Down-regulatory effect of alpha1-acid glycoprotein on bovine neutrophil degranulation

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Abstract

In this paper, the possible involvement of the acute phase protein α1-acid glycoprotein (AGP) in the local immunomodulation of inflammation was investigated. The dose response of bovine neutrophils to AGP as to mobilization of primary and secondary granules was studied. It was found that AGP fulfills a protective role against spontaneous exocytosis of secondary, but not primary, granules. This downregulatory effect is much more evident when degranulation is challenged with Zymosan activated serum (ZAS). AGP activity is dose-dependent, the acute phase concentration being more active than the physiological one. Carbohydrate moiety of AGP was found to be critical, since experimentally desialylated protein does not maintain its exocytosis-modulatory activity.

The fact that AGP may modulate the degranulation of neutrophils confirms the hypothesis that AGP is heavily involved in the fine tuning of neutrophil activity in the inflammatory environment.

Keywords: Acute phase reaction; Granulocytes; α1-Acid glycoprotein; Exocytosis

Résumé

Dans cet article nous avons étudié la possible implication de l’alpha1-acide glycoprotéine (AGF) dans l’immunomodulation de l’inflammation. La dose réponse des neutrophiles bovins à l’AGP par...
rapport à la mobilisation des granules primaires et secondaires a été examinée. Il a été identifié que
AGP remplit un rôle de protection contre l’exocytose spontanée des granules secondaires, mais non
primaires. Cet effet inhibite est encore plus évident lorsque la dégranulation est stimulée avec le
sérum activé par le Zymosan. L’activité de l’AGP est dose dépendante, la concentration de la phase
aigue étant plus active que la phase physiologique. La partie hydrate de carbone de l’AGP est
essentielle, puisque la protéine désialylée de façon expérimentale ne maintient pas son activité de
modulation sur l’exocytose. Le fait que l’AGP puisse moduler la dégranulation des neutrophiles,
confirme l’hypothèse que l’AGP est impliquée au niveau du processus inflammatoire dans l’activité
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Mots clés : Phase aigue de l’inflammation ; Granulocytes ; α1-Acide glycoprotéine ; Exocytose

1. Introduction

Innate immunity strongly relies on the activity of polymorphonuclear (PMN) neutrophil
leukocytes. They represent the first line of defence against invading pathogens by
migrating to sites of infection within minutes from any injury, in order to search and
destroy foreign intruders [23]. Neutrophils fulfil their role mainly by internalizing and
exposing pathogens to the destructive action of reactive oxygen species (ROS) and
hydrolyzing enzymes stored in their granules [20]. Bovine neutrophils, like other species,
contain secretory vesicles, primary (azurophilic) and secondary (specific) granules. In
addition, bovine and other small ruminants, such as goats and sheep, share unique, large,
granules that are not present in human cells [8,14]. While the organization of granules in
bovine neutrophils is known, there is a relative lack of knowledge about the molecules
which modulate their mobilization. Neutrophil exocytosis occurs due to the translocation
of granules. This process is hierarchical, secondary granules being mobilized more readily
than primary ones, in humans as well as in bovines [1,14,30], and it is likely to be
dependent on specific remodelling events. Due to their very high destructive potential, both
PMN and organism as a whole have developed tight controlled mechanisms whose role is
down regulating and limiting the destruction of surrounding cells and tissues [21]. The
resolution of the neutrophil phase of inflammation depends on the fine tuning of their
lifespan, regulated by apoptosis, but also by the presence of molecules that may act as down
regulators of the PMN action. The immunocalin α1-acid glycoprotein (AGP) possesses an
immunomodulatory activity and it is believed to play an important role in the regulation of
local inflammatory reaction, for example by reducing the tissue damages caused by an
excessive activation of complement [27]. The concentration of AGP rises in plasma from
three to five folds during systemic reaction of inflammation [10,15], and therefore is
considered, at least from a clinical perspective, a minor acute phase protein. AGP is mainly
synthesized by liver, but it can be localized in several other human [10] and bovine [12]
tissues. AGP exerts a sort of protective activity by reducing the apoptosis rate in some
inflamed tissues [28] and by increasing the lifespan of monocytes, at least in the bovine
species [2]. Defensive functions of neutrophils can be affected as well: for example, AGP
has been reported to influence neutrophil chemotaxis [11,29], aggregation and generation
of reactive oxygen species [6]. The activity of AGP on neutrophil exocytosis has not been
investigated so far, neither in human nor in veterinary medicine. Our hypothesis is that, due to its well known immunomodulatory function, AGP acts as a local regulatory mechanism that modulates the exocytosis of granules from bovine neutrophils after their recruitment in the inflammatory focus. Therefore, the major aim of this study was to investigate the capability of purified bovine AGP to influence the degranulation of neutrophils by studying the possible relationship between AGP and the exocytosis of primary and secondary granules. The hypothesis that AGP may modulate neutrophil degranulation by competing with inflammatory mediator receptors, which in our experimental design included ZAS, was also explored. Finally, since AGP is one of the most glycosylated protein in the organism, and its glycan pattern strongly influences its biological activity [3], in the last part of this study the relationship between the terminal sialic acid residues of AGP and its exocytosis-modulating activity was investigated by using in parallel a desialylated glycoform.

