# The sensitivity to plasmin digestion of human IgG proteins of different heavy chain subclasses[1,2]

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*Summary.* Normal IgG and purified IgG proteins of all 4 subclasses were digested with plasmin. As expected, $IgG_3$ proteins were highly susceptible to degradation. Usually, activation with streptokinase resulted in faster and more accentuated degradation, but normal IgG was more intensely degraded by nonactivated plasmin. The presence of plasmin activators in IgG preparations might account for this observation.

In the early 1960's several authors reported spontaneous degradative changes in human gamma globulin during storage, interpreted as similar to the splitting into Fab and Fc fragments induced by papain[3–5]. Plasmin was assumed to be the responsible enzyme, and Sgouris et al. were the first authors to confirm this possibility by adding plasmin to human gamma globulin[6]. Their results were later confirmed by Connell and Painter[7]. 10 years later we reported the increased lability of $IgG_3$ proteins during storage, which could be potentiated by mild reduction[8]. The sensitivity of $IgG_3$ to plasmin digestion was confirmed by Skvaril et al. in a study of plasmin-treated normal human IgG[9]. One problem with this last investigation was the long digestion period (3–4 weeks at 37 °C), in contrast with the results reported by Connell and Painter, who found obvious changes at 24 h. This discrepancy raised questions about the specificity of the changes observed by Skavril et al., and it seemed pertinent to study the sensitivity to digestion with plasmin of monoclonal proteins of different IgG subclasses under carefully controlled conditions. The results of such experiments are reported here.

*Materials and methods.* Purified normal IgG was obtained from Cohn's Fraction II[10] by chromatography in DE-52[11] using sodium phosphate buffer (pH 6.5, 0.01 M) for elution. 10 monoclonal $IgG_2$ proteins (3 $IgG_1$ and $IgG_3$, 2 $IgG_2$ and 2 $IgG_4$) were purified by precipitation in 33% saturated ammonium sulfate, followed by chromatography in DE-52. Plasmin was purified from Cohn's Fraction III using Lysine-Sepharose 4B[12], following the method of Chibber et al.[13], as modified by the manufacturers[14]. The eluted plasminogen was separated by gel filtration in AcA 34[15],

1 This is publication no. 125 from the Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina.

2 Acknowledgments. The authors wish to express their gratitude to Mr Charles L. Smith for excellent editorial assistance and to Mrs Louise B. Just for photographic work. Research supported in part by ACS Grant IM-16I.

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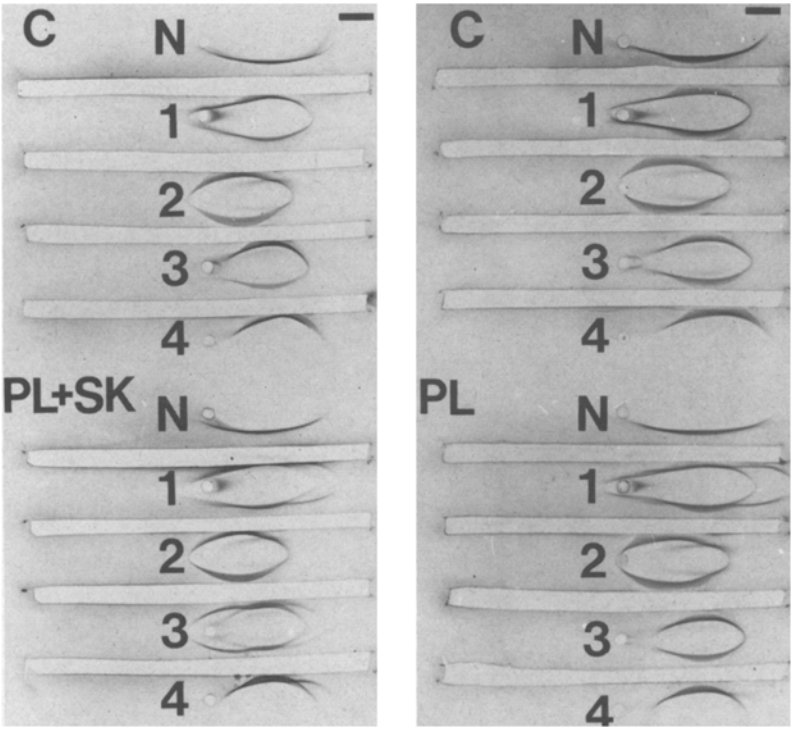


