Vol. 119, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1984 Pages 185-192

# INVOLVEMENT OF HYPOSIALYLATED IMMUNOGLOBULINS IN THE INACTIVATION OF α1-PROTEINASE INHIBITOR

M. Duc Dodon and L. Gazzolo

Unité de Virologie Fondamentale et Appliquée,
I.N.S.E.R.M. Unité 51, Groupe de Recherche C.R.S. N°33 ,
1, Place Professeur Joseph Renaut , 69371 LYON CEDEX 08, FRANCE

Received January 10, 1984

The elastase inhibitory capacity of α1-proteinase inhibitor (α1-PI) was measured, using a direct and reproducible method, with phagocytic cells maintained in the tissue culture plate through the assay. The oxidative inactivation of α1-PI is known to be mediated by the action of myeloperoxidase (MPO). The fact that hyposialylated IgG (hs IgG) induce the release of MPO prompted us to investigate the effects of such hs IgG on the inhibitory capacity of α1-PI. The results show that 1-PI inactivation was observed only when phagocytic cells were activated by aggregated hs IgG, and not by unaggregated hs IgG. These observations indicate that hyposialylation should be completed by aggregation to perpetuate the oxidative reactions characteristic of inflammatory diseases.

A post-translational modification of immunoglobulins (Ig), such as hyposialylation, can markedly affect the structural conformation of the molecule, and thus its antigenicity and its immunogenicity (1). We have recently shown that hyposialylated IgG, aggregated or not, can serve as stimuli in releasing myeloperoxidase (MPO) from phagocytic cells (2). This enzyme, in combination with $H_2O_2$, catalytically inactivates the α1-proteinase inhibitor (α1-PI), by mediating the oxidation of its methionine residues into methionine sulfoxide ones (3,4,5). This oxidative inactivation of α1-PI is linked to a decrease in its capacity to inhibit elastase, thus resulting in the degradation of structural proteins, such as elastin, collagen and proteoglycans. The importance of this process has been underlined in the pathogenesis of both emphysema and rheumatoid arthritis (RA) (6,7,8).

0006-291X/84 $1.50

Since hyposialylated Ig (hs IgG) have been shown to be constituents of human rheumatoid factors (9), it seemed relevant to determine whether hs IgG, aggregated or not, might be implicated in the modification of the elastase-α1-PI homeostasis.

The present study was designed to this question. The results obtained are providing a better definition of the involvement of hs IgG in the induction of the inflammatory processes.

## MATERIALS AND METHODS

Chemicals : Neuraminidase, type V from Clostridium perfringens, α1-proteinase inhibitor (α1-PI) from human plasma, Elastase, type III from porcine pancreas, Elastin-like substrate (N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide or Suc-$(Ala)_3$-NA), Human γ-globulins (Cohn Fraction II) were obtained from Sigma Chemical Co, (Saint Louis, MO). Phorbol-12-myristate-13-acetate (PMA) was supplied by Consolidated Midland Co, (Brewster, NY). Enzacryl- AH was purchased from Koch-Light Laboratories (Colnbrook). Culture media were purchased from GIBCO (Grand Island, NY).

Cells : Mononuclear phagocytic cells were prepared from citrated (44mg/ml) peripheral blood from human donors. Whole blood was then diluted 1/4 in RPMI 1640 medium and centrifuged over Ficoll Hypaque (p=1.078 $g/cm^3$) at 20°C, according to the method described by Boyum (10). Cells present at the interphase layer were collected, washed three times in RPMI 1640 medium, counted and resuspended in PBS. They were seeded in wells of Microtest II Tissue culture plate N° 3042 (Falcon, Oxnard, CA), at a ratio of $1.0 \times 10^6$ cells/50µl/well. Thereafter, cells were incubated at 37°C in 5% $CO_2$, in the presence of normal or hyposialylated IgG.

Hyposialylated IgG : Hyposialylated IgG (hs IgG) were obtained by incubating normal IgG with neuraminidase immobilised onto Enzacryl-AH, according to a procedure described previously (1). The modulation in the degree of hyposialylation and in that of aggregation was performed by modifying the time of incubation in the presence of neuraminidase. The sialic acid content was determined by a fluorimetric assay. It was found that hs IgG contained about three times less sialic acid than normal IgG (2.25+0.05 µg SA/mg), whatever the time of treatment with neuraminidase (7, 60 or 360 min).

