Upregulation of Antiphospholipid Antibodies Following Cyclophosphamide Therapy in Patients with Systemic Lupus Erythematosus

PANAYIOTIS G. VLACHOYIANNOPOULOS, SOPHIE P. TOYA, GHIKAS KATSIFIS, ELIAS ZINTZARAS, ATHANASIOS G. TZIOUFAS, and HARALAMPOS M. MOUTSOPOULOS

ABSTRACT. ***Objective.*** We have observed several cases of patients with systemic lupus erythematosus (SLE) who developed antiphospholipid antibodies (aPL) or full blown antiphospholipid syndrome (APS) after being successfully treated with cyclophosphamide (CYC). To further evaluate the significance of this phenomenon we undertook a retrospective study of our patient population with SLE.

Methods. The charts of 320 patients with SLE, either CYC treated (n = 117) or non-treated (n = 203), were reviewed. The disease activity over time was evaluated using the European Consensus Lupus Activity Measurement (ECLAM) scoring system, as well as initial and cumulative anti-dsDNA antibody titers and C3, C4 complement levels. aPL titers (IgG and IgM) were documented for all patients. Seroconversion was defined as the *de novo* appearance of aPL antibodies at a titer higher than the 99th percentile of 100 normal sera, tested on 2 occasions 12 weeks apart.

Results. Seroconversion occurred in 22 out of 117 patients treated with CYC as compared with 2 out of 203 non-CYC treated patients [odds ratio (OR) = 23.27, 95% confidence interval (CI) 5.36-101.01]. Six patients from the seroconverted CYC treated group were diagnosed with APS compared to none in the non-CYC treated group. The association between seroconversion and CYC remained significant after adjustment for ECLAM score after treatment, prednisone dose and disease duration (OR = 13.4, 95% CI 2.67-67.50). Seroconversion occurred despite successful disease remission as judged by significant decrease of: anti-dsDNA antibody titers ($p < 0.01$), ECLAM scores ($p < 0.01$), and C3 ($p < 0.01$) and C4 levels ($p < 0.01$).

Conclusion. Our data suggest that CYC therapy might be associated with upregulation of aPL and development of antiphospholipid syndrome despite suppression of SLE activity. (First Release July 15 2008; J Rheumatol 2008;35:1768–75)

Key Indexing Terms:
ANTIPHOSPHOLIPID ANTIBODIES
SYSTEMIC LUPUS ERYTHEMATOSUS
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UPREGULATION

Cyclophosphamide (CYC) is an immunosuppressant agent widely used for the treatment of systemic autoimmune diseases[1,2]. Its use is associated with reduction in the numbers of circulating B and T lymphocytes[3] and alteration in the functionality of immune cells[4], with subsequent suppression of antibody responses[5,6]. Data from the US National Institutes of Health studies[7,8] have shown that CYC is superior to steroids for the treatment of systemic lupus erythematosus (SLE) related nephritis, and has been considered the preferred treatment for this purpose.

From the Department of Pathophysiology, University of Athens School of Medicine, and Laiko University Hospital, Athens; and the Department of Biomathematics, University of Thessaly School of Medicine, Larissa, Greece.

P.G. Vlachoyiannopoulos, MD; S.P. Toya, MD; G. Katsifis, MD; A.G. Tzioufas, MD; H.M. Moutsopoulos, MD, FACP, FRCP, Department of Pathophysiology, University of Athens School of Medicine, and Laiko University Hospital; E. Zintzaras, PhD, Department of Biomathematics, University of Thessaly School of Medicine.

Address reprint requests to Dr. P. Vlachoyiannopoulos, Department of Pathophysiology, University of Athens Medical School, 75, Mikras Asias St, 11527, Athens, Greece. E-mail: pvlah@med.uoa.gr

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CYC has been known to have considerable side effects including secondary infections and impaired fertility. However, it has never, to our knowledge, shown to have immune enhancing effects in humans. For this reason, we were surprised to observe that use of CYC in our patients with SLE was often associated with new development of antiphospholipid antibodies (aPL). Equally surprising was the fact that these autoantibodies appeared despite successful treatment of SLE as indicated by remission of clinical manifestations, reduction of anti-dsDNA levels, and increase of C3 and C4 complement levels. To further clarify this clinical impression, we retrospectively evaluated our SLE patient cohort and divided them into 2 groups: (1) patients who received CYC, and (2) patients who never received CYC. The clinical picture, the autoantibody responses, and the development of clinical antiphospholipid syndrome (APS) were recorded and compared between the 2 groups.

