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Amidase and Peptidase Activities of Polyclonal Immunoglobulin G Present in the Sera of Patients with Rheumatoid Arthritis

KINJI MATSUURA,[†,1] SHINYA IKOMA,[2] MASAFUMI SUGIYAMA,[2] MASANORI FUNAUCHI,[2] AND HYOGO SINOHARA*[,3]

Departments of [1]Biochemistry and [2]Internal Medicine, and [3]Life Science Institute, Kinki University School of Medicine, Osaka-Sayama, Osaka 589-8511, Japan

Abstract

Polyclonal Immunoglobulin (Ig) G from patients with rheumatoid arthritis (RA) and healthy subjects hydrolyzed carbobenzoxy-Val-Gly-Arg *p*-nitroanilide and D-Pro-Phe-Arg *p*-nitroanilide. RA IgG exhibited higher activity against the former substrate, but not the latter. On the other hand, RA IgG showed reduced activity against D-Pro-Phe-Arg methylcoumarinamide, when compared with those of the healthy controls. These results suggest that RA IgGs differ from normal IgGs in the substrate specificity of amidase activity. Preliminary studies have shown that two out of three RA IgG samples cleaved a pentapeptide—Gln-Arg-Arg-Ala-Ala—which is assumed to be associated with the risk of developing RA (Gregersen, P. K. et al. (1987), *Arthritis Rheum.* **30,** 1205–1213). By contrast, virtually no cleavage of the same peptide was observed with IgG from healthy controls. A peptide analog, Gln-Arg-Arg-Trp-Ala, was not cleaved at all by any IgGs examined either from RA patients or healthy controls.

Index Entries: Amidase activity of IgG; peptidase activity of IgG; rheumatoid arthritis; shared epitope.

Introduction

About 20 yr ago, Erhan and Greller pointed out the structural similarity between the complementarity-determining region (CDR) of

*Author to whom correspondence and reprint requests should be addressed.
[†]Present address: The University of Texas Houston, Medical School, Department of Pathology and Laboratory Medicine, Houston, TX 77225.

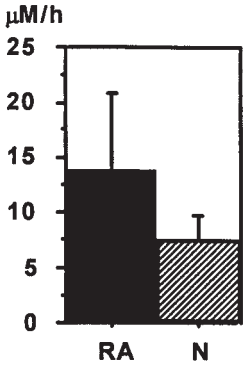

Fig. 1. Cleavage of carbobenzoxy-Val-Gly-Arg-pNA by IgGs from RA patients (n = 23) as compared with those of healthy controls (n = 10). IgGs were prepared using protein G-Sepharose as described previously *(9)*. Ten microliters of each IgG sample was mixed with 10 μL of 10 m*M* amidase substrate and 10 μL of 50 m*M* Tris-HCl (pH 7.7) in 96-well plates, and incubated for 18 h in a humidified incubator. Hydrolysis of the substrate was determined by reading the absorbance at 405 nm in a microplate reader. The vertical bar represents standard error of the mean. The difference between the two groups is statistically significant ($p < 0.05$).

immunoglobulin (Ig) light (L) chain and a catalytic center of serine proteinase, and suggested that the L chain might have proteinase activity *(1)*. Recently, Paul et al. showed that some autoantibodies directed to vasoactive intestinal peptide (VIP) and thyroglobulin were capable of cleaving their own antigens *(2–4)*. Furthermore, L chains derived from these autoantibodies and most Bence Jones proteins were found to cleave the synthetic amidase substrates and biologically active peptides *(5–7)*. Recently, Kalaga et al. reported that IgG samples from patients with rheumatoid arthritis (RA) had about twofold decreased amidase activity compared to those of healthy controls *(8)*. Our findings indicate that RA IgGs have greater amidase activities than those of control *(9)*, the opposite of Kalaga's results. Clarification of the cause of this discrepancy has led to a new hypothesis that IgGs of RA patients differ from those of healthy controls in the substrate specificity of peptidase activity, the former preferring the small-sized hydrophobic residues to large-sized residues at P'1 subsite—i.e., the residue immediately C-terminal to the scissile bond. As an extension of this work, we also found that RA IgGs cleaved the pentapeptide Gln-Arg-Arg-Ala-Ala, which is associated with the risk of developing RA according to the so-called shared epitope hypothesis *(10)*. By contrast, virtually no cleavage of the same peptide was observed with IgGs from healthy controls.