2. Materials and methods

2.1. Reagents

All reagents used in these experiments were purchased from Sigma-Chemicals Co., unless otherwise specified. Hanks balanced saline solution with 0.5 mM CaCl₂ and 1 mM MgCl₂ (HBSS+) was used throughout all the experiments. NaCl solutions were diluted starting from sterile cell tested 5 M NaCl.

Cell culture tested, endotoxin free, albumin was purchased from GIBCO (Invitrogen S.R.L., Milano, Italy).

Bovine serum was activated with 15 mg/ml of Zymosan A from S. cerevisiae at 37 °C for 60 min. The mixture was then incubated at 56 °C for 30 min to destroy complement components with the exception of C5a. Zymosan particles were removed by centrifuging the mixture for 15 min at 1000 × g at 4 °C. The obtained supernatant was filtered through a 0.22 μm filter membrane (Millipore, Segrate, Italy), stored at −80 °C and used within three months. Degranulation was induced using the ZAS stock solution diluted 1:5 vol/vol with HBSS+.

All the experiments carried out in this study used bovine AGP aliquots purified from plasma as previously reported [2]. Two different concentrations of purified AGP were used: low concentration, similar to that physiologically found in bovine plasma of healthy subjects (0.3 mg/ml) and high concentration, similar to that found in bovine plasma during acute phase response to several inflammatory statuses (0.9 mg/ml) [24].

2.2. Bovine neutrophils isolation

Clinically healthy lactating Holstein cows between 2 and 7 years of age were used throughout these studies as blood donors for all experiments. Blood was obtained from the jugular vein and collected into blood bag containing acid-citrate-dextrose (Terumo, Belgium). Samples containing less than 5% eosynophils were used. Neutrophils were isolated using a Percoll®-gradient as previously described [19], with
slight modifications. Briefly, 40 ml of blood were transferred to 50 ml polypropylene conical tubes and centrifuged (1000 \( g \)) for 20 min at 4 °C. The plasma and buffy coat were aseptically aspirated and discarded. The remaining cells were suspended in 35 ml final volume of ice-cold PBS and the suspension slowly pipetted down the side of a clean 50 ml polypropylene conical tube containing 10 ml of 1.087 g/ml Percoll®. Samples were centrifuged (400 \( g \)) for 40 min at 20 °C. The supernatant, mononuclear cell layer, and Percoll® were aseptically aspirated and a pellet composed of PMN and erythrocytes was retained. Erythrocytes were lysed by mixing 1 volume of cells with 2 volumes of an ice cold 0.2% NaCl solution and inverting the tube for 1 min. Tonicity was restored by the addition of one-half volume of a 3.7% NaCl solution. The samples were centrifuged at 500 \( g \) for 2 min at 4 °C. Lysis were usually repeated, sometimes twice, using pre-warmed (37 °C) Red Blood Cells Lysis Buffer. The cell pellet was washed twice by resuspension in PBS and recentrifugation for 2 min at 4 °C. Cells were enumerated using an automated cell counter. Cell viability and differential cell counts were determined by trypan blue exclusion and Wright staining, respectively. Neutrophils purity was >95% and viability >90%. Cells concentration was adjusted with HBSS+ and maintained on ice until used in the various assays described below.

