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Vandana Pradhan¹ , Pallavi Pandit¹ , Prathamesh Surve¹ , Maxime Lecerf^{2,3,4,5} , Anjali Rajadhyaksha⁶ , Milind Nadkar⁶ , Prasad V. Khadilkar¹ , Durga A. Chougule¹ , Aalaap A. Naigaonkar¹ , Sébastien Lacroix-Desmazes^{2,3,4,5} , Jagadeesh Bayry^{2,3,4,5} , Kanjaksha Ghosh¹ , Sridhi V. Kaveri^{2,3,4,5} 

Abstract

Objective: Antibodies with catalytic (hydrolytic) properties to DNA or RNA have been reported in systemic lupus erythematosus (SLE). However, it is well known that ethnicity plays an important role in the presentation of SLE and severity of the disease; hence, these data may not truly represent a general feature of all SLE patients. Therefore, we have analyzed the hydrolyzing activity of immunoglobulin G (IgG) of SLE patients from the Indian population with an aim to decode whether the catalytic antibody response represents part of an active disease process.

Methods: IgGs were isolated from the sera of 72 consecutive patients diagnosed with SLE. As a control, IgGs from healthy donors were used. The catalytic activity of IgG was measured by PFR-MCA and affinity-linked oligonucleotide nuclease assay.

Results: IgGs from patients with SLE from the Indian subcontinent displayed significantly higher hydrolysis rates of both the surrogate substrate, PFR-MCA, and the DNA than IgG from healthy individuals. Intergroup comparisons of the IgG-PFR-MCA interactions with clinical manifestations of the disease demonstrated a significantly increased level of hydrolysis among the patients with renal involvement who tested positive for anti-dsDNA antibodies. The PFR-MCA hydrolysis also appears to be associated with the active disease ($p=0.0988$, vs. inactive group).

Conclusion: The prevalence of catalytic antibodies represents a general feature of SLE patients, irrespective of their origin.

Keywords: Catalytic antibodies, hydrolytic antibodies, systemic lupus erythematosus, PFR-MCA



ORCID IDs of the authors:

V.P. 0000-0001-9990-8059;
P.P. 0000-0002-5913-3778;
P.S. 0000-0001-6106-1286;
M.L. 0000-0002-1477-2162;
A.R. 0000-0001-8161-6701;
M.N. 0000-0003-1790-6127;
P.V.K. 0000-0002-6146-9545;
D.A.C. 0000-0003-2717-3123;
A.A.N. 0000-0002-1976-6108;
S.L.D. 0000-0001-5625-8447;
J.B. 0000-0003-0498-9808;
K.G. 0000-0002-0645-6565;
S.V.K. 0000-0002-0837-4053.

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¹ Department of Clinical & Experimental Immunology, National Institute of Immunohaematology, King Edward Memorial Hospital, Parel, Mumbai, India

² Institut National de la Santé et de la Recherche Médicale Unité 1138, Paris, France

³ Centre de Recherche des Cordeliers, Equipe - Immunopathologie et Immunointervention Thérapeutique, Paris, France

⁴ Sorbonne Université, UMR S 1138, Paris, France

⁵ Université Paris Descartes, Sorbonne Paris Cité, UMR S 1138, Paris, France

⁶ Department of Medicine, King Edward Memorial Hospital, Mumbai, India

Address for Correspondence:

Vandana Pradhan, National Institute of Immunohaematology, King Edward Memorial Hospital, Parel, Mumbai, India

E-mail: pradhanv69@rediffmail.com

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Introduction

Immunoglobulins (Ig) or antibodies are known for their versatile functions in physiology. They are one of the key molecules of adaptive immune compartment and are important for the maintenance of immune homeostasis, neutralization of pathogens, and activation of effector cells. In addition to this wide array of functions, antibodies under certain conditions also exhibit an enzymatic or catalytic activity and are called “abzymes” (antibody enzymes). It has been proposed that catalytic antibodies are involved in the clearance of biochemical waste and immune complexes by hydrolyzing them. Thus, this natural clearance of immune complexes can lower the deleterious secondary effects such as inflammation and infiltration of various molecules of the immune system. Catalytic antibodies are described both in physiology as well as under various pathological conditions, such as asthma, hemophilia A, multiple sclerosis, rheumatoid arthritis, Hashimoto’s thyroiditis, sepsis, graft versus host disease in transplantation, and others (1-18). Depending on the target antigen, both pathogenic and beneficial role of catalytic antibodies have been described (10, 19).