Assay of inhibitory capacity of α1-PI : Mononuclear phagocytic cells ($10^6$/well) were incubated in the presence of different stimuli (PMA, hs IgG, normal IgG) and α1-PI (50 nmoles), for 45 min at 37°C, to a final volume of 150 µl. At the end of this incubation time, cells were kept at 20°C. Then, 50 µl 200mM Tris pH 8.0, 25 µl elastase (50nmoles) were added. Two minutes later, 50 µl of substrate (500 nmoles) were added (11). After another 4 minutes, the amount of degraded substrate per well was detected colorimetrically by using a Microelisa autoreader (MR580 Dinatech) at 405 nm.

## RESULTS

### Oxidative inactivation of human α1-PI : assay conditions.

Previous methods devised to measure α1-PI oxidation were often dependent on the cumbersome purification of this protein (8).

In order to get a more useful and reproducible assay, we develop a method to determine the loss of $\alpha$1-PI inhibitory activity towards elastase, directly in the culture wells. To this end, exogenous $\alpha$1-PI was added to the culture of phagocytic cells, just after the addition of the stimulating agent. Elastase and elastin-like substrate were added 45 min after the addition of $\alpha$1-PI.

When $\alpha$1-PI is active (unoxidised), the $\alpha$1-PI complexes elastase, and inhibits its activity towards elastin. To detect a modification in $\alpha$1-PI activity, reactions were performed by increasing the amount of $\alpha$1-PI with a constant amount of elastase and substrate (50 and 500 nmoles, respectively), in a final volume of 275 µl. Figure 1 shows that the inhibition of elastase activity by $\alpha$1-PI increased up to an amount of 150nmoles. In the experiments reported in this study, the amount of $\alpha$1-PI introduced in the reaction mixture was 50nmoles. Under these conditions, 40% of the elastase activity was inhibited.

When $\alpha$1-PI is oxidised, it does not complex elastase, which is active towards elastin. Consequently, the amount of degraded

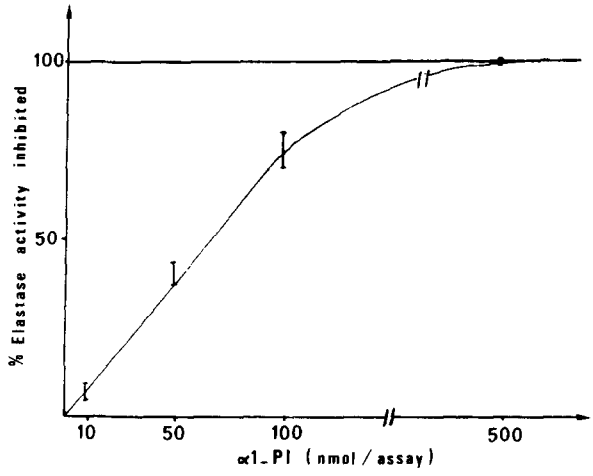


Fig. 1. Inhibition of porcine pancreatic elastase : dependence on the concentration of human $\alpha$1-PI. Assay conditions : 0.22mM elastase, 0.2M Tris-HCl pH 8.0, 1.81mM Suc-$(Ala)_3$-NA. Experiments were performed in duplicate, and results are expressed in percent of the elastase activity inhibited by human $\alpha$1-PI.

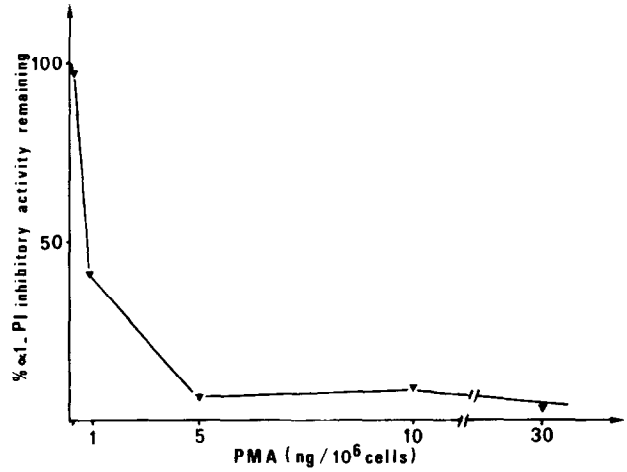


Fig. 2. Effect of PMA concentrations on the human α1-PI inhibitory activity. The assay conditions were the same as in Fig. 1, with 0.33mM human α1-PI. Results, expressed in percent of the inhibitory activity of the controls, are the mean of 3 experiments.

substrate by elastase will account for a loss of its inhibitory capacity. To verify these assay conditions, we used phorbol myristate acetate (PMA) known as a membrane perturbing agent inducing a respiratory burst (12). Concentrations of PMA over the range 0.1 to 30 ng/$10^6$ cells were tested. As shown in Figure 2, half-loss of the α1-PI inhibitory activity required 1ng/$10^6$cells. Maximal loss was seen with 5ng/$10^6$cells.