2.3. Immunocytochemistry studies related to AGP binding to bovine neutrophils

In order to verify if the activated bovine neutrophils were capable to bind AGP on the membrane surface, 1.5 \( \times 10^5 \) neutrophils were stimulated for 30 min with AGP at 0.3 mg/ml and 0.9 mg/ml.

The cells were centrifuged at 500 \( g \) for 8 min at 4 °C, washed twice with cold PBS in order to remove unbound AGP molecules and stabilized with 1% formalin. Cells were allowed to settle on a glass slide and processed to assess AGP localization by immunofluorescence. After an incubation of 30 min at 26 °C in PBS containing 1% BSA and 1% NDS (Normal Donkey Serum), the slides were incubated overnight at 4 °C with a polyclonal rabbit anti-bovine AGP (17 μg/ml in Dulbecco-modified PBS) [2]. After the incubation, the samples were washed twice in PBS and stained with a fluorescein isothiocyanate (FITC) conjugated donkey anti-rabbit IgG antibody (1:200) for 30 min at 26 °C. Chromatin DNA was stained with DAPI (0.05 μg/ml in PBS).

As negative control, the primary antibody against AGP was omitted in one slide for each experiment. All samples were mounted with an anti-fade medium and observed with a conventional epifluorescence microscope (Nikon, Eclipse E 600).

2.4. Neutrophil degranulation studies

2.4.1. Experimental design for the stimulation of the cells

Degranulation responses of neutrophils were investigated by studying the effect of AGP on the exocytosis of primary (azurophil) and secondary (specific) granules. All the experiments were carried out at 37 °C in a sterile 96-well flat bottom ELISA plates (Becton Dickinson). Cells were incubated in different conditions as described below. The
supernatant of each well was then transferred in an Eppendorf tube, centrifuged at $400 \times g$ for 7 min at room temperature (RT) and divided in two aliquots for the evaluation of granules exocytosis.

Primary and secondary granules release was determined by measuring the enzymatic activity of myeloperoxidase (MPO) [16] and alkaline phosphatase (ALK-P) [18], respectively, which were assayed from the supernatants obtained from $2 \times 10^6$ cells in 200 µl. The assay of alkaline phosphatase enzymatic activity exocytosed by specific granules was carried out following a slight modification of a previously established protocol [4]. To 33 µl of supernatant, 100 µl of p-nitrophenyl phosphate (pNPP) ready made solution were added in 96 wells non sterile ELISA plates, and the mixture was incubated for 10 min at RT. The reaction was finally blocked by the addition of 50 µl NaOH 3 M, and the plates were read at an absorbance of 410 nm. MPO activity contained in primary granules was assayed on 50 µl of supernatant: 200 µl of tetramethylbenzidine (TMB) ready made solution were added. The reaction was carried out for 30 min at RT, and finally blocked by adding 50 µl H$_2$SO$_4$ 1 M. The plates were read at an absorbance of 450 nm.

Background values were calculated from wells containing pNPP and TMB, in their respective assay, added with 33 µl and 50 µl of HBSS+ respectively, and the results were automatically subtracted from all values.

The data were expressed as the percentage of ALK-P, or MPO, activity compared to the total enzyme content of the cells, as determined after incubation of the same amount of cells with 0.5% hexadecyltrimethylammonium bromide (CTAB).

Supernatants were then transferred into 96-well flat bottom non sterile ELISA plates and enzymatic activities were measured on automatic microtiter plate reader Multiscan MS (Labsystem, Helsinki, Finland).

The experimental design was planned as follows:

**Experiment 1.** In a first series of experiments, the cells were incubated with the two different concentrations of purified AGP in order to study the effect of two different concentrations of AGP on spontaneous degranulation of cells. Negative controls were neutrophils incubated with an equal volume of HBSS+ instead of AGP. MPO and ALK-P activities were measured after 120 min of incubation at 37 °C and at 5% CO$_2$ as previously described.

**Experiment 2.** In this second series of experiments, isolated neutrophils were incubated with two different concentrations of purified AGP and, in the meanwhile, challenged with ZAS. Positive controls were neutrophils incubated with ZAS and HBSS+ instead of AGP. Negative controls were non activated neutrophils in which equal volume of HBSS+ was added instead of ZAS and AGP.

