avramidis et al., 2002). The AGP producing cells were detected using specific anti-AGP antibodies (1:500). As an independent 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. After repeated washes with PBS, slides were incubated for 5 min in 0.5 mg/ml 3,3-diaminobenzidine-tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) in PBS (pH 7.4 for air pouch lining tissue specimens or pH 6 for air pouch exudate PMN cells), containing 0.01% H₂O₂. Finally, the slides were counterstained with Giemsa solution, and positive cells were determined microscopically. Two independent investigators performed these microscopic studies, and the consensus of the two investigators was used as the final scoring.

Cytokine production

For IL-1 or IL-6 production plastic adherent cells (5×10^6) isolated from the air pouch exudates of untreated normal rats were cultured in RPMI 1640 with or without a suboptimal dose of LPS (5 μ g/ml) for 48 h in the presence or absence of AGP (100 μ g/ml). The supernatants were passed through a 0.22 μ m filter and stored at -30 °C.

IL-1 and IL-6 assessment

The co-stimulatory thymocyte assay was used to detect the IL-1 activity as previously described [30]. IL-6 levels in LPS treated air pouch exudate cells were detected also using the Quantikine M Murine (rat IL-6) kit (R&D Systems) according to the manufacturer's instructions. Cytokine levels were determined by reciprocal analysis as previously described and expressed as percentage of normal controls (Yiangou and Hadjipetrou-Kourounakis, 1989; Yiangou et al., 1998).

ThinCert™ (Greiner Bio-One GmbH, Germany) culture inserts with translucent membranes and 0.4 µm pores were used to co-culture air pouch exudate cells isolated from non-treated air pouches of normal mice with the air pouch exudate PMN cells or the air pouch membrane cells isolated from the air pouches of mice 30 min post-LPS injection. The cells forming the air pouch membrane were isolated by mechanical homogenization using forceps. Briefly, 0.6×10^6 normal non-treated air pouch exudate cells were allowed to attach to the bottom side of the ThinCert for 6 h in complete RPMI 1640 medium containing 5% FCS. The ThinCert was then inverted in a culture plate in the presence of RPMI 1640 containing a suboptimal dose of LPS (5 µg/ml). The cells (1.5×10^6) forming the air pouch membrane or the air pouch exudate PMN cells isolated from the LPS treated air pouches were then added to the upper side of the ThinCert. Co-culture was maintained for 24 h at 37 °C and 5% CO₂ followed by immunocytochemistry on the underside of the ThinCert to detect IL-6-positive cells.

Immunocytochemistry was performed as described above using specific anti-IL-6 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz CA, USA) as primary antibody and anti-goat FITC conjugated antibodies (Sigma, St. Louis, MO, USA) as secondary antibody. The number of positive cells was determined by fluorescence microscopy and counted in 10 fields at 400 magnification. To confirm specificity of the immunodetection the samples were incubated only with the secondary antibody, and the number of positive cells was excluded from the number of cells estimated for testing samples. The final scores are the results of readings rendered by two independent investigators.

Statistical analysis

The results are reported as the mean±SEM. Multiple comparisons were performed by one-way ANOVA followed by Tukey's test, and statistical significance was accepted at values of $P < 0.05$.

Results

Effect of LPS, FCA or HgCl₂ on AGP production in air pouch and serum

Exudates isolated from LPS treated air pouches exhibit a response in which the 40–43 kDa and 70–90 kDa AGP isoforms were elevated at 1–3 h (Fig. 1A). On the other hand, exudates from FCA treated air pouches showed a lag period in which the 40–43 kDa and 70–90 kDa AGP isoforms are detected after 6 h and peak at 24 h (Fig. 1A). The exudates from HgCl₂ treated pouches, however, exhibited a strong biphasic response involving just the 40–43 kDa AGP, the first being at 1–3 h and the second at 12–24 h. Thus, the responses to LPS and FCA are similar in that both AGP isoforms are induced whereas they differ in that the FCA exhibits a 6 h lag period. The 6-day-old air pouch fluids of control rats contained low AGP pool levels ranging from 2.2 to 5.3 µg/ml. At the maximum of induction (Fig. 1A, C) the responses to LPS (12.1–17.8 µg/ml) and HgCl₂ (17.3–22.1 µg/ml), and the response to FCA (14.2–21.3 µg/ml) exhibit a similar level of AGP. Similar concentration of total AGP was obtained when the same samples tested by radial immunodiffusion assay (data not shown). However, in the serum, only the 40–43 kDa AGP levels increase in response to LPS, HgCl₂ or FCA (Fig. 1B). Serum of normal or treated rats contained higher levels of AGP than the respective in air pouch. Serum of normal rats contained 0.32–0.67 mg/ml AGP while serum isolated from