Systemic lupus erythematosus (SLE) is a chronic, potentially fatal, autoimmune disease characterized by exacerbations and remissions. SLE is presented with various clinical manifestations affecting multiple organs, including skin, kidney, joints, and cardiovascular and nervous system. Clinical and experimental studies have shown that both cellular and soluble inflammatory mediators are implicated in the pathogenesis of lupus (20-25). The hallmark of SLE is the presence of an array of IgG and IgM autoantibodies against one or more nuclear components, particularly double-stranded DNA (dsDNA). In addition, the autoantibodies to histones and nucleosome have also been also reported.

Antibodies with catalytic properties to DNA or RNA have been reported in SLE (2, 26, 27). However, it is well known that ethnicity plays an important role in the presentation of SLE and the severity of the disease (28). Particularly, the disease is more severe in Asian and non-Caucasian patients with SLE and displays a poorer outcome (29). Therefore, previous reports on the catalytic antibodies in patients with SLE might not truly represent a general feature of all patients with SLE. Also, these reports did not provide an indication on whether catalytic antibodies are linked to disease severity. Therefore, in the present study, we analyzed the hydrolyzing activity of SLE patients' IgG from the Indian population with an aim to decode whether the catalytic antibody response represents part of an active disease process.

Methods

Patients with SLE

Sera from 72 consecutive patients diagnosed with SLE were obtained from the Department of Medicine, King Edward Memorial Hospital, Parel, Mumbai, India. Written informed consent was obtained from all the patients and subjects who participated in this study. Patients were enrolled in the study after obtaining a requisite ethical committee permission from the ethics committee for research on human subjects, National Institute of Immunohaematology, Mumbai, India. All these patients were classified by the American College of Rheumatology (ACR) criteria for SLE (30). The disease activity was assessed at the time of evaluation using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (31). The female-to-male ratio was 13.8:1, and the mean age at the time of evaluation was between 12 and 60 years with a mean \pm standard deviation (SD) of 28.0 \pm 9.8 years and the disease duration was 12.3 \pm 19.2 months. The SLEDAI scores ranged between 4 and 38. The patients were categorized into two groups: (a) patients with <12 months of steroid treatment and (b) patients with >12 months of steroid treatment.

Peripheral blood was collected in a plain tube (BD Vacutainer) and centrifuged at 3000 rpm for 10 min at 4°C. The serum was stored in aliquots at -20°C until use. Forty-five age- and sex-matched healthy individuals were included as controls.

Antibody screening

Patients' sera were tested for autoantibody titers. Anti-nuclear antibodies (ANA; Bio-rad) and anti-dsDNA antibodies (Immco) were tested by an indirect immunofluorescence assay. The threshold for the sample to be considered as

positive was 1:80 titer for both anti-ANA and anti-dsDNA antibodies.

Purification of IgG

IgGs were isolated from the serum by affinity chromatography, using the protein A-Sepharose (GE healthcare) column, where 100 μ L of serum was diluted in 200 μ L of binding buffer (20 mM sodium phosphate, pH 7.8) and eluted with elution buffer (0.1M citric acid, pH 2.7). This was followed by immediate size-exclusion chromatography on a superose-12 column equilibrated with 50 mM Tris, 8 M urea, and 0.02 % NaN₃ (pH 7.7) to exclude potentially contaminating proteases, as detailed in our previous reports (6, 10, 28). IgG-containing fractions were then pooled and dialyzed against PBS-0.02% NaN₃ for 2 days with four changes of buffer at 4°C, followed by dialysis against catalytic buffer containing 5 mM CaCl₂ (pH 7.7) for 1 day with two buffer changes at 4°C, as previously described (32). The purity of IgG preparations was confirmed by SDS-PAGE and immunoblotting under non-reducing conditions. IgG was quantified by ultraviolet spectrophotometry (32).