In order to rule out any unspecific protein activation, cells were further challenged with 0.9 mg/ml albumin. Time course incubation was carried out for 0, 15, 30, 60, 120 and 240 min.

**Experiment 3** was designed in order to verify if AGP’s activity was due to interaction of the protein with neutrophil membrane. Isolated neutrophils were therefore pre-incubated with two different concentrations of purified AGP for 10 min. Unbound protein was washed away by centrifugation ($200 \times g$ for 7 min) and cells were
resuspended in HBSS+ and incubated with ZAS for 120 min. MPO and ALK-P activities were measured as described. 

Experiment 4 was aimed to assess the importance of the glycan pattern of AGP for its degranulation-modulatory activity. De-sialylated AGP (as-AGP) was prepared by treating purified AGP with 200 mU/ml neuraminidase (streptococcus 6646K, EC 3.2.1.51) in 0.01 M sodium phosphate buffer, pH 6.0, for 2 h at 37 °C, exactly as previously reported [2]. Isolated neutrophils were then incubated with two different concentrations of purified as-AGP. In the last group of experiments, neutrophils exocytosis was also challenged with ZAS. Aliquots of protein which were not submitted to neuraminidase treatment were used as controls. ALK-P and MPO activities were measured after 120 min, following the protocols previously described.

2.5. Statistical analysis

All statistical procedures were computed by using statistical software (SPSS 15.0, SPSS Inc., Chicago, USA). Results are expressed as mean values plus or minus standard error of the mean values. Different treatments were compared using a non parametric Wilcoxon test for paired samples. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. AGP binds to the surface of neutrophils

With the aim to investigate if AGP may react with the surface of cells, resting granulocytes were labelled for AGP immunoreactivity by using the polyclonal anti-bovine AGP antibody. Results are presented in Fig. 1. The incubation of the cells with AGP resulted in a homogeneous fluorescent staining of PMN surface. This anti-AGP immunoreactivity apparently increases when neutrophils are treated with acute phase concentrations of AGP (0.9 mg/ml) (Fig. 1: panel C). Fig. 1 therefore shows that exogenous plasma AGP is capable to bind the surface of bovine neutrophils.

3.2. AGP modulates spontaneous degranulation (Experiment 1)

The first series of experiments was designed to determine whether AGP may modulate spontaneous degranulation of secondary (specific) and primary (azurophilic) granules. Results are presented in Fig. 2. The treatment of cells for 120 min with AGP fulfils a protective effect against spontaneous exocytosis of secondary granules (Fig. 2A), since ALK-P activity, which was used as a marker of secondary granules exocytosis, dropped from $7.04 \pm 0.92\%$ (mean $\pm$ S.E.M.) of the HBSS treated cells to $1.89 \pm 1.03\%$ of AGP incubated cells ($P < 0.05$). This effect is dose-dependent, the physiological concentration being non effective. The effect of AGP on primary granules spontaneous exocytosis is apparently opposite (Fig. 2B), since MPO activity, which was used as a marker of secondary granules exocytosis, increases from $2.85 \pm 1.22\%$ of the HBSS treated cells to
Due to the very high individual variability, this effect is not statistically significant. This effect of AGP on primary granules spontaneous degranulation can be appreciated only when cells are treated with acute phase concentration of AGP. Treating the cells with physiological concentration of AGP had apparently no effect.

7.97 ± 4.01% of 0.9 mg/ml AGP-treated neutrophils. Due to the very high individual variability, this effect is not statistically significant. This effect of AGP on primary granules spontaneous degranulation can be appreciated only when cells are treated with acute phase concentration of AGP. Treating the cells with physiological concentration of AGP had apparently no effect.
3.3. AGP modulates the exocytosis of secondary granules induced by ZAS (Experiment 2)

The effects of AGP on secondary granules exocytosis are presented in Fig. 3A. The isolation procedure that was carried out to obtain the cells from clinically healthy animals was sufficient to induce a slight activation of neutrophils, as shown by the release of 5.05 ± 0.51% of the total ALK-P content from non-stimulated cells at $T_0$. When neutrophils are stimulated with ZAS, which contains soluble C5a complement fraction derived from normal serum after activation with Zymosan, the enzymatic activity of ALK-P released from positive controls increases from 12.92 ± 2.95% ($T_0$) to 45.17 ± 3.67% ($T_{240}$).