LPS, HgCl₂ or FCA treated rats at the maximum of induction contained similar levels of AGP ranging from 1.35 to 2.56 mg/ml. Furthermore, we observed a delayed appearance of AGP in the serum, which is detected at 6–24 h (Fig. 1B and C). The above data suggest that the 70–90 kDa AGP is specific to the air pouch exudates while the 40–43 kDa AGP is observed in both the exudates and serum. The data also suggest a lag period in the appearance of the 40–43 kDa AGP in the serum (6–24 h) which is characteristic of a synthesis–secretion system.

The identification of sites of AGP isoform synthesis

The data in Fig. 2A suggest that resting PMN cells in the air pouch may be the source of the high molecular weight AGPs. Western blot analysis of lysates of non-treated air pouch PMNs contain the high molecular weight AGPs not seen in the exudates (Fig. 2A). In addition, immunoblots of AGP forms in blood PMN cell lysates isolated 3 h post-LPS injection resemble that of normal air pouch PMN cell lysates (data not shown). On the other hand, the 40–43 kDa AGP is detected mainly in homogenates of air pouch lining tissue while it is undetectable in PMN cell lysates isolated from non-treated or LPS treated air pouches (Fig. 2A). Furthermore, the PMN cell lysates isolated from LPS treated air pouches contain a unique 50–60 kDa AGP that is not detected in air pouch exudates. All AGPs detected in normal or LPS treated air pouch exudates are digested by PNGase F indicating that they represent fucosylated AGP glycoforms (Fig. 2B). Thus, our data suggest that the AGPs detected in treated air pouches may be due to the differential glycosylation of locally produced AGP. We conclude that the air pouch lining tissue produces mainly the 40–43 kDa AGP while the air pouch PMN cells produce the 70–90 kDa AGPs.

Detection of AGP producing cells in air pouch

The accumulation of the 70–90 kDa AGP in air pouch exudates (Fig. 1A) correlates with the increase in the number of PMN cells accumulated in the air pouches early post-LPS or FCA treatment (Fig. 3A). In addition, immunocytochemistry revealed that the maximum number of AGP-positive PMN cells (Fig. 3A–C) in air pouch exudates after LPS or FCA treatment correlates with both the maximum number of PMN cells and the increased level of 70–90 kDa AGPs in the air pouch exudates.

Immunohistochemistry on paraffin sections of unchallenged air pouch lining tissue revealed low numbers of AGP-positive cells (Fig. 3D). At 2 h post-LPS treatment or at 24 h post-FCA treatment, monocyte–macrophage lineage clusters of AGP-positive cells are observed only inside the lining tissue and not in vascular endothelial cells (Fig. 3E). The morphology of the air pouch exudate cells and the air pouch lining tissue (Fig. 3B–E) is similar with that previously described (Isazi et al., 1989; Isaji and Naito, 1992). These data suggest that, at the early stages of the inflammatory response, LPS activates both PMN and macrophage-monocyte lineage cells present in the air pouch exudate or lining tissue, respectively, to produce and secrete AGP.