These first experiments indicate that the assay conditions described above will be instrumental in the detection of oxidised α1-PI by determining the level of its activity directly in the culture plates, with phagocytic cells in situ.

Hyposialylated IgG and inhibitory capacity of human α1-PI.

To assess the effect of hs IgG on the oxidative inactivation of α1-PI, human phagocytic cells were incubated for 45min. at 37°C with increasing amounts of hs aggregated IgG or with normal IgG (concentrations ranging from 10 to 200 µg/well).

Results presented in Figure 3 clearly show that the inhibitory capacity of α1-PI decreased for a concentration as low as 10 µg of hs aggregated IgG. For 20 µg, 70% of the α1-PI inhibitory

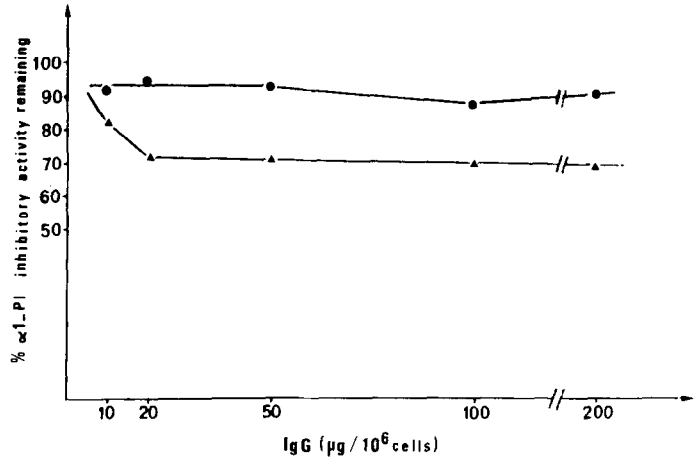


Fig.3. Effect of IgG concentrations on the human α1-PI inhibitory activity. Cells were incubated either with normal IgG ( ● ) or with aggregated hs IgG ( ▲ ). Assay conditions as in Fig. 2. Results are the mean of 5 experiments. They are expressed in percent of the inhibitory activity of the control cells, incubated with any added IgG.

activity were still detected and for higher concentrations (50 to 200μg/$10^6$ cells), no further decrease was noted. No significant modification can be observed, when phagocytic cells were incubated with normal IgG.

Consequently, inactivation of extracellular α1-PI was occuring in a dose-dependent manner when phagocytic cells were incubated with hs aggregated IgG.

Results presented above do not distinguish the effect of aggregation from that of hyposialylation. To assess an eventual difference, phagocytic cells were incubated with hs IgG varying in their degree of aggregation. Such types of IgG were obtained by modulating the time of treatment with neuraminidase (hs IgG-7min, hs IgG-60min, hs IgG-360 min). In addition, hs unaggregated IgG, obtained after centrifuging hs IgG 360 min at 105,000g for 2hr, were added to cells in culture (50 μg/$10^6$ cells). Controls were performed either using normal IgG after high speed centrifugation or without any addition.

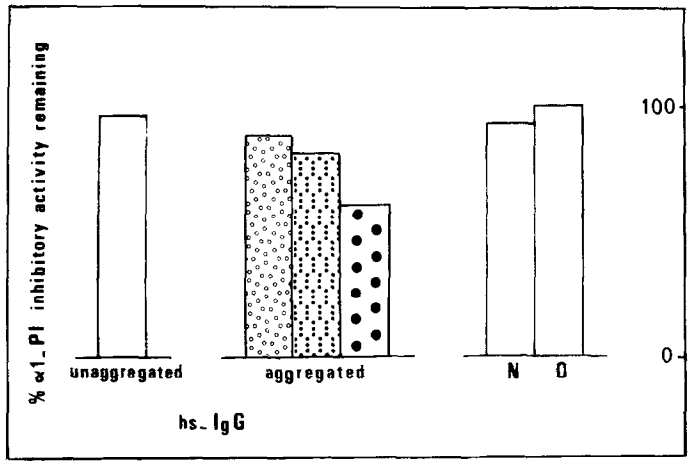


Fig. 4. Effect of aggregated or unaggregated hs IgG on human α1-PI inhibitory activity. Phagocytic cells were incubated with 50 μg of each type of IgG. Assay conditions as in Fig. 2. Aggregated IgG were : hs-7min, hs-60min, hs-360min. Normal IgG (N). Results (mean of 2 experiments) are expressed as percent of the inhibitory activity of control cells incubated without any added IgG (O).

