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Case Report Open Access

# The Appearance of *In vivo* ANA Positivity after a Period of 17 years in a Patient with Lupus Nephritis who had Received both Methylprednisolone and Cyclophosphamide Pulse Therapy

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## Abstract

*In vivo* anti-nuclear antibody positive phenomenon in the renal tissues except for necropsy/autopsy cases or very severe cases of systemic lupus erythematosus is a controversial issue until today. However, we experienced a case of lupus nephritis that showed *in vivo* anti-nuclear antibody-positive cells in the renal tissues. Four successive renal biopsies over 17 years were studied with immunofluorescence as well as immunohistochemical and immune-electron microscopic staining. The first, second and third biopsies did not reveal any *in vivo* anti-nuclear antibody-positive cells. However, the fourth biopsy in relapse showed positive cells, and the transfer of the patient's serum into the mice provoked *in vivo* anti-nuclear antibody-positive cells in the mouse intestines. We present this case in detail and discuss the implications of these observations.

**Keywords:** Lupus nephritis; *In vivo* ANA; Cyclophosphamide therapy; SLE

## Introduction

Positivity of *in vivo* anti-nuclear antibody (ANA) in the renal tissues of systemic lupus erythematosus (SLE) has so far only been observed in severe or autopsy cases [1-3]. However, we experienced a case of a 56 year old female who received 4 courses of intravenous cyclophosphamide (IVCY) therapy as well as 3 sessions of methylprednisolone (mPSL) pulse treatment for diffuse proliferative lupus nephritis (LN). She remained in clinical remission for 16 years after these treatments but eventually developed relapse of LN.

The initial renal biopsy (Bx) and the subsequent two renal Bxs, which were performed in remission, did not show any *in vivo* ANA-positive cells, whereas the fourth Bx during moderate relapse revealed apparent *in vivo* ANA-positive cells in the tissue. We present this rare case along with additional experimental data and also review previous pertinent articles [4-9].

## Case Report

History of illness around each renal biopsy (Figure 1).

A 43 year old female presented with livedo reticularis, facial erythema, arthralgia, Raynaud phenomenon, proteinuria as well as hematuria and BP 200/100 mmHg. The laboratory data are shown in Table 1.

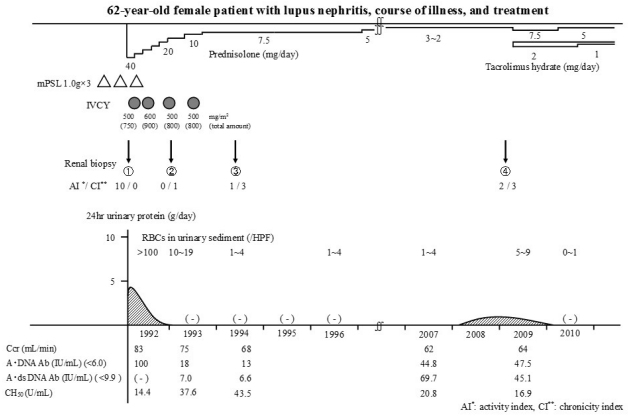


**Figure 1:** Course of the illness and treatment.

She was diagnosed with SLE based on her clinical features and laboratory data. A renal Bx revealed Class IV-G (A) LN with subendothelial deposits. First, mPSL pulse therapy was administered followed by oral prednisolone (PSL) at 0.7 mg/kg of body weight. Thereafter, the second and third rounds of mPSL pulse therapy were given monthly. One, two and six months after the last mPSL pulse therapy, IVCY therapy at a dose of 750 mg (500 mg/m$^2$ of body

surface), 900 mg (600 mg/m$^2$), and 800 mg (500 mg/m$^2$) was administered, respectively. With these treatments and the use of antihypertensive drugs, she became normotensive, negative for proteinuria, 20-30 red blood cells (RBCs)/HPF in her urinary sediment and nearly a normal level of immunological abnormalities.