Effect of DEX on AGP production in air pouch and serum

Glucocorticoids are involved in the regulation of hepatic AGP gene expression and are required for its maximum expression *in vivo* and *in vitro* (Baumann et al., 1983). Treatment of the air pouch with DEX results in a significant increase of only the 40–43 kDa AGP at 1 h post-treatment (Fig. 4A). In addition, DEX inhibits PMN cell accumulation in the air pouch (Fig. 4B), which is consistent with the data presented in Fig. 2A and Fig. 3A showing correlation between PMN cells and the 70–90 kDa AGP accumulation in the air pouch. Simultaneous administration of LPS and DEX in air pouch results in the inhibition of both PMN cell accumulation and 70–90 kDa AGP production and in a significant increase of the 40–43 kDa AGP (Fig. 4A). DEX administration increased the number of AGP-positive cells only in the air pouch lining tissue (Fig. 4C) and not in air pouch PMN cells (data not shown). These

results further support our previous observations that, upon activation, cells forming the air pouch lining tissue secrete the 40–43 kDa AGP while PMN cells infiltrating the air pouch secrete the 70–90 kDa AGPs.

Effect of LPS on IL-1 and IL-6 production in air pouch

IL-1 bioactivity (Fig. 5A) and IL-6 levels (Fig. 5B) in LPS treated air pouch exudates gradually increased reaching a maximum at 6 h post-treatment and then declined. Furthermore, DEX administration in the air pouch does not induce an increase of IL-1 bioactivity or IL-6 levels. Cytokines such as IL-6 and IL-1 are regulators of the AGP gene expression in the liver (Fey and Fuller, 1987) while AGP modulates cytokine production *in vitro* (Boutten et al., 1992). Our data show that there is no direct correlation between AGP accumulation (Fig. 1A) and IL-1 or IL-6 in LPS treated air pouch exudates since AGP was produced earlier than these cytokines. These results suggest that AGP may play a role in the induction of cytokine production in the air pouch.

Effect of AGP on cytokine production in LPS treated air pouch cells

We investigated whether AGP affects IL-1 or IL-6 production by LPS activated air pouch cells. Our data (Fig. 6A) show that AGP alone does not increase cytokine activity although it significantly increased the LPS induced IL-6 production in the air pouch. Co-cultivation experiments (Fig. 6B–D) revealed that only the cells forming the air pouch lining tissue exhibit the capacity to secrete factors that in turn increased the LPS mediated production of IL-6 by the normal air pouch resident cells. These observations suggest that the increased cytokine production in air pouch exudates (Fig. 5) may be due to AGP secretion early after LPS administration and that the 40–43 kDa AGP activates the production of IL-6. Hence, local production of AGP may play a crucial role in pro-inflammatory processes.

Determination of AGP levels in serum, air pouch and synovium fluid of normal and arthritic rats

The air pouch membrane resembles the synovial membrane (Sedgwick et al., 1983) and since in previous studies (Yiangou et al., 1993b) we found that AGP might be involved in the development of AA, we determined the AGP levels accumulated in air pouches and the synovial fluid of adjuvant arthritic rats at the early stages of disease development. AGP (40–43 kDa) levels in serum, air pouch exudate (Fig. 7A) and synovial fluid (Fig. 7B) of AA rats increased significantly at day 7 post-FCA treatment compared to the respective AGP levels in normal control rats. However, administration of HBV to the air pouch or in the muscles of AA rats resulted in a significant reduction of arthritis development (data not shown) and in serum 40–43 kDa AGP levels compared to the respective levels of AGP in the serum of non-treated AA rats (Fig. 7A and B). Administration of HBV in air pouch does not affect the elevated 40–43 kDa AGP levels compared to the respective levels in the air pouch of non-treated AA rats (Fig. 7A). Moreover, intramuscular HBV administration in AA rats results in increased 40–43 kDa AGP levels in the synovial fluid compared to the respective levels in the synovial fluid of non-treated AA rats (Fig. 7B). Furthermore, HBV administration to normal rats resulted in an increase of AGP levels in serum, synovial fluid and air pouch (Fig. 7). The HBV-mediated regulation of AGP in the synovial fluid of AA rats is similar with that we previously described for α 1-antitrypsin while differs from that obtained in serum using the same samples (Pantazidis et al., 2005) suggesting local production of AGP.

Discussion

The data presented in this study show differences between the local vs. the systemic inflammatory response initiated in the synovial-like air pouch model after LPS administration as indicated by a rapid accumulation of AGP in air pouches early post-treatment. These results

represent the first report showing such a rapid *in vivo* induction of AGP in this model. We propose that this rapid induction of AGP is a strong biomarker of the inflammatory response and may be involved in the physiological activation of a local acute phase response.