Results shown in Figure 4 clearly indicate that the oxidative inactivation of α1-PI was indeed dependent on the aggregation and correlated with the extent of aggregation. Conversely, hs unaggregated IgG were unable to induce this phenomenom. In fact, the same results were obtained when concentrations over the range 50 to 200μg/$10^6$ cells of hs unaggregated IgG were used, and when the incubation time of phagocytic cells with α1-PI was extended to 3 hr (data not shown).

In conclusion, hyposialylated IgG are involved in the oxidative inactivation of α1-PI only when present under aggregated form.

## DISCUSSION

Oxidation of the methionine residues of α1-PI results in a loss of its inhibitory capacity towards elastase. Inactive α1-PI purified from human inflammatory synovial fluid has been shown to contain methionine sulfoxide residues, owing probably to the action of the MPO-$H_2O_2$ system which is released from activated phagocytic cells (5,8).

Results reported in this article were obtained, taking advantage of the assay we have developed to measure oxidised $\alpha$1-PI, directly in the culture plates with cells in situ. These results show that in vitro oxidation of $\alpha$1-PI is obtained only when phagocytic cells are activated by aggregated hs IgG. Conversely, $\alpha$1-PI is not oxidised, when phagocytic cells were incubated with unaggregated hs IgG, although we have demonstrated that both types of IgG were equally able to induce MPO release, albeit to a different level (2).

The low amount of MPO activity released after incubation of phagocytic cells with unaggregated hs IgG may not be sufficient to reach the threshold necessary to the oxidation of $\alpha$1-PI. In fact, unaggregated hs IgG may be considered as membrane perturbing agents, inducing only a minimal release of MPO. Unaggregated hs IgG should be relayed by aggregated hs IgG to carry out the consecutive steps of oxidative reactions, leading to tissue damage. It has been recently reported that the MPO-$H_2O_2$ system is able to generate aggregates of normal IgG in vitro (13). Preliminary experiments indicate that hs IgG seem to be more sensitive to this aggregating effect than normal IgG. It is therefore possible that the amount of MPO released, in the presence of unaggregated hs IgG, may be sufficient for aggregating hs IgG. The results reported in this study provide new information on the precise role of hs IgG as active stimuli in the induction of inflammatory processes through the initiation and the maintenance of oxidative reactions.

## ACKNOWLEDGEMENT

This work was supported by a grant fom I.N.S.E.R.M. (CRL 81 20 27).

## REFERENCES

1. Duc Dodon, M., and Quash, G.A. (1981) Immunology 42, 401-408.
2. Duc Dodon, M., Gazzolo, L., and Quash, G.A. (1983) Submitted to Immunology

3. Tsan, M.-F. (1982) J. Cell Physiol. 111, 49-54.
4. Brot, N., and Weissbach, H. (1983) Arch. Biochem. Biophys. 223 271-283.
5. Matheson, N.R., Wong, P.S., and Travis, J. (1979) Biochem. Biophys. Res. Commun. 88, 402-409.
6. Carell, R.W., Jeppson, J.-O., Laurell, C.-B., Brennan S.O., Owen, M.C., Vaughan, L., and Boswell, D.R. (1982) Nature 298, 329-334.
7. Carp, H., Miller, F., Hoidal, J.R., and Janoff, A. (1982) Proc. Nat. Acad. Sci. U.S.A. 79, 2041-2045.
8. Wong, P.S., and Travis, J. (1980) Biochem. Biophys. Res. Commun. 96, 1449-1454.
9. Duc Dodon, M., and Quash, G.A. (1983) Rheumatology International 3, 97-100.
10. Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21 (Suppl. 97) 77-89.
11. Bieth, J., Spiess, B., and Wermuth, C.G. (1974) Biochem. Med. 11, 350-357.
12. Janoff, A., Carp, H., Laurent, P., and Raju, L. (1983) Am. Rev. Respir. Dis. 127, S31-S38.
13. Jasin, H.E. (1983) J. Immunol. 130, 1918-1923.