The patient had been followed up with daily PSL (15 mg/day) at our out-patient clinic. Her negative proteinuria and 10-19 RBCs/HPF in the urinary sediment persisted with almost normal immunological data (Table 1).

| No. of Renal Biopsy | No. 1 | No. 2 | No. 3 | No. 4 |
|---|---|---|---|---|
| Date of Biopsy | 1992, Feb | 1993, Feb | 1994, Oct | 2009, Jan |
| BP mmHg | 200/100 | 122/70 | 140/80 | 166/108 |
| Edema | (+) | (-) | (-) | (-) |
| Urinalysis | | | | |
| Proteinuria | 3.3 gm/day | (-) | (-) | (++) |
| RBCs/HPF | 30-49 | 10-19 | 1-4 | 5-9 |
| Cast* | RBC Cast | (-) | (-) | (-) |
| Hemoglobin (g/dL) | 11.1 | 11 | 12.8 | 13 |
| WBCs (/μL) | 5,800 | 8,000 | 6,800 | 5,400 |
| Platelets (×10$^4$/μL) | 20.2 | 30.5 | 25.9 | 30.5 |
| Serum Cr (mg/dL) | 0.9 | 0.9 | 0.9 | 0.6 |
| CH50 (U/mL) | 14.4 | 37.6 | 43.5 | 16.9 |
| ANA (X) | 160 | 40 | 160 | 1,280$^{\gamma}$ |
| A-DNA Ab (RIA) (IU/mL) (<6.0) | >100 | 18 | 13 | 47.5 |
| A-ds DNA Ab (EIA) (IU/mL) (<9.9) | n.d$^{\gamma}$ | 7 | 6.6 | 45.1 |
| IC† (μg/mL) (<3) | 5.7 | 1.8 | 1.3 | 7.5 |
| Anti-cardiolipin IgG Ab | (+) | (-) | (-) | (-) |
| TP/Alb (g/dL)/(g/dL) | 6.3/3.1 | 7.0/3.8 | 7.3/3.7 | 8.7/3.8 |
| HbA1C (%), blood sugar(mg/dL**) | 5.4%, 88 | 5.4%, 86 | 5.5%, 90 | 5.6%, 109 |
| 120/70: On a hypertensive drug (nicardipine hydrochloride LA 20 mg × 2/day), Cast*: Most significant cast, 1,280$^{\gamma}$: Homogenous pattern, IC†: C1q binding assay, n.d$^{\gamma}$: Not determined, mg/dL**: Fasting blood sugar | | | | |

**Table 1:** Main physical findings and laboratory data at each renal biopsy.

A second renal Bx was performed 12 months later and disclosed chronic inactive focal sclerosing LN (Class III [C]). A fourth round of IVCY at 800 mg (500 mg/m$^2$) was infused 2 months after the third one. With these treatments, her daily PSL dose was gradually decreased to 7.5 mg. A urinalysis continued to show negative proteinuria and 1-4 RBCs/HPF in the urinary sediment with only a few remaining immunological abnormalities (Table 1). A third renal Bx was carried out 32 months after the first IVCY treatment showing inactive sclerosing LN (Class III [C]), too. The laboratory data remained the same as at the second renal Bx (Table 1). The patient continued to receive PSL (7.5-2.0 mg per day) after this renal Bx.

The patient did well for the following 16 years. However, she started to have (+)-(++) proteinuria and 5-9 RBCs/HPF in the urinary sediment. A flare-up in the immunologic data was noted (Table 1) with ANA positivity of 1,280X and a homogenous pattern. Because these data suggested the relapse of LN, a fourth renal Bx was performed 17 years after the initial treatment and showed focal proliferative and sclerosing LN (Class III [A/C]). An immunofluorescence (IF) study demonstrated *in vivo* ANA positivity in glomerular cells as well as also cells in the interstitium, tubuli and tubular lumen.