Fig. 1. Immunoelectrophoretic study of the results of 24-h incubation of several IgG proteins with plasmin. The following proteins were separated in each well: normal IgG (N); GIA, $IgG_1$ (1); PLE, $IgG_2$ (2); MOG, $IgG_3$ (3); and LOW, $IgG_4$ (4). C, control samples incubated for the same period of time without enzyme; PL, samples incubated with plasmin; PL+SK, samples incubated with streptokinase-activated plasmin. All troughs were filled with polyvalent anti-immunoglobulin serum.

and 3 overlapping peaks were obtained. The fractions constituting the descending limb of the second peak and the third peak in its entirety (found to contain maximal plasmin activity by electrophoresis in fibrin-containing agar according to the method of Heimburger and Schwick[16]) were pooled, concentrated to an OD (280 nm) of 1.4 and used for digestion of IgG proteins.

The purified proteins were adjusted to a concentration of 10 mg/ml with phosphate-buffered saline (PBS). In the basic experimental protocol, 3 aliquots of 500 µl of the proteins adjusted to 10 mg/ml were prepared. To one of them was added 100 µl of the plasmin preparations; to the second aliquot we added the same volume of plasmin plus 50 µl of a solution containing at least 5000 U of streptokinase[17,18]; the third aliquot was a control to which nothing was added. The different aliquots of all proteins included in the study were then incubated at 37 °C, and results of digestion were checked every 24 h for 2 to 4 days by immunoelectrophoresis[19], using a polyvalent anti-human immunoglobulin antiserum[20]. A special experiment was carried out to determine whether $IgG_3$ proteins would be protected from digestion when ε-aminocaproic acid was added to the incubation mixture, using 3 samples prepared in addition to the ones already mentioned. One consisted of the protein diluted in PBS containing 0.2 M ε-aminocaproic acid; the second was identical to the first except for the addition of 100 µl of purified plasmin solution; the third contained the protein diluted in PBS containing ε-aminocaproic acid plus plasmin and streptokinase, in the same amounts used in the standard experiment. The digestion of the $IgG_3$ proteins was then followed for 6 days.

The results of the different incubations, as judged immunoelectrophoretically, were scored from 0 to 3 according to the following criteria; 0, no changes; 1, single split (cathodal or anodal) of the precipitin arc; 2, double split (cathodal and anodal) of the precipitin arc; 3, obvious release of Fab- and Fc-like fragments.

*Results and discussion.* As illustrated by figure 1, and summarized in the table, $IgG_3$ proteins were consistently found to be more affected by incubation with plasmin than were proteins of other subclasses. This was particularly evident in the case of protein MOG, an exceptionally stable $IgG_3$ protein that resisted prolonged incubation with plasmin without evidence of digestion but underwent extensive splitting into Fab- and Fc-like fragments after 24 h of incubation with streptokinase-activated plasmin. Other $IgG_3$ proteins showed spontaneous degradation to various degrees, but the splitting into Fab- and Fc-like fragments was always more evident in the samples incubated with plasmin or with streptokinase-activated plasmin.

Results with proteins of other subclasses seemed to confirm the higher sensitivity to proteolysis of $IgG_1$ and $IgG_4$ proteins than $IgG_2$ proteins. However, there was some heterogeneity worthy of notice. One of the $IgG_1$ proteins was very susceptible to plasmin, while 2 others seemed very resistant. Although one of the $IgG_4$ proteins was clearly affected by plasmin, the other appeared to undergo spontaneous degradation, which was not increased in the presence of plasmin. We also found that normal IgG was clearly more affected by plasmin than by streptokinase-activated plasmin; this may suggest that the protein preparation contained some activating substance more effective than streptokinase.