Our studies have demonstrated a unique sequence of events that comprise the localized acute phase response in the arthritic pouch model. The differences in AGPs produced by resting air pouch PMN cells vs. LPS stimulated PMN cells are due to glycosylated and fucosylated AGPs of an apparent molecular weight of 70–90 kDa. The significance of this specific post-translational modification is not well understood although it may involve AGP's immunoregulatory function.

Our studies have shown cell specific differences in the AGP isoforms produced by cells of the air pouch and serum. The response to DEX as well as differences in cellular localization played a role in the isoform produced and are indicative of the importance of the biological environment. Thus, the DEX mediated inhibition of PMN cell infiltration and rapid specific increase of the 40–43 kDa AGP further demonstrate the role of environment and cell type, *e.g.*, high molecular weight AGPs are synthesized and then secreted by cells that infiltrated the air pouch while the 40–43 kDa AGP is produced by cells forming the air pouch lining tissue. Since fucosylated structures of AGP are suitable ligands for E-selectin (Simon and Goldsmith, 2002) a potential function of PMN cell AGP glycoforms released in the air pouch may directly antagonize adhesion of newly arriving PMN cells by neutralizing E-selectin on the inflamed endothelium (Fournier et al., 1999; Poland et al., 2005). Thus, the 70–90 kDa AGPs may be responsible for a feedback type inhibition of further cell accumulation, in LPS treated air pouches, as observed at 3–6 h post-treatment (Fig. 3). Alternatively, the latter may also be due to other anti-inflammatory effects of total AGP or specific AGP glycoforms such as inhibition of chemotactic responses of PMN cells to C5a (Hochepied et al., 2003; Poland et al., 2005).

The complexity of the LPS mediated acute phase response involving activation and differential glycosylation of AGP is clearly seen by the heterogeneity of the glycosylated forms that occur in the resting vs. stimulated environment as well as the localization of the PMNs, *i.e.*, air pouch vs. serum. Thus, the significance of the synthesis and secretion of the 70–90 kDa form in PMNs that is not secreted is suggestive of differential functions. Cell specific differences occur between human PMN cells which synthesize and secrete fucosylated AGP (50–60 kDa) *in vitro* whereas human monocytes produce and secrete only the 40–43 kDa AGP (Poland et al., 2005). Thus, the specific biological environment, *i.e.*, air pouch vs. serum, may be a major factor in the differences in glycosylation and in secretable vs. non-secretable forms of AGP. These data suggest that specific functions of AGP may be affected by the biological environment in which the acute phase response is activated.

The fact that the major non-secreted 50–60 kDa AGP form is produced by PMN cells in LPS treated air pouches and by PMN cells that have infiltrated the myocardium of patients that have died of acute myocardial infarction (Poland et al., 2005) suggest that this AGP may be a marker of acute inflammatory response. Alternatively, this glycoform may exhibit an intracellular protective role similar to that of heat shock proteins or metallothioneins (Borghesi and Lynes, 1996).

The ability of HgCl₂ to specifically activate the synthesis and secretion of the 40–43 kDa AGP in PMNs serves as another example of the diversity of response of the AGP gene to various activating factors (Yiangou et al., 1991, 2002). This diversity of mechanism of activation is further emphasized by the report that HgCl₂ induces AGP in the mouse liver in the absence of cytokines or glucocorticoids (Yiangou et al., 1991). Since the HgCl₂ (and other heavy metals) can stimulate oxidative stress, oxidative protein modification, and protein

aggregation, the mechanism of this alternative activation of AGP may be due to these physiological responses to heavy metals.