The incubation of the cells with 0.9 mg/ml albumin, carried out to rule out an unspecific protein activation of the cells, gave no significant difference when compared to positive control, since it ranges from 10.97 ± 1.32% ($T_0$) to 42.57 ± 6.44% ($T_{240}$). When neutrophils are incubated with physiological concentration of AGP the response is biphasic and time-dependent: after the first 15 min lag it cannot be observed any statistically
significant difference between AGP-treated cells, which ranges between 10.88 ± 2.59% \((T_{0'})\) and 13.93 ± 1.34% \((T_{30'})\), and positive controls, which ranges between 12.92 ± 2.95% \((T_{0'})\) and 21.28 ± 2.67% \((T_{15'})\). On the contrary, starting from \(T_{30'}\), it becomes evident that AGP, even when administered at physiological concentration, can reduce the activity of ALK-P. This inhibitory activity reaches its highest point at \(T_{240'}\) (26.98 ± 2.49% versus 45.17 ± 3.67% of positive controls).

The inhibitory activity of AGP toward secondary granules exocytosis is still more evident when neutrophils are treated with acute phase concentration of protein, similar to those found in plasma during the systemic response to inflammation. The degranulation of secondary granules is almost immediately inhibited when compared with positive controls.

Fig. 3. The effect of AGP treatment on secondary and primary granules exocytosis. Neutrophils were isolated from 7 cows and incubated with AGP at two different concentrations: physiological concentration (0.3 mg/ml) and acute phase concentration (0.9 mg/ml). Albumin (0.9 mg/ml) was used in order to rule out any unspecific protein activity. Degranulation of secondary granules was induced with ZAS as described in Section 2. Positive controls were neutrophils treated with ZAS and HBSS instead of AGP. Negative controls were HBSS treated cells without ZAS. Panel A presents alkaline phosphatase (ALK-P) activity in the neutrophil culture supernatant, used as marker of secondary granules exocytosis. Panel B presents myeloperoxidase (MPO) activity in the neutrophil culture supernatant, used as marker of primary granules exocytosis. Values are expressed as percentages of ALK-P and MPO activity compared to total ALK-P and MPO activity in neutrophils lysed with CTAB (mean ± S.E.M.). Statistically significant differences between the two concentrations of AGP and positive control (neutrophils activated with ZAS and treated with HBSS instead of AGP) were indicated as * \((P < 0.05)\). The differences between positive and negative controls were all statistically significant, and were not indicated in the figure.
(at $T_0$, ZAS/AGP = 6.71 ± 1.87% versus positive controls = 12.93 ± 2.95%). This inhibitory activity is steadily increasing over time, and reaches its highest point at $T_{240}$ (ZAS/AGP = 9.15 ± 1.26% versus 45.17 ± 3.67% of positive controls). The down-regulating effect of acute phase concentration of AGP on secondary granules exocytosis is statistically significant starting from $T_{15}$. The effects of AGP on primary granules exocytosis are presented in Fig. 3B.

Again, the isolation procedure carried out to obtain the cells from clinically healthy animals was sufficient to induce a slight activation of neutrophils, as shown by the release of 3.19 ± 0.60% (mean ± S.E.M.) of the total MPO content from nonstimulated cells at $T_0$. The challenging of bovine neutrophils with ZAS induces an immediate exocytosis of primary granules, as indicated by the high MPO enzymatic activity at $T_0$ (37.91 ± 8.13% of the total MPO activity). Incubation of ZAS-activated neutrophils with albumin (0.9 mg/ml) and AGP at both concentrations apparently has no effect on the primary granules exocytosis. Moreover, the release of MPO is not time-dependent, since it does not change from $T_0$ to $T_{240}$.

In conclusion, the results presented in Fig. 3 demonstrated that the treatment of ZAS-activated neutrophils with AGP purified from bovine serum selectively reduces the release of secondary granule content in a dose-dependent way, and apparently has no effect on that of primary granules.

The third series of experiments presented in this paper were intended to better understand the mechanism of action of AGP’s modulation of exocytosis.