## Materials and Methods

All renal Bxs were performed to determine the best treatment for the patient and informed consent was obtained from the patient each time. The staining procedures have been described in our previous articles [10,11]. Each biopsy specimen from the first to fourth Bxs was stained routinely with IF-labeled polyclonal rabbit anti-human antibodies to IgG, IgA, IgM, C1q, C3, C4 and fibrinogen (Dako, Denmark). The specimens from the fourth Bx were also studied by an immunohistochemical (IHC) method including single staining of IgG as well as double staining for anti-IgG antibody and PAS. The IgG antibody was a mouse monoclonal antibody to human IgG (Clone: A57H; Nichirei, Tokyo, Japan) as the first antibody. Counter staining was performed with PAS. In addition, immunoelectronmicroscopy (IEM) was performed with rabbit anti-human IgG, followed by anti-rabbit colloidal gold-conjugated donkey IgG (Jackson Immuno Research Lab, PA, USA). The publication of her renal pathology as well as the laboratory data and illness history was permitted by the patient via written sheets.

An experiment involving the transfer of the patient's serum into mice was performed in order to confirm whether or not the serum had the ability to produce *in vivo* ANA positivity. The experiment was done in a C57BL/6J strain of mouse weighing 20 gm (8 weeks old). Six male mice were used for the study (three for the experiment and three as controls). Briefly, 0.3 mL of the patient's serum obtained at the fourth Bx was injected intraperitoneally (IP) in the three experimental mice, and 0.3 mL of a healthy subject's serum was injected IP in the three control mice. All 6 mice were sacrificed 72 hrs later and their kidneys, intestines and livers were subjected to IF as well as PAS staining. The IF study was performed with polyclonal rabbit antibody of F (ab)2 to human IgG (Dako, Gostrup, Denmark; Code: F0185). This case report including the animal experiment, was approved by the Faculty of Medicine Research Ethics Committee and Experimental Animal Committee, Kyorin University.

## Result

Pathologic findings of each biopsy and the patient's serum transfer study.

**Light microscopic, immunofluorescent and electron microscopic findings on the renal biopsy specimens.**

First biopsy (Figures 2A-2C).

(A) Low magnification reveals three glomeruli demonstrating diffuse proliferative nephritis as well as no infiltration of inflammatory cells in the TI or a tubulitis appearance (PAS staining, X100). (B) High magnification of a glomerulus shows global proliferative GN, subendothelial deposits (short arrows), a thrombosis in a glomerular

intracapillary lumen (an arrow head), and karyhorrhexis in a segment (a long arrow) (PAS staining, X400). (C) The IF study reveals IgG granular deposits along the GCLs, in the mesangium, and in the subendothelium of glomerular capillaries (anti-IgG Ab staining, X400). No *in vivo* ANA-positive cells are noted.

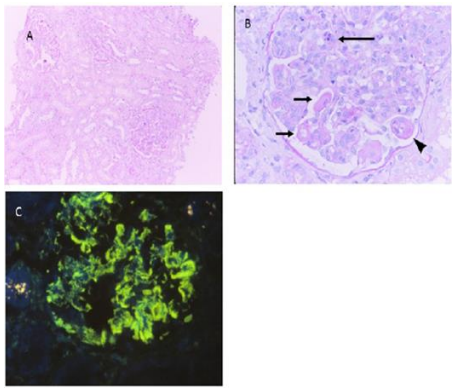


**Figure 2:** The first biopsy.

The Bx specimens contained nine glomeruli and they showed active and diffuse global endocapillary proliferative glomerulonephritis (GN). They were accompanied by subendothelial deposits, karyorrhexis, and the infiltration of inflammatory cells in the capillary lumens of the glomeruli (Figures 2A and 2B). A few glomerular capillary lumens also contained thrombotic materials. The tubulo-interstitium (TI) revealed neither the infiltration of inflammatory cells nor tubulitis (Figure 2A). An IF study showed IgG, C1q, C3 and C4 deposits along the glomerular capillary loops (GCLs) and in the mesangial areas (Figure 2C, IgG), as well as on the vessel walls of the arterioles. Subendothelial deposits of GCLs were also positive for these factors. However, no *in vivo* ANA-positive cells were observed. Electron microscopy revealed massive electron-dense deposits beneath the capillary basement membrane (BM), as well as in the subepithelial and mesangial areas. Furthermore, inflammatory cells in the capillary lumens and marked fusion of the foot processes in the glomeruli were observed (data not shown). These findings indicated a diagnosis of active diffuse global proliferative LN (Class IV-G [A]).