Amidase Activities of RA IgGs

Polyclonal IgGs of RA patients had significantly greater amidase activities than those of healthy controls, when carbobenzoxy-Val-Gly-Arg *p*-nitroanilide (pNA) was used as substrate (*see* Fig. 1). This result differs

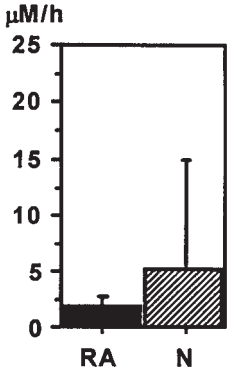

Fig. 2. Cleavage of Pro-Phe-Arg-MCA by IgGs from RA patients (n = 23) as compared with those of healthy controls (n = 10). The assay conditions were the same as those described in the legend for Fig. 1, except that carbobenzoxy-Val-Gly-Arg-pNA was replaced by Pro-Phe-Arg-MCA, and the activity was estimated as the fluorescence (emission 470 nm; excitation 370 nm) of the generated aminomethylcoumarin. The vertical bar represents standard error of the mean.

from the findings of Kalaga et al. *(8)*, who showed that IgG amidase activities from RA patients were about half those of the healthy controls. On the other hand, essentially the same results were obtained when we used their substrate—i.e., Pro-Phe-Arg methylcoumarinamide (MCA). However, the difference in our study was not statistically significant, possibly because the healthy controls showed a large scatter of values leading to high standard error (*see* Fig. 2). When Pro-Phe-Arg-pNA was used as substrate, virtually no differences were observed between RA and normal IgGs (*see* Fig. 3). These results suggest that RA IgGs differ somewhat in their substrate specificity from those of healthy subjects.

Relationship Between Amidase Activities of RA Patients Assayed by Different Substrates

The amidase activities of RA IgGs were compared with each other using three different substrates. As shown in Fig. 4, RA IgGs showed relatively low activity with little variation against Pro-Phe-Arg-MCA (*A* group), confirming the previous result *(8)*. The relative amidase activities of individual patients against the two pNA substrates were roughly correlated with each other (*B* and *C* groups). These results further support the notion that the amidase activity of individual IgG differs in its substrate specificity.

Catalytic Constants for IgG-Catalyzed Amidolysis

The K_m values (0.035–0.078 m*M*) of RA IgGs for carbobenzoxy-Val-Gly-Arg-pNA were one order of magnitude lower that those (0.32–0.64 m*M*) of IgG from healthy controls *(9)*. On the other hand, the K_m values

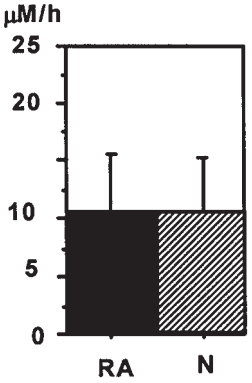

Fig. 3. Cleavage of Pro-Phe-Arg-pNA by IgGs from RA patients (n = 23) as compared with those of healthy controls (n = 10). The activity was determined as described in the legend for Fig. 1, and the vertical bar represents the standard error of the mean.