IgG-mediated hydrolysis of PFR-MCA

Purified IgG from 72 SLE patients and 45 healthy individuals was analyzed for the hydrolysis of PFR-MCA using a protocol established in the laboratory (32). In brief, IgG (66.67 nM) was mixed with 100 mM PFR-MCA (Peptide Institute, Inc.) in 40 μ L of catalytic buffer containing 5 mM CaCl₂ (pH 7.7) in white 96-well U-bottom plates (Thermo Scientific) and incubated in the dark for 24 h at 37°C. Hydrolysis of the PFR-MCA substrate was determined by the fluorescence of the leaving group (aminomethylcoumarin; λ_{em} 465 nm, λ_{ex} 360 nm) using a spectrofluorometer (Infinite M 200 PRO; Tecan). Fluorescent values were compared with the standard curve of free MCA, and the corresponding quantities of released MCA were computed. At each time point, background release of MCA, measured in wells containing the substrate alone, was subtracted from the release value observed in the presence of antibodies. Data were expressed as the quantity of released MCA computed at time 0, subtracted from the quantity of released MCA computed at a given time point per amount of time per amount of IgG.

Screening for potential catalytic anti-DNA antibodies by the affinity-linked oligonucleotide nuclease assay (ALONA)

Thirty SLE patients, who underwent the steroid therapy for <12 months, and 30 healthy individuals were further analyzed by the ALONA assay using the protocol by Mouratou et

al. (33), with appropriate modifications. Purified antibodies were first dialyzed against 25 mM Tris-HCl buffer, pH 7.5, 10 mM MgCl₂, and then subjected to the ALONA assay. Microtiter plates (Maxisorp, Nunc) were coated with 100 μ L/well of a 1 mg/mL solution of streptavidin (Roche) in Tris-buffered saline (TBS), overnight at 4°C. Plates were then washed three times with TBS-Tween 0.1% (TBS-T) buffer, and 100 μ L of a 0.01 mg/mL solution of 5'digoxigenin and 3'biotin-labeled ssDNA or dsDNA was added to each well. The plates were incubated for 30 min at 37°C and then washed three times with TBS-T. The wells were filled with 300 μ L of washing solution, and the plates were incubated for further 30 min at 37°C. Wells were washed three times with TBS-T, and 200 μ L of purified IgG was added to each well. The reactions were carried out at 37°C for 24 hours. The uncleaved substrate was detected by anti-digoxigenin ELISA. The plates were washed three times, and alkaline phosphatase-labeled, anti-digoxigenin Fab fragments (Roche), diluted 1:5000 in TBS-T, were added to the wells. The plates were incubated for 1 h at 37°C and washed five times with TBS-T. The 200 μ L of p-nitrophenyl phosphate (pNPP) substrate solution was added to each well, and microtiter plates were further incubated at 37°C until the yellow product was accumulated. The absorbance of the solution in each well was then determined at 405 nm with an ELISA plate reader using a reference wavelength of 630 nm. The hydrolytic activity was inversely proportional to the absorbance.

Statistical analysis

The mean \pm SD value was calculated for continuous variables and proportions for categorical variables. The means between two groups were analyzed using unpaired Student's t-test (Graph Pad). The Mann-Whitney U-test was used to determine the association between the laboratory investigations, clinical manifestations, and production of autoantibodies in patients with SLE. The Pearson correlation test was used to analyze the correlations between the PFR-MCA assay/DNA hydrolysis assay and SLEDAI scores. A $p \leq 0.05$ was considered as statistically significant.