Second and third biopsies (Figures 3A and 3B).

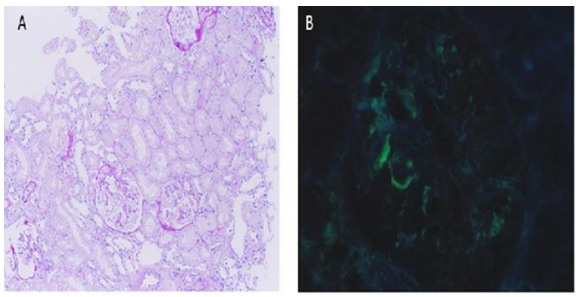


**Figure 3:** The third biopsy.

(A) Low magnification shows almost normal glomeruli with minor focal tubular atrophy (PAS staining, X100). (B) The IF study demonstrates almost complete disappearance of immunoglobulins on the glomerulus and no *in vivo* ANA-positive cells (anti-IgG Ab staining, X400).

The second Bx showed minimal widening of the mesangial matrix but no cell proliferation in eight glomeruli. TI showed only focal distribution of tubular atrophy. An IF study revealed a decrease in the immunoglobulin and complement of IgG, C1q and C3 along the GCLs and mesangial areas as well as no *in vivo* ANA-positive cells (data not shown).

The third Bx specimens revealed two global sclerotic glomeruli and the remaining seven glomeruli showing similar findings of mild mesangial matrix expansion. TI revealed focal tubular atrophies and was accompanied by the infiltration of mononuclear cells (Figure 3A). IF revealed almost no depositions of immunoglobulins or complements in the specimens as well as no *in vivo* ANA-positive cells (Figure 3B). The pathological diagnosis of the second and third Bxs was chronic inactive focal sclerosing LN (Class III [C]).

Fourth biopsy (Figures 4A-4E).

(A) Low magnification shows only segmental proliferation of cells and widening of the mesangium in the glomeruli, along with infiltration of a few inflammatory cells in the TI (PAS staining, X200). (B) High magnification of the TI demonstrates the infiltration of inflammatory cells (short arrows), tubulitis (long arrows), and floating cells in the tubular lumen, including giant nuclear cells (PAS staining, X600). (C) An IF study shows IgM deposition in the mesangial area without positivity in the TI area (anti-IgM staining, X200). (D, E) An IF study shows some *in vivo* ANA-positive cells (arrows) in a glomerulus (D), tubuli and TI (E) (anti-IgG Ab staining, X400).

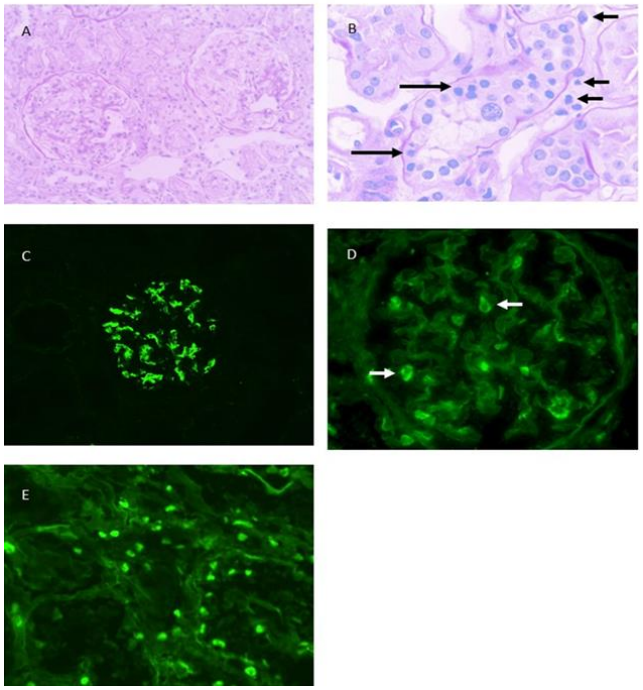


**Figure 4:** The fourth biopsy.

The Bx specimens contained 11 glomeruli. One glomerulus showed global sclerosis, and the other 10 had a similar appearance with focal