### 3.4. The pre-incubation of cells with AGP reduces the exocytosis of neutrophil granules induced by ZAS (Experiment 3)

In order to verify if one of the possible mechanisms of action of AGP was the interaction with the molecules that are believed to be involved in the binding of ZAS-generated by-products, e.g. C5a receptor (i.e. CD88), isolated neutrophils were pre-incubated with AGP for 10 min, after which exocytosis was induced by ZAS. Results are presented in Fig. 4. The pre-incubation of cells with AGP does not modify the exocytosis of primary granules (Fig. 4B). On the contrary, Fig. 4A shows that the enzymatic activity of ALK-P used as marker for secondary granules exocytosis is decreased, from 20.30 ± 4.55% of the positive controls to 9.46 ± 1.64% of the AGP’s (0.9 mg/ml) pre-incubated cells after 120 min of incubation with ZAS ($P < 0.05$) While there is a decrease of secondary granules exocytosis also when cells are pre-treated with physiological concentration of AGP (14.64. ± 2.18%), this result is not statistically significant when compared to positive control.

### 3.5. AGP effects on degranulation are related to its sialic acid content (Experiment 4)

The last set of experiments was set out to determine the importance of the sialic acid residues exposed on the surface of AGP. Exocytosis studies were then performed using in parallel AGP aliquots purified from bovine serum and neuraminidase-treated AGP (as-AGP), in which terminal sialic acid residues were enzymatically removed. The reaction was carried out for 120 min. When neutrophils were activated with ZAS, as-AGP lost every
inhibitory activity, which is retained by the olo-protein (Fig. 5A). The desialylation of AGP has no effect on primary granules (Fig. 5B). The spontaneous exocytosis down-regulatory effects of AGP on secondary granules is equally lost when sialic acid terminal residues are removed, as shown in Fig. 6A as well as the apparently up-regulatory activity on primary granules exocytosis, as shown in Fig. 6B.

In conclusion, both inhibitory and up-regulatory effects on secondary and primary granules exocytosis, respectively, cannot be detected by using the desialylated protein.

4. Discussion

The activity of neutrophils is essential for the development of the first phase of innate immunity. Yet, they are very aggressive cells, and the extracellular release of ROS
products, as well as the proteolytic enzymes contained in their granules, may cause massive tissue injuries during acute and chronic inflammation. Many acute phase proteins fulfill anti-inflammatory functions [7] directed toward the inhibition of these collateral damages of inflammation. In the first part of this study we provide evidence that one of them, the minor acute phase protein α1-acid glycoprotein, can modulate one of the most important functions of bovine neutrophils, the mobilization of their granules. Spontaneous degranulation is affected in a dose-dependent way by AGP. The biological significance of this result is unknown, but we may speculate that at least one of the functions of the rise in serum concentration of AGP during acute phase reaction might be that of counteract a praecox degranulation of neutrophils while they are still in the blood stream. The apparently opposite effect on primary granules has still to be elucidated. The capability of AGP to down-regulate the mobilization of secondary granules was more evident when the cells were challenged with ZAS.

Fig. 5. The effects of desialylation of AGP on its modulatory activity of neutrophil exocytosis. Isolated neutrophils (7 cows) were incubated with physiological (0.3 mg/ml) and acute phase (0.9 mg/ml) concentrations of AGP before and after treatment with neuraminidase, which specifically removes terminal sialic acid residues. Degranulation of secondary (Panel A) and primary (Panel B) granules was induced with ZAS as described in Section 2. HBSS and ZAS alone, without adding AGP, were used as positive control. Negative controls were HBSS treated cells without ZAS. After 120 min, alkaline phosphatase (ALK-P) (Panel A) and myeloperoxidase (MPO) (Panel B) enzymatic activities were measured in the supernatant of neutrophil culture as markers for secondary and primary granules exocytosis, respectively. Values are expressed as percentages of ALK-P and MPO enzymatic activity compared to total ALK-P and MPO activity in neutrophils lysed with CTAB (mean ± S.E.M.). Statistically significant differences between the two indications of AGP and HBSS treated activated neutrophils (positive control) indicated as * (P < 0.05). The differences between positive and negative controls were all statistically significant, and were not indicated in the figure.
The pathways regulating the specific mobilization of neutrophil granules are far to be clarified; nonetheless, granules translocation is known to depend on highly specific signals, i.e., their mobilization shows to be hierarchical, since primary granules are translocated after the secondary ones [25]. Still it is to be queried how AGP might interfere with the molecular pathways driving to the exocytosis of neutrophil granules.