Results

Systemic lupus erythematosus is a complex disease, with the involvement of multiple organs and tissues. Thirty patients in our study (41.7%) had received steroid therapy for <12 months, and the remaining 42 patients (58.3%) were under the steroid therapy for over 12 months. All the patients in our cohort tested positive for ANA (threshold, $\geq 1:80$). However, the anti-dsDNA antibody positivity (threshold, $\geq 1:80$) was

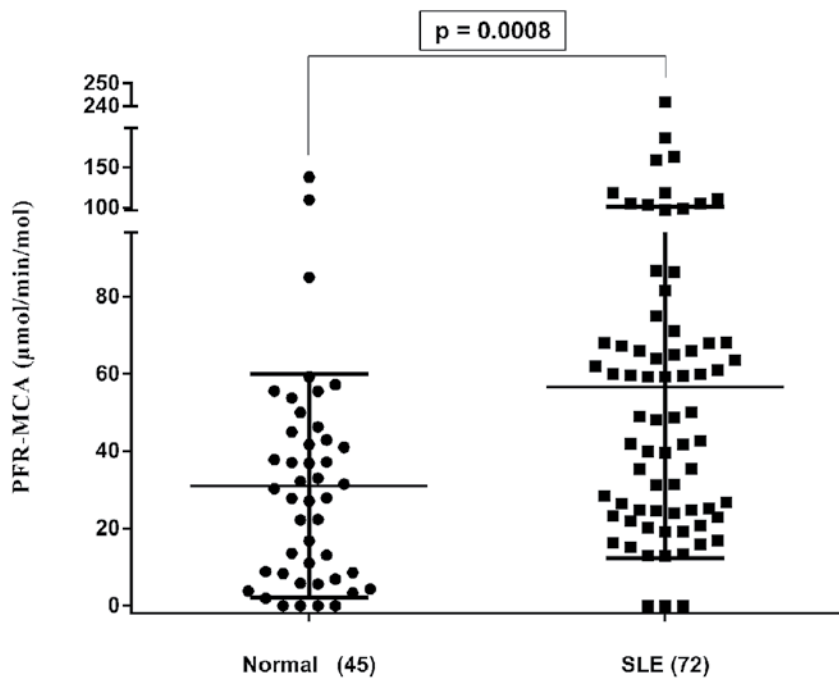


Figure 1. Rates of the PFR-MCA hydrolysis ($\mu\text{mol}/\text{min}/\text{mol}$, mean \pm SD) by IgG of 72 patients with SLE and 45 healthy controls (normal). The mean (\pm SD) hydrolysis in the normal group was 31.03 (\pm 28.92) $\mu\text{mol}/\text{min}/\text{mol}$, whereas in patients with SLE, it was 56.62 (\pm 44.41) $\mu\text{mol}/\text{min}/\text{mol}$. The statistical significance as determined by the Mann-Whitney U-test is indicated.