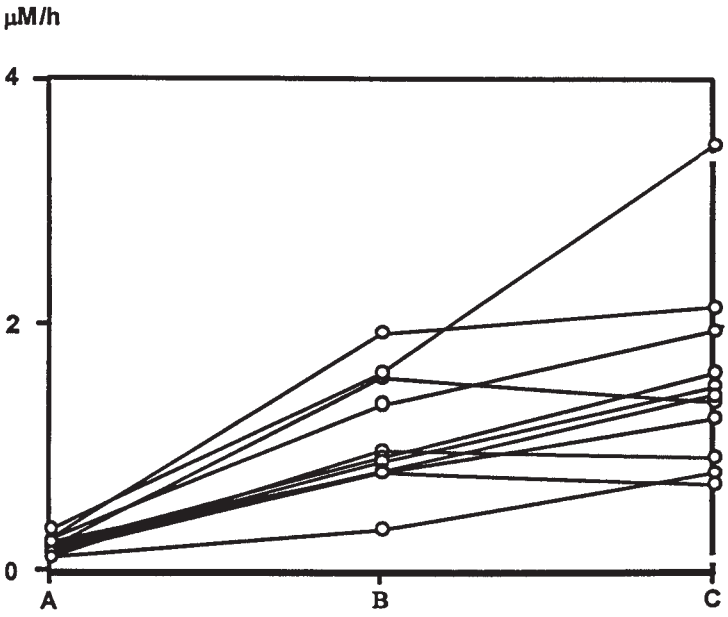

Fig. 4. IgG amidase activities from individual RA patients were compared with each other using three different substrates: **(A)** Carbobenzoxy-Val-Gly-Arg-pNA; **(B)** Pro-Phe-Arg-MCA; **(C)** Pro-Phe-Arg-pNA. The activity was determined as described in the legends for Figs. 1–3.

for Pro-Phe-Arg-MCA (0.45–0.77 m*M*) were higher than those of the healthy groups (0.11–0.18 m*M*). By marked contrast, no statistically significant differences were observed in the K_m values between any pair of lupus erythematosus, Sjögren's syndrome, and normal controls for either substrate *(9)*. These results are also consistent with the notion that the substrate speci-

ficity of RA IgGs differs from that of IgGs from the healthy controls or the two autoimmune diseases described here.

Hydrolysis of Pentapeptides

Recent immunogenetic analysis of RA patients revealed that susceptibility to this disease is associated with an epitope formed by several amino acids of the third hypervariable region of DRβ1 molecules *(10–13)*. There are several *DRB1* genes that express the same amino-acid sequence around codon 71, and these are associated with RA in different human populations. The maximal risk of developing RA is associated with the so-called shared epitope, i.e., Gln-Arg(Lys)-Arg-Ala-Ala, located at positions 70–74 *(10)*. At present, virtually nothing is known of the role played by this epitope for determining the RA susceptibility. However, the substrate specificity of catalysis by the RA IgGs suggests that some IgGs raised against this epitope may have acquired the potential to hydrolyze it. To test this possibility, a synthetic pentapeptide, Gln-Arg-Arg-Ala-Ala, was incubated with RA and control IgGs. The original substrate with no incubation contained two peaks (*a* and *b* (*see* Fig. 5*A*), and their sequence analysis yielded the same sequence, Gln-Arg-Arg-Ala-Ala. One of the two peaks probably represents a peptide fraction from which the protecting groups were incompletely removed. After incubation, two new peaks (*c* and *d*) appeared (*see* Fig. 5*B*). The sequence analysis of peak *c* indicated that it consisted of tripeptide, Arg-Ala-Ala (*see* Table 1). The analysis of peak *d* suggested that it was a mixture of Gln-Arg-Arg, Gln-Arg, and Ala-Ala. These results suggest that the pentapeptide was cleaved mainly between Arg-Arg, yielding Arg-Ala-Ala (peak *c*) and Gln-Arg (present in peak *d*). The presence of Gln-Arg-Arg (in peak *d*) also suggested that the pentapeptide was cleaved between Arg and Ala, yielding Gln-Arg-Arg (in peak *d*) and Ala-Ala (in peak *d*). Of three IgG samples from RA patients, two showed essentially the same cleavage pattern, but one had no activity. On the other hand, the control pentapeptide, Gln-Arg-Arg-Trp-Ala, was not cleaved at all by any IgGs from RA or healthy controls *(9)*. These results are consistent with the data obtained with synthetic amidase substrates—i.e., RA IgG favors the small-sized residue at P'1 subsite.