In the experiment designed to test the possibility of protecting $IgG_3$ proteins from plasmin-induced (or spontaneous) degradation, the only preparation affected after incubation for up to 96 h was the one incubated with plasmin, streptokinase, and ε-aminocaproic acid, showing that this compound was not protective under these conditions. After incubation for 1 week, all samples (controls and samples incubated with plasmin or with plasmin plus

Effects of incubation of IgG proteins with plasmin, with or without activation with streptokinase

| Protein | Incubation 24 h | | | 48 h | | | 72 h | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | PL | PL + SK | C | PL | PL + SK | C | PL | PL + SK |
| Normal IgG | 0 | 1 | 0 | 0 | 2 | 1 | 0 | 2 | 1 |
| WEI ($IgG_1$) | – | – | – | – | – | – | 0 | 2 | 2 |
| GIA ($IgG_1$) | 0 | 1 | 1 | 0 | 3 | 3 | – | – | – |
| STO ($IgG_1$) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HIT ($IgG_2$) | – | – | – | – | – | – | 0 | 0 | 1 |
| PLE ($IgG_2$) | 0 | 0 | 0 | 0 | 0 | 0 | – | – | – |
| WOO ($IgG_3$) | – | – | – | – | – | – | 1 | 3 | 3 |
| MOG ($IgG_3$) | 0 | 0 | 3 | 0 | 1 | 3 | 0 | 1 | 3 |
| MAX ($IgG_3$) | 2 | 3 | 3 | 2 | 3 | 3 | 2 | 3 | 3 |
| FRA ($IgG_4$) | – | – | – | – | – | – | 1 | – | 1 |
| LOW ($IgG_4$) | 0 | 1 | 1 | 0 | 2 | 2 | – | – | – |

Abbreviations and symbols: C, control; PL, plasmin; PL+SK, streptokinase-activated plasmin; –, not done; 0, no change; 1, cathodal or anodal split of the precipitin arc; 2, cathodal and anodal splits of the precipitin arc; 3, evident split into Fab and Fc fragments.

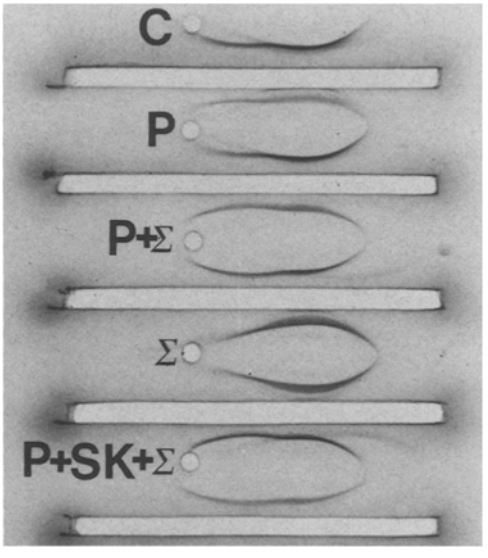


Fig. 2. Immunoelectrophoretic study of several aliquots of protein MOG ($IgG_3$) incubated for 7 days at 37 °C. C, control sample; P, sample incubated with plasmin; P+ε, sample incubated with plasmin and ε-aminocaproic acid; ε, sample incubated with ε-aminocaproic acid; P+SK+ε, sample incubated with streptokinase-activated plasmin plus 0.1 M ε-aminocaproic acid. The troughs were filled with a polyvalent anti-immunoglobulin serum.

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streptokinase, with or without ε-aminocaproic acid) showed evidence of degradation, except for 1 sample where the $IgG_3$ protein was incubated with 0.1 M ε-aminocaproic acid (figure 2). It thus seems that this compound can have a protective effect when small amounts of plasmin are present in the sample, as is probably the case for most purified $IgG_3$ preparations.

In conclusion, our results show that plasmin induces the same type of denaturatives changes in monoclonal IgG proteins as seen during storage, or after incubation with reducing agents[8], confirming the postulated role of this enzyme. The speed of the reaction, in our observations, was intermediate between that reported by Connell and Painter[7] and the very slow degradation described by Skvaril et al.[3]. The amount of enzyme, its state of activation, and the presence of activating or inhibiting substances as contaminants of the IgG preparations may be of relevance in determining differences in the activity of the enzyme, beyond the individual degrees of susceptibility of each IgG subclass.