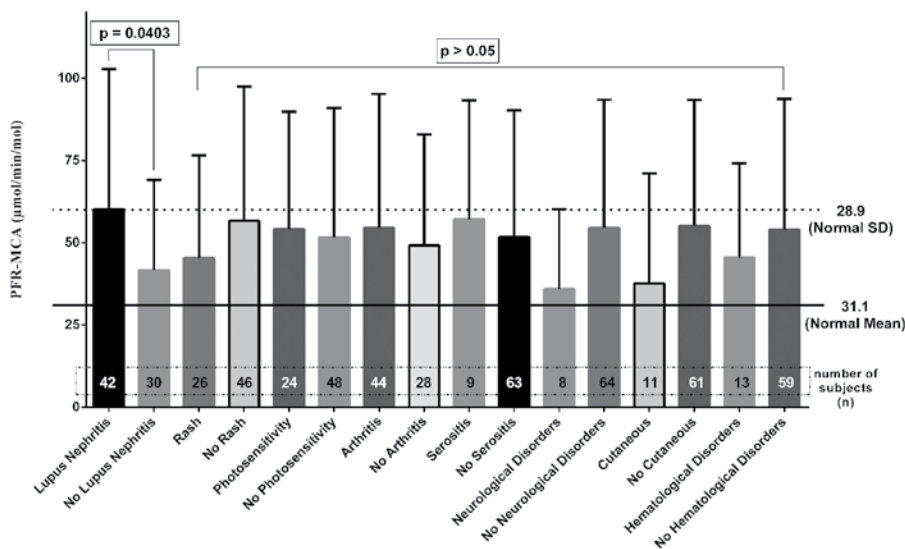


Figure 2. Relationship between the SLE patients' IgG-mediated hydrolysis of PFR-MCA ($\mu\text{mol}/\text{min}/\text{mol}$, mean \pm SD) and clinical manifestations. The number of patients in each category is indicated. The mean and SD values of PFR-MCA for IgG from the normal individuals are also marked. Statistical significance as determined by the Mann-Whitney U-test is indicated.

observed in 52 patients (72.2%). Clinical manifestations as per the ACR criteria revealed that 36.1% of the patients in our cohort had rash, 33.3% had photosensitivity, 61.1% had arthritis, 12.5% had serositis, 58.3% had renal disorders, 11.1% had neurological disorders, 15.3% presented cutaneous involvement, and 18.1% had hematological disorders. In view of a diverse clinical presentation, it is likely that IgG might display heterogeneity in the properties and

hydrolysis of target antigens. Therefore, we first analyzed the hydrolytic activity of IgG from the patients with SLE from India by using the surrogate substrate PFR-MCA.

IgG from healthy individuals demonstrated a marginal hydrolysis of PFR-MCA with an activity of 31.03 \pm 28.92 $\mu\text{mol}/\text{min}/\text{mol}$ (mean \pm SD, n=45). On the contrary, IgG from patients with SLE demonstrated significantly higher

hydrolysis rates of PFR-MCA than IgG from the healthy individuals, and the hydrolysis rate was 56.62 \pm 44.41 $\mu\text{mol}/\text{min}/\text{mol}$ (mean \pm SD, n=72, Figure 1). Intergroup comparisons of the IgG-PFR-MCA interactions with clinical manifestations of the disease demonstrated a significantly raised level of hydrolysis among the patients with renal involvement ($p=0.0403$). Other clinical manifestations had non-significant differences (Figure 2).

Previous data in patients with hemophilia from the laboratory have revealed that the IgG catalytic activity shows an evolution with the appearance of pathogenic antibodies (4, 8). Therefore, we analyzed whether the catalytic activity of SLE IgG based on the PFR-MCA assay shows a correlation with the disease activity. We found that the PFR-MCA values and SLEDAI scores of patients with SLE were not significantly correlated ($r=0.0731$; $p=0.5416$) (Figure 3). However, the hydrolytic activity appears to be associated with the active disease ($p=0.0988$, vs. inactive group).

As DNA is one of the antigenic targets of SLE IgG, we aimed to validate the PFR-MCA results using ALONA. The DNA-hydrolyzing activity was assessed among the 30 cases of SLE, who were under the steroid therapy for <12 months, and 30 age- and sex-matched controls. We found that SLE patients' IgGs had a significantly higher DNA-specific hydrolyzing activity (mean \pm SD, 0.206 \pm 0.194 ng hydrolyzed DNA/ μg IgG in 24 h) than that of normal subjects (0.071 \pm 0.074 ng hydrolyzed DNA/ μg IgG; $p=0.0008$). The DNA-hydrolyzing activity was more significant in patients with positive anti-dsDNA antibodies (n=24; $p=0.0001$) (Figure 4a). However, the extent of DNA hydrolysis ($r=-0.04176$; $p=0.8266$) did not correlate with the SLEDAI scores of SLE patients (Figure 4b). Also, unlike the PFR-MCA assay, the hydrolyzing activity of IgG by ALONA was similar among the patients with active vs. inactive disease ($p=0.2366$) and lupus nephritis vs. non-lupus nephritis patients ($p=0.1266$) (Figure 5). This highlights the heterogeneity of autoimmune response in patients with lupus.