Conclusion

Recent evidence indicates that the expression of antigen-specific peptidase activities is increased in some autoimmune diseases, such as lupus erythematosus, Hashimoto's thyroiditis, and asthma *(14)*. Although little is presently known of the autoantigen of RA, the risk of developing RA has been shown to be associated with the oligopeptide sequence consisting of Gln-Arg (Lys)-Arg-Ala-Ala (the shared epitope) *(10–13)*. We found that this pentapeptide was cleaved by some RA IgGs, suggesting that the expression of antigen-specific peptidase activity was also increased in RA

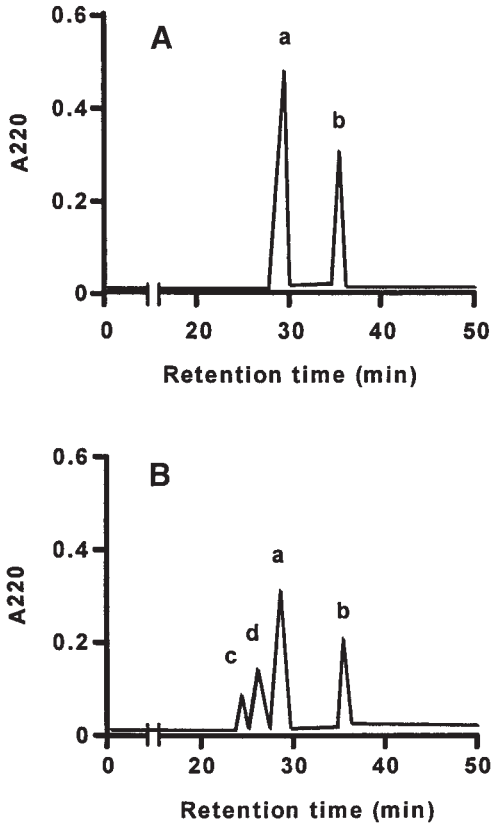

Fig. 5. HPLC of Gln-Arg-Arg-Ala-Ala treated with IgG from a patient with RA. The pentapeptide was synthesized as described previously *(9)*. Forty micrograms of the peptide was incubated with IgG (40 μg) in 150 μl of 50 m*M* Tris-HCl (pH 8.2) containing 0.15 *M* NaCl, at 37°C for 0 h **(A)** and 18 h **(B)** in a humidified incubator. After addition of 0.1 mL of 0.1% heptafluorobutyric acid, the entire reaction mixture was applied to a C_{18} column (2.1 × 30 mm, Applied Biosystems, San Jose, CA), which had been equilibrated with 0.1% heptafluorobutyric acid. The sample was eluted with a linear gradient of acetonitrile (0–90%) in 0.1% heptafluorobutyric acid. The eluate was monitored by the absorbance at 220 nm. Peaks *a–d* were collected and subjected to amino-acid sequence analysis with a gas-phase sequencer (470A protein sequencer, Applied Biosystems) as described previously *(9)*.

patients. These results further support and extend the generalization that antigen-specific peptidase activities are increased in autoimmune diseases *(14)*. It is likely that this catalytic activity modifies the pathogenesis or clinical process of RA and causes some beneficial or harmful effects, depending on the function of the autoantigen. It is difficult to predict the clinical significance of this peptidase activity at the present time, since the nature of RA autoantigen is unclear. Further elucidation of this property will lead to a better understanding of pathogenesis, and to the development of new treatment and/or prevention of RA.

Table 1
Amino Terminal Sequences of Two Peaks Obtained from the Pentapeptide after Incubation with RA IgG

Cycle number	Peak *c* PTH[a] (pmol)	Peak *d* PTH[a] (pmol)
1	Arg (5)	Gln, Arg, Ala (14, 4, 2)
2	Ala (21)	Arg, Ala (8, 1)
3	Ala (10)	Arg, Ala (7, 1)

[a]Amino acids were identified as PTH (phenylthiohydantoin) derivatives.

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Discussion

Kirby: The IgG seems to have some specificity, although it doesn't mind the protecting group too much. In an 18-h incubation, the half-life is of the order of 18 h, and something like half of the starting material is gone. The rate seems somewhat slow.

Matsuura: Yes. This was a total IgG fraction from serum. The catalysts are a subpopulation in the total IgG.

Tramontano: Have you ever tried to see if these IgGs or antibodies are still active in the presence of serum? We suspect serum has inhibitors of proteolytic antibodies.

Matsuura: No, I have not done this experiment.

Paul: Unfortunately, cleavage of short peptides by serum is difficult to study because of the presence of conventional proteases, giving a high background.