## The early response of $^{59}Fe$ incorporation in the bone marrow of irradiated rats[1]

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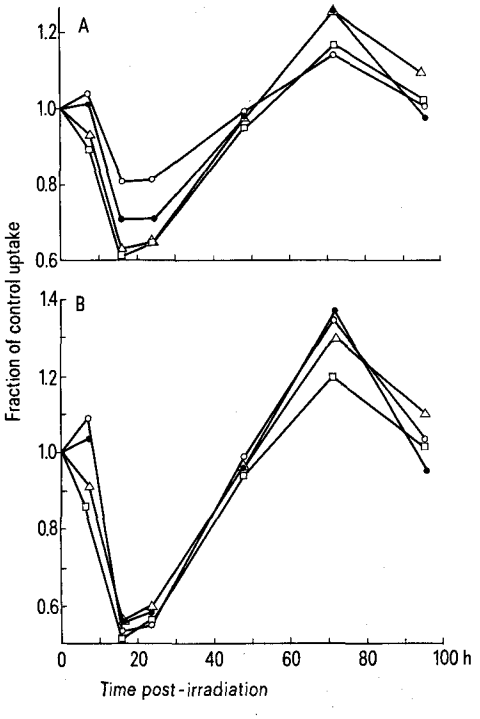
*Summary.* The change in radioiron uptake in selected bone marrow samples in irradiated rats is representative of the total skeletal uptake only if the baseline or radioresistant uptake has been subtracted.

Holá et al.[2] have stated that for a given radiation exposure to mice, the degree of reduced $^{59}Fe$ uptake in various bone marrow compartments depended upon the particular bone under study. This conflicts with the common practice of using the uptake of radioiron in an individual bone, e.g., the femur or the tibia, as being representative of the erythropoietic activity of the total skeleton[3]. The study reported here was designed to examine this conflict and to seek a method for circumventing it.

*Method.* Female Sprague-Dawley rats, weighing between 170 and 200 g were used. The animals were anesthetized with sodium pentobarbitol and irradiated through their ventral surfaces, 4 at a time, on a rotating table. A 1000 kVp General Electric Industrial X-ray unit was used under the following conditions: 1000 kVp, no additional filtration, HVL of 3.0 mm Pb, and a target to midline distance of 66.0 cm. The exposure rate was 15.8 R/min. and a total whole-body exposure of 50 R was given. At varying times post-exposure and under light halothane anesthesia, the animals were injected via the femoral vein with 5 μCi of $^{59}Fe$ labeled ferrous citrate. 6 h later, the the rats were sacrificed via cervical separation. Approximately 1 h prior to sacrifice, $^{51}Cr$ labeled red cells were injected via the femoral vein. Just prior to sacrifice, a 25 μl blood sample was drawn from the tail vein. This blood sample was used to subtract any bone activity which was due to radioiron in the circulating blood. The eviscerated animals were ashed at 595 °C and cleansed bone samples were counted individually in an autogamma counter. The data were taken for 16 control animals and 4 irradiated rats at each point. An additional 24 rats were irradiated with exposures of 700, 850 and 1000 R and the $^{59}Fe$ uptake measured at 24 h post-exposure. It has been shown that with exposures of these levels, the uptake




Fraction of control uptake vs time in h post-irradiation. A. Ratio of total uptakes; B. Ratio of net uptakes. ○, Scapula; □, Femur; ▲, Cervical spinal segements 1–5; △, Sternum.

1 This paper is based on work performed under contract with the US Energy Research and Development Administration at the University of Rochester Biomedical and Environmental Research Project, and has been assigned Report No. UR-3490-949.

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Net $^{59}Fe$ uptake in control and baseline rats (% of injected $^{59}Fe$)

| Bone sample | Control | Baseline |
|---|---|---|
| Scapula | 0.617 ± 0.20* | 0.371 ± 0.022 |
| Femur | 5.43 ± 0.28 | 1.14 ± 0.06 |
| Cervical Vertebrae (1–5) | 0.913 ± 0.034 | 0.301 ± 0.016 |
| Sternum | 1.05 ± 0.04 | 0.172 ± 0.011 |

*Mean ± 1 SE.