Discussion

Systemic lupus erythematosus is a complex autoimmune disorder with a potential to harm multiple organs. In this report, we provide another facet of autoimmune response in patients with SLE. Although the DNA-specific hydrolytic activity of IgG did not distinguish between the active and inactive form of SLE, the PFR-MCA assay suggests that the hydrolytic activity of IgG is associated with the active disease and lupus nephritis. Therefore, we could

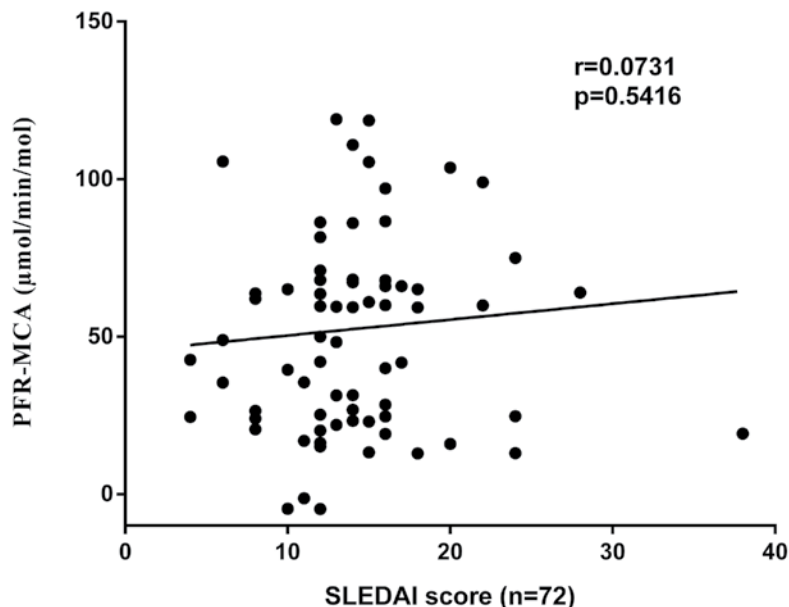


Figure 3. Correlation between the SLE patients' IgG-mediated PFR-MCA hydrolysis ($\mu\text{mol}/\text{min}/\text{mol}$) and SLEDAI scores of 72 patients with SLE, as determined by the Pearson correlation test.