The rapid accumulation of AGP at the site of LPS administration may reflect its potential anti- and pro-inflammatory role. Our data show that AGP acts synergistically with suboptimal doses of LPS and activates *in vitro* air pouch exudate cells to produce IL-6 while IL-1 bioactivity is unaffected. Furthermore, our findings indicate that the increase in IL-6 production is activated only by the cells forming the air pouch lining tissue. These cells produce only the 40–43 kDa AGP glycoform suggesting that this glycoform may play a role in the potentially increased IL-6 production by the air pouch exudate cells. AGP induced IL-1Ra secretion and the inhibitory effect of such factors on IL-1 activity is well documented (Bories et al., 1990; Tilg et al., 1993). AGP increases the *in vitro* cytokine secretion by LPS activated human alveolar or peritoneal macrophages and blood monocytes (Isazi et al., 1989; Borghesi and Lynes 1996). In addition, pulmonary exposure of mice to LPS induced rapid acute phase response expression in lung, which precedes IL-6-mediated systemic elevation of acute phase reactants (Vernooy et al., 2005). Taken together, all the above observations suggest that local production of AGP *in vivo* at the early stages after initiation of an inflammatory response may activate cytokine secretion.

Our studies showed that the serum of mice at 3 h post- LPS treatment of air pouches contains only the 40–43 kDa AGP although blood PMN cells at the same time point contain high molecular weight of AGPs suggesting that the liver may be the only main potential source of serum AGP. Since high molecular weight AGPs are secreted by PMN cells infiltrating the air pouch we suggest that a certain factor present only in air pouch exudates and not in serum might activate the secretion of these AGP isoforms. Alternatively, the high molecular weight AGPs exhibit short half-life after their secretion in the serum. Treatment of the air pouches with LPS increased the TNF α , IL-1 and IL-6 (Miller et al., 1997; Fig. 5) but only IL-6 reached the circulation in biologically significant amounts (Miller et al., 1997). The latter indicates that the air pouch retains factors involved only in local inflammatory responses. On the other hand air pouch produced IL-6 may act locally for example as initiator of the febrile response (Miller et al., 1997) as well as an important systemic mediator of the acute phase response and AGP production by the liver.

The strongly fucosylated human 50–60 kDa AGP secreted by activated PMN cells is suggested to provide an endogenous feedback-inhibitory response to excessive inflammation (Poland et al., 2005). Our data also suggest that the local rapid accumulation of AGP in LPS or HBV treated air pouches may reflect its protective role against the deleterious effect of inflammatory mediators. Several studies suggest that AGP may protect from the deleterious effects of infection or apoptosis (Van Molle et al., 1997; Hocheppied et al., 2000) while it is protective only when its concentration is rapidly increased (Libert et al., 1998). Our data emphasize the importance of the biological environment for AGP production and activity. Adjuvant arthritis is a systemic autoimmune disease model and early as well as recent studies demonstrated that treatment of AA rats by intramuscular, intradermal, subcutaneous or intraperitoneal administration of HBV results in remission of AA development (Billingham et al., 1973; Hadjipetrou-Kourounakis and Yiangou, 1984; Kwon et al., 2002; Kim et al., 2008). Furthermore, when low dose of HBV was intramuscularly injected to only hind paws, severe arthritis developed only in the front paws, suggesting potential local activity of HBV in the joint synovium (Hadjipetrou-Kourounakis and Yiangou 1984). The findings in this study show that intramuscular HBV treatment increased the AGP levels in synovial fluid of AA rats suggesting that the AGP produced in synovial cavity may exhibit antiarthritic activity. Moreover, remission of AA by HBV is associated with reduced serum AGP levels (Fig. 7) or liver AGP mRNA (Yiangou et al., 1993b) at the early stages of AA development suggesting that systemically produced AGP rather activates than inhibits arthritis. These data further

support that specific functions of AGP may be affected by the biological environment in which the acute phase response is activated indicating that AGP has a dual anti- and pro-inflammatory activity that may depend on whether it is locally or systemically produced. The water-soluble extract of HBV that contains several peptides such as mellitin, mast cell degranulating peptide and adolapin as well as enzymes such as phospholipase A2 produced anti-nociceptive and anti-inflammatory effects on AA rats (Kwon et al., 2002). Microarray analysis of human chondrosarcoma cells (Yin et al., 2005) revealed that co-stimulation with LPS and HBV reverses the LPS induced upregulation of genes such as that of the IL-6 receptor, matrix metalloproteinase 15 (MMP-15), TNF (ligand) super family 10, caspase 6, and tissue inhibitor of metalloproteinase-1 TIMP-1. Several isoforms of C/EBP transcription factors are involved in the regulation of AGP gene expression in the liver or bone marrow PMN cells (Yiangou et al., 2002; Theilgaard-Mönch et al., 2005). Park et al. (2004) demonstrated that HBV and mellitin inactivate NF- κ B transcription factor by direct binding to the p50 subunit and suggested that this is an important mechanism of their antiarthritic effects. Local activation of the inflammasome danger signaling pathway that is mediated by NF- κ B and IL-1 β , TNF α and IL-6 production results in decreased osteoblast function and in increased osteoclast activity (Hallab et al., 2009). These observations may offer possible explanations concerning the mechanism of antiarthritic activity of HBV.