AGP binds on the surface of neutrophil membrane, as shown by immunocytometry studies. It is therefore at least conceivable that the activity of AGP is due to the interaction with the protein on the surface of the cells. In order to verify the functional significance of this interaction, neutrophils were pre-treated with AGP, and then activated with ZAS. Since priming of neutrophils with AGP reduces the secondary granules exocytosis, it can be hypothesized that AGP might, at least partially, act by competing with the specific pathways which are activated by the pro-inflammatory challengers used to stimulate the cells. Essentially, the treatment of serum with Zymosan generates C5a that, in turn, activates the cells by interacting with CD88, its specific receptor. It would be very

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**Fig. 6.** The effects of desialylated AGP on neutrophil spontaneous degranulation. Isolated neutrophils (5 cows) were incubated with two different concentrations of AGP (0.3 and 0.9 mg/ml) before and after treatment with neuraminidase, which specifically removes terminal sialic acid residues. HBSS instead of AGP was used as negative controls. After 120 min, alkaline phosphatase (ALK-P) (Panel A) and myeloperoxidase (MPO) (Panel B) enzymatic activities were measured in the supernatant of neutrophil culture as markers for secondary and primary granules exocytosis, respectively. Values are expressed as percentages of ALK-P and MPO enzymatic activity compared to total ALK-P and MPO activity in neutrophils lysed with CTAB (mean ± S.E.M.). Statistically significant differences between AGP and HBSS treated neutrophils (negative control) were indicated as * ($P < 0.05$).
interesting to determine whether the reduced exocytosis induced by AGP is due to the interaction of the protein with CD88, which may contribute to down-regulate the secondary signals necessary to integrate the inflammatory response triggered by C5a.

And through still undisclosed mechanisms, AGP might modulate neutrophil granule exocytosis by binding to its specific receptors exposed on the surface of the cells. AGP’s binding sites on neutrophils are still poorly understood: at least two different binding sites have been identified on human granulocytes surface, with different binding affinities [22]. The molecular pathways activated when these receptors interact with AGP are still unknown. AGP’s receptors on the surface of neutrophils are likely involved in cytoskeletal remodelling events that drive to the selective mobilization of granules. This hypothesis is supported by a recent finding [9] which reported that AGP triggers Ca\(^{2+}\) mobilization in human neutrophils. Since the hierarchical mobilization of granules is due to different cytoskeletal activation, which is calcium dependent, we may speculate that the effects of AGP on the modulation of secondary granules translocation are related to its capability of inducing the intracellular Ca\(^{2+}\) mobilization.

Another possible mechanism of action of AGP can be assumed. As a binding protein, AGP might also act by sequestering some of the mediators, thus reducing their biological availability in the inflammatory environment. Further experiments should support such hypothesis; anyway, AGP can bind some inflammatory mediators such as PAF [13], as already shown in humans.

All the exocytosis modulating activities of AGP shown in this paper are strongly related with its glycan moiety, since all of them were suppressed by the removal of sialic acid terminal residues exposed on the protein surface. These results are consistent with other reports [2,5] demonstrating that the sialic acid residues are essential to some functions of the protein. A remarkable knowledge, since the glycosylation of AGP is strongly dependent on its physiological and pathological status. Furthermore, in accordance with above findings, other authors [9] had previously demonstrated that sialic acid molecules exposed on the surface of the AGP are essential to increase Ca\(^{2+}\) concentration. Comparing the effect on granule exocytosis of plasma AGP and the hypersialylated and hyperfucosylated glycoforms present in secondary granules of neutrophils [26,17], or in serum during several inflammatory diseases [3] would be an interesting matter of investigation. In conclusion, our findings reported for the first time that AGP is able to specifically down-regulate the secondary granule exocytosis. Signalling mechanisms underlying the involvement of AGP in modulating neutrophil functions are still not known, and further studies are necessary to go insight the signalling capability of this immunomodulatory protein.

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