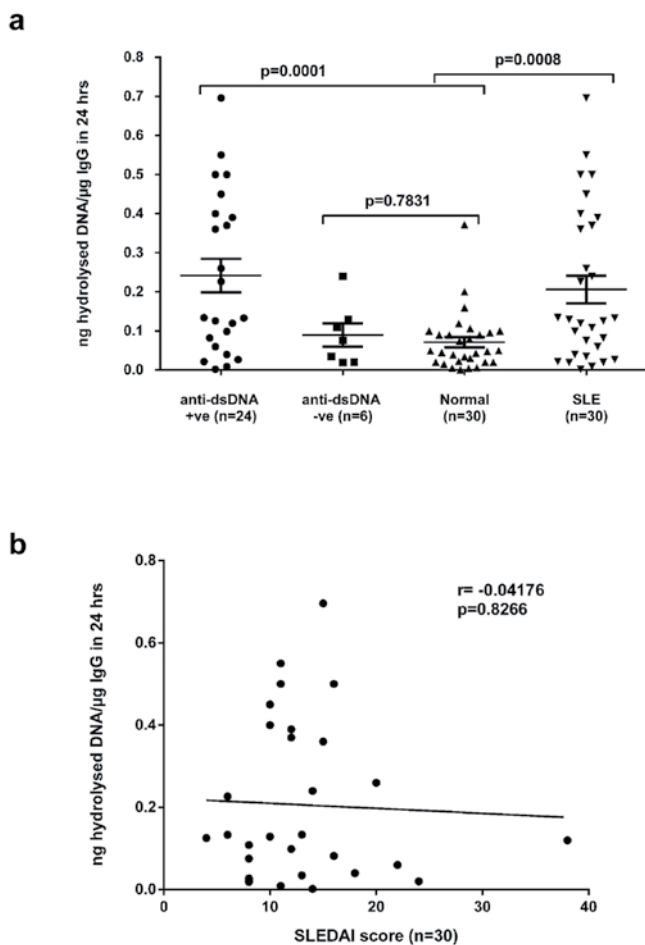


Figure 4. a, b. Hydrolysis of DNA (mean \pm SD) by IgG in patients with SLE [30 active cases of SLE who were under the steroid therapy for <12 months, classified into anti-dsDNA-positive (n=24) or -negative (n=6), and healthy controls (normal, n=30)]. The statistical significance as determined by the Mann-Whitney U-test is indicated (a); Correlation between the DNA hydrolysis by IgGs of patients with SLE (30 active cases of SLE who were under the steroid therapy for <12 months) and the SLEDAI score of patients, as determined by the Pearson correlation test (b).

conclude that the catalytic IgG response in SLE is part of a pathogenic autoimmune response in patients.

A similar DNA hydrolytic activity of IgG in both active and inactive SLE patients, but a higher PFR-MCA activity in active disease, suggests that these patients have a hydrolytic IgG response against other antigens as well such as C1q or various ribonucleoproteins, including Ro, small nuclear ribonucleoprotein (snRNP), La, Sm, ribosomes, or nucleosomes (34). In this context, PFR-MCA serves as a useful surrogate parameter to distinguish the active disease from the inactive disease based on the catalytic activity of IgG. The utility of generic tri-peptide synthetic substrate PFR-MCA for screening hydrolytic activity of IgG has been validated by various studies, including ours (3, 35-37). The hydrolysis of this peptide at the amide bond between arginine and the fluorescent moiety, MCA, leads to the release of fluorescent MCA tag and, hence, allows determining the rates of hydrolysis. In the present study, the absence of contamination of IgG samples by adventitious proteases was ensured by the use of a double-step purification procedure that involves a step of purification based on the affinity and a step of purification based on the protein size under the 8M urea-containing denaturing condition.

The occurrence of catalytic antibodies has been described under various pathological conditions, including autoimmune and allo-immune responses. Even in our report, the rate of hydrolysis of PFR-MCA by SLE IgG is significantly higher than that in healthy donors. Therefore, emergence and enhanced titers of hydrolytic antibodies might represent a universal phenomenon of inflammatory response. During early 1990s, Gabibov and colleagues reported that autoantibodies purified from the sera of patients with SLE and other autoimmune diseases cleave phosphodiester bonds (2, 38, 39). Polyclonal IgG antibodies purified from the sera of several patients with SLE and patients with hepatitis B showed the RNA-hydrolyzing activities that differed from the weak RNase A-type activities of IgG from the healthy donors (40). Not surprisingly, based on the fact that abzymes are slow catalysts as compared to the kinetic values determined for conventional DNases, the reaction turnover values for DNA-abzymes were two times lower (40). Nevinsky et al. (41) reported that the oligomeric forms of IgG antibodies, their Fab fragments, and isolated light chains exhibit endonuclease activity. They also reported that the catalytic activity of isolated light chains was higher than that of the oligomeric forms, wherein the light chains of