Other acute phase proteins such as α 1-antitrypsin, C reactive protein (CRP) and serum amyloid A (SAA) are expressed extrahepatically (Schultz and Arnold, 1990; Yiangou et al., 1991; Yiangou and Papaconstantinou, 1993a). We also found increased levels of α 1-antitrypsin in exudates from LPS treated air pouches (Pantazidis et al., 2005) or in HBV treated synovial fluid suggesting an antiprotease activity. Furthermore, HBV regulates in a different manner local and systemic AGP or α 1- antitrypsin production. HBV was found to possess considerable anti-oxidant and hydroxyl radical scavenging activity (Rekka et al., 1990). Taken together all the above suggest that early expression of AGP and other acute phase proteins in the air pouch space or in synovial fluid exert a local protective effect by limiting the inflammatory response and its deleterious effects in the air pouch or synovial fluid.

The mechanisms regulating recruitment of leukocytes in the joint in inflammatory arthritis models are not fully understood. Thus, further clarification of the modulatory effects of either AGP or HBV in rat arthritic joints must be addressed. Since inflammation initiated in the air pouch is similar to that in the synovium and since the air pouch model provide more cells, tissue as well as exudate than joints, it could be used as a model for further studies of the mechanism of the inflammatory responses in arthritic rat joints.

In conclusion, in this study we have evaluated the effect of LPS, FCA, HgCl₂ as well as DEX and honeybee venom on the induction of local acute phase response in the rat dorsal air pouch. Our results demonstrate differential regulation of AGP production in air pouch by the above stimulants. Our data provide *in vivo* evidence that early post- LPS treatment, air pouch lining tissue cells or PMN cells present in the air pouch exudate produce different glycoforms of AGP, which in turn may modulate IL-1 and IL-6 production. We observed that the cells forming the air pouch produce only the 40–43 kDa glycoform that in turn may activate IL-6 production and secretion . Furthermore, our data show that the antiarthritic effect of HBV is accompanied by differential regulation of systemic or local AGP and α 1- antitrypsin production in the air pouch or synovial fluid of arthritic rats.

In a system as complex as the local inflammatory response in the dorsal rat air pouch or synovial tissue the multiple effects of AGP may involve several mechanisms of action. The complexity of AGP activity is emphasized in our report of the rapid induction of AGP production and accumulation of several AGP glycoforms in the rat dorsal air pouch in response to LPS treatment and subsequent modulation of IL-1 and IL-6 production. The significance of the

events lies in understanding the systemic or local activity of AGP in the initiation or suppression of inflammatory responses such as adjuvant arthritis in rats.

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Fig. 1.

Detection of AGP forms in rats after injection of LPS, FCA or HgCl₂ in the rat dorsal air pouch. Panels A–B: Representative Western blot analysis showing the time course of the accumulation of AGP forms in air pouch exudates (panel A) or serum (panel B). Lane AGP represents purified rat AGP (100 ng) and lane N represents a sample isolated from normal (N) control rats (untreated air pouch). Panel C: The Western blot bands were scanned and subjected to reciprocal analysis to determine total AGP levels in each sample. The histogram shows the time course accumulation of AGP in air pouch exudates or serum. The results represent one of two similar experiments and each bar represents the average of three rats. *Statistically significant difference in comparison with the control group, $P < 0.05$. #Statistically significant difference in comparison with time point giving maximum response, $P < 0.05$.

Fig. 2.