mesangial proliferation (Figure 4A). TI manifested tubulitis with giant nuclear cells and small condensed nuclear cells, as well as the infiltration of mononuclear cells among the tubuli (Figure 4B). IF revealed IgM deposits and less-intensified IgA as well as IgG deposits in the mesangial areas (Figure 4C, IgM). C1q, C3 and C4 findings were negative. Furthermore, the positive staining of IgG in the nucleus was noted in the glomerular cells, tubular cells and interstitial cells (Figures 4D and 4E), although IgA, IgM, C1q, C3 and C4 staining were negative in these nuclei. The pathological diagnosis was focal proliferative and sclerosing LN (Class III-[A/C]) associated with an *in vivo* ANA-positive phenomenon.

**Identification of *in vivo* ANA-positive cells at the fourth renal biopsy.**

Anti-IgG Ab staining (Figures 4D and 4E and Figures 5A-5C).

(A) IHC anti-IgG Ab staining shows that one glomerulus has a few positive cells on the epithelial side of the GCLs, and these cells are presumed to be podocytes (arrows) (X600). IHC staining for anti-IgG Ab and PAS shows a positive cell in one part of the glomerulus, which is clearly demonstrated to be a podocyte, as it is located outside of the GCL (an arrow) (inset, X600). (B) An IEM study reveals the existence of colloid particles mainly in the nucleus of epithelial cells (N) and a few particles in its cytoplasm. (C) (X8,000). The inset indicates the localization of the observation area. The arrowhead indicates the glomerular BM (X2,000). (C) Positive cells are noted in the intra-tubulus and TI, as well as in the tubular lumen. The positive cells in the intra-tubulus seem to be degenerated tubular cells (long arrows). The positive cells in the TI are thought to be infiltrating inflammatory cells, as they have nuclear shapes suggesting polymorph-neutrophils or macrophages and are located in the vicinity of the peritubular capillaries (short arrows). In addition, the floating cells in the tubular lumen also have positive findings (arrowhead) (X600).

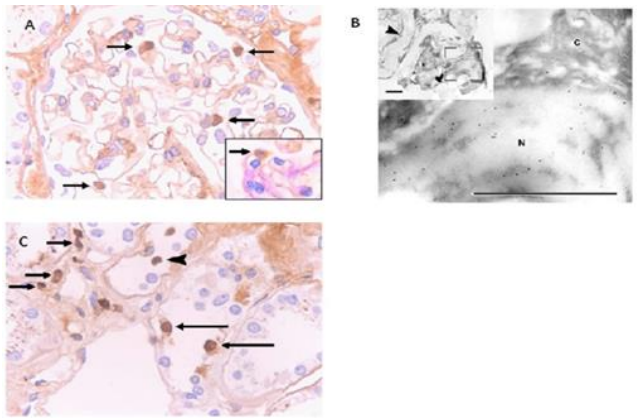


**Figure 5:** Anti-IgG Ab staining.

IF and IHC staining as well as IEM study demonstrated positive cells in the glomeruli, tubular epithelium, and interstitium (Figures 4D, 4E and Figures 5A-5C). IHC staining revealed positivity for anti-IgG Ab in the nuclei belonging to glomerular epithelial cells, intratubular cells of the epithelium, the cells in the interstitium and cells in the tubular lumen (Figure 5A, inset, 5C). The positivity was also confirmed with an IEM study, as showing the colloid particles mainly present in the nucleus of one epithelial cell with a few particles in its cytoplasm (Figure 5B).

Transfer of the patient's serum into mice (Figures 6A and 6B).