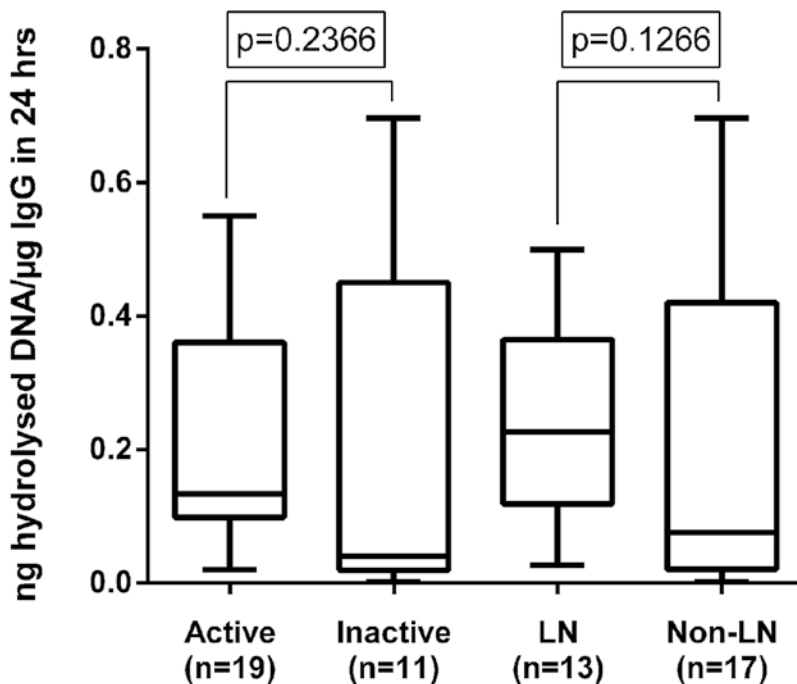


Figure 5. DNA hydrolysis by IgG purified from patients with SLE [active vs. inactive; lupus nephritis (LN) vs. non-LN] who were under the steroid therapy for <12 months (n=30). The values are depicted as box plots, where the boxes represent the 25th-75th percentiles, and the lines within the boxes represent the median. The statistical significance as determined by the Mann-Whitney U-test is indicated.

immunoglobulins had a lower affinity to DNA and were mainly responsible for DNase activity (41, 42).

In an attempt to understand the role of DNA-hydrolyzing antibodies, Kozyr et al. (43) demonstrated the cytotoxicity of anti-DNA autoantibodies isolated from the sera of SLE patients. This report thus demonstrates a pathogenic role of DNA-hydrolyzing antibodies. Alternatively, these catalytic antibodies might represent part of disease-controlling mechanism of the immune system by causing DNA degradation and hence reducing the autoantigen pool. The knowledge on the DNA-hydrolyzing antibodies in patients with SLE is still in its infancy, and based on the current knowledge, it is clear that the biological role of catalytic antibodies in SLE is particularly complex, as catalytic antibodies to nuclear antigens are also described in other autoimmune diseases (44, 45). The mechanisms underlying the origin of catalytic antibodies and the precise role that these antibodies play in the pathogenesis of lupus should be studied in a large cohort of patients with SLE. The follow-up studies aimed at a direct relationship between the catalytic antibodies and the clinical manifestations in patients with SLE might also lead to possible development of novel biomarkers and therapeutic strategies for SLE (46-48).

Ethics Committee Approval: Ethics committee approval was received for this study from the National Institute of Immunohaematology, Mumbai, India.

Informed Consent: Written informed consent was obtained from all the patients and subjects who participated in this study.

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Author Contributions: Concept - V.P., S.L.D., J.B., K.G., S.V.K.; Design - V.P., M.L., K.G., S.V.K.; Supervision - V.P., M.L., K.G., S.V.K.; Resources - V.P., S.L.D., J.B., K.G., S.V.K.; Materials - V.P., M.N., A.R., K.G., S.V.K.; Data Collection and/or Processing - V.P., P.P., P.S., M.L., A.R., M.N., P.V.K., D.A.C., A.A.N.; Analysis and/or Interpretation - V.P., P.P., P.S., M.L., A.R., M.N., P.V.K., D.A.C., A.A.N.; Literature Search - V.P., S.L.D., J.B., S.V.K.; Writing Manuscript - V.P., J.B., S.V.K.; Critical Review - V.P., P.P., P.S., M.L., A.R., M.N., P.V.K., D.A.C., A.A.N., S.L.D., J.B.

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