Detection and characterization of AGP forms in lysates of air pouch PMN cells and in lining tissue forming the air pouch. Panel A : Western blot of 10% SDS-PAGE gels showing several AGP forms in air pouch exudates and homogenates prepared from air pouch PMN cells as well as air pouch lining tissue isolated from control (N) or LPS treated rats. Panel B: Western blot of 7.5 % SDS-PAGE gel is showing PNGase F digestion of exudates isolated from LPS treated and untreated air pouches.

Fig. 3. PMN cell accumulation and characterization of AGP-positive cells in air pouch exudates or air pouch lining tissue after treatment with LPS or FCA. Panel A: Time course of the accumulation of PMN cells (left axis, solid circles) and of AGP-positive cells (immunocytochemically determined – right axis, open circles) in LPS or FCA treated air pouch exudates. Each value represents the mean $\pm$ SEM from three rats. The results represent one of three similar experiments. All values are significantly different from control with at least $P < 0.05$. Panels B–C: Representative immunocytochemistry showing the morphology of AGP-positive PMN cells in exudates of (B) non treated and (C) LPS or FCA treated air pouches. Panels D–E: Representative immunohistochemistry showing the cluster of AGP-positive cells

in air pouch lining tissue from (D) non treated rats and (E) from rats treated 2 h previously with 3 mg / kg BW LPS. Arrows show AGP-positive cells of the monocyte-macrophage lineage and arrowheads show negative staining for AGP in surrounding vessels endothelial cells.

Fig. 4. Effect of DEX on AGP and PMN cell accumulation in air pouch exudate. Panel A: Representative Western blot of 7.5% SDS-PAGE gels showing the accumulation of AGP in the rat air pouch exudates 1 h post- DEX, LPS or DEX+LPS treatment. Lane AGP represents purified rat AGP (100 ng) and lane N represents a sample isolated from normal (control) rats. Panel B: Determination of AGP levels (left axis) and number of cells accumulated in air pouch exudate (right axis) after treatment with DEX, LPS or DEX+LPS. Each bar represents the mean $\pm$ SEM of five rats. *Statistically significant difference in comparison with the control (normal) group, $P < 0.05$. #Statistically significant difference in comparison with time point giving

maximum response, $P < 0.05$. Panel C: Representative immunohistochemistry on air pouch lining tissue showing clusters of AGP-positive cells (1 h post- treatment).

Fig. 5. Effect of LPS and DEX on IL-1 and IL-6 in exudates isolated from dorsal rat air pouch. Time course experiment showing the IL-1 bioactivity (panel A) and IL-6 levels (panel B) in LPS (solid squares) or DEX (open circles) treated air pouches. Each value represents the mean $\pm$ SEM of five rats and significant differences from control values are indicated by * $P < 0.05$.

Fig. 6.

Effect of AGP on cytokine production by mouse dorsal air pouch PMN exudate cells. Panel A: The histogram shows the effect of AGP on IL-1 and IL-6 production on air pouch exudate cells activated *in vitro* by suboptimal dose of LPS. Panels B –D: Determination of IL-6 producing cells after co-cultivation of *in vivo* LPS-activated air pouch exudate PMN cells or air pouch lining tissue cells with normal air pouch exudate PMN cells in the presence of suboptimal dose of LPS. Cells producing IL-6 were detected by immunofluorescence microscopy. Notice the low (panel C) and high (panel D) number of IL-6 producing cells after co-culture with air pouch exudate PMN cells (panel C) and the air pouch membrane cells (panel D). Arrows show IL-6-positive cells.

Fig. 7.

Comparison of the effect of HBV administration on AGP levels in serum vs. air pouch (panel A) and serum vs. synovial fluid of normal (N) or 7-day AA rats (panel B). HBV (0.5 mg / kg BW) administered in air pouches (panel A) or intramuscularly (panel B) every other day and for 5 days (three doses). Each bar represents the mean $\pm$ SEM of five rats and significant differences from control values are indicated by * $P < 0.05$ while from AA values are indicated by # $P < 0.05$. At the bottom of panel B a representative Western blot analysis showing the AGP levels in serum and synovial fluid of normal or AA rats treated or not with HBV.