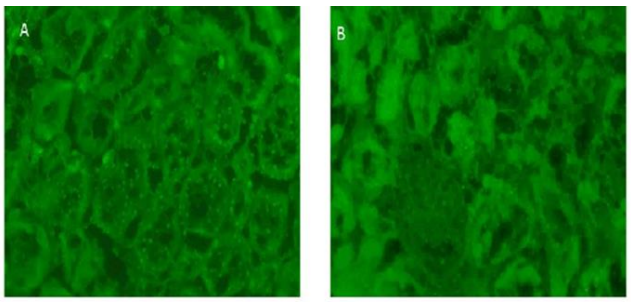


**Figure 6:** The serum transfer study in mice shows *in vivo* ANA-positive cells in the intestine (A) But not in the kidneys (B) (X400).

The specimens obtained following the patient's serum transfer revealed *in vivo* ANA-positive findings on the intestinal cells (in all three mice) (Figure 6A) but not in cells of the kidneys (Figure 6B) or liver. The control mice showed negative findings in the tissues of all three examined organs (data not shown).

## Discussion

*In vivo* ANA positivity has frequently been observed in the subcutaneous tissues of patients with mixed connective tissue disease (MCTD) and SLE [12,13]. This phenomenon was originally described in the renal tissues of necropsy cases with LN [1]. In addition, several articles have documented this phenomenon in experimental studies on mouse renal tissues as well as in human mononuclear cells drawn from the peripheral blood [4-9]. We have also observed this phenomenon in the renal tissues of patients with very severe cases of SLE [3]. The occurrence of the *in vivo* ANA phenomenon in autopsies or severe cases with SLE is presumed to be associated with the leakage of serum from the surrounding blood vessels, eventually penetrating into nearby cells. The cells of blood vessels and nearby tissues are thought to have damaged cellular membranes, which results in the leakage and penetration of serum into these cells. This penetration subsequently results in the *in vivo* ANA phenomenon [2]. In addition, the fixation process for renal Bx tissues might result in the penetration of serum from the vessels into nearby cells, thereby resulting in false *in vivo* ANA positivity [14].

In the present case, we experienced a patient who had severe LN at presentation, received the first renal Bx and subsequently underwent four rounds of IVCY therapy as well as mPSL pulse treatment. After these treatments, she remained stable for the following 16 years and underwent two additional renal Bxs. The initial Bx showed severe diffuse proliferative LN but no *in vivo* ANA-positive cells. The second and third Bxs, which were performed during the remission of SLE activity, did not reveal any *in vivo* ANA-positive cells, either. She eventually developed a relapse of LN and the immunological findings showed a markedly elevated ANA titer as well as an anti-ds DNA antibody level in addition to (++) proteinuria. Therefore, a fourth renal Bx was performed, which revealed *in vivo* ANA positivity in the glomerular, tubular and interstitial cells. This staining pattern was homogenous, with the same staining appearance of the serum for the ANA test. The cells of the glomeruli were diagnosed as glomerular epithelial cells. The cells of the tubuli were thought to be tubular cells or infiltrating cells and the interstitial cells were presumed to be

resident or infiltrating cells. Furthermore, this *in vivo* ANA positivity was confirmed by a transfer study involving the injection of the patient's serum into mice as demonstrated in the cells of the intestines.

These observations occurred in a patient with SLE relapse but who was not near-death. Several mechanisms underlying the development of *in vivo* ANA positivity have been reported, including a very high titer of anti-RNP antibody [4], cells possessing DNA on their cellular surface [5], specific anti-DNA antibodies [6,7], cells expressing specific molecular arrangements with an affinity to anti-DNA antibody [8], and cells having myosin or caveolin receptors [9]. However, the precise mechanism of action in this present case was unclear but some characteristics of anti-DNA antibody might exist like as Okudaira or Vlahakos articles [6,7]. It might be related to the four courses of IVCY therapy performed in this patient. Experimental data in mice suggest that some abnormalities occurring in various cell components or the anti-DNA Ab nature induced by IVCY therapy may lead to the appearance of this *in vivo* ANA phenomenon [15-18].

## Conflict of Interests

None

## Acknowledgement

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