MATERIALS AND METHODS

Patient cohort. The files of 320 consecutive patients with SLE, followed in the department of Pathophysiology of the University of Athens Medical

School from January 1995 until December 2005, either CYC treated (n = 117) or non-treated (n = 203), were reviewed. The data collected included the initial and cumulative European Consensus Lupus Activity Measurement (ECLAM) scores, serological measures such as anti-dsDNA, aPL responses, C3 and C4 complement component levels, and therapeutic interventions. Disease duration, length of followup, and time elapsed from treatment initiation to seroconversion were also recorded. Anti-dsDNA and aPL (ELISA) as well as C3 and C4 complement levels were recorded at least 3 times yearly for each patient. From January 2000 until the end of the study aPL positivity was verified by also testing antibodies against ß$_2$-glycoprotein-I (ß$_2$-GPI) IgG (ELISA).

The CYC therapeutic schedule consisted of monthly pulses of 0.75 to 1.00 g/m^2 for up to 6 months, bimonthly for 12 months, and every 4 months thereafter. Indications for initiation of CYC were: nephritis histopathologically classified as WHO class III and IV, systemic vasculitis, or central nervous system disease. Alternative treatment modalities, for patients not fulfilling the above criteria, were hydroxychloroquine, methotrexate, or azathioprine. Five patients from the non-CYC treated group were treated with mycophenolate mofetil, and 3 patients with anti-CD20 monoclonal antibodies (rituximab) for a short period of time as compared to their entire followup period. All the above were combined as needed with a high (10 mg or more daily) or low (less than 10 mg daily) dose of prednisone or equivalent dose of methylprednisolone. This dose corresponded to the lowest effective dose of prednisone and was determined within the first 6 months after initial presentation.

Definitions. The diagnosis of SLE was established in accordance with the American College of Rheumatology diagnostic criteria[9], which were further updated in 1997[10], while the diagnosis of APS was made according to recently revised criteria[11]. Seroconversion was defined as the new acquisition of serum aPL documented at least twice, 12 weeks apart, and in titers higher than the 99th percentile of 100 normal sera obtained from individuals younger than 55 years[11]. Disease duration was defined as the time elapsed from the first SLE related symptom to the last followup. Time to seroconversion was defined as the length of the followup prior to seroconversion. Neurologic disorder was defined as the presence of seizures without any other cause and/or the presence of psychosis without any other cause. APS nephropathy was defined according to recently revised criteria for APS and related features[11,12]. The disease activity was appraised with the ECLAM score[13], C3 and C4 complement component, and anti-dsDNA levels.

Statistical analysis. The cumulative prevalence rates of clinical and serologic characteristics of CYC treated and non-CYC treated patients were compared by contingency tables using Fisher's exact test. The association between seroconversion and treatment group (CYC vs non-CYC treated patients) was tested using a simple logistic model and was expressed with an odds ratio (OR) and its corresponding 95% confidence interval (CI). The above association was also examined after adjustment for the ECLAM score (≤ 2, > 2), the intensity of prednisone treatment (high or low) and disease duration, using a multiple logistic model. Further, the incidence of seroconversion versus time was compared between the 2 groups by Kaplan-Meier survival analysis and the log-rank test. The Wilcoxon matched pairs test was used in the seroconverted group of CYC treated patients to compare the values of the ECLAM score, C3 and C4 levels, anti-dsDNA, and aPL IgG and IgM antibody titers before and after treatment with CYC. Fisher's exact test was used to compare APS incidence in the 2 treatment groups. Receiver-operating characteristic (ROC) analysis was used to determine diagnostic accuracy of ECLAM score and prednisone dose in regards to seroconversion. Statistical significance required a $p < 0.05$. The statistical analysis was performed using SPSS v13 and Statistica v6.

RESULTS

Clinical and serological characteristics of the patients

The profile of the patients in the 2 treatment groups is summarized in Table 1. The female to male ratio, the prevalence of arthritis, photosensitivity, Raynaud's phenomenon, serositis, vasculitis, thromboembolic events, livedo reticularis, autoimmune hemolytic anemia, and autoantibodies to dsDNA and to extractable nuclear antigens were comparable between the CYC treated and the non-CYC treated group. The CYC treated group was characterized by a higher prevalence of severe disease (ECLAM score 5.81 ± 1.68 vs 2.5 ± 1.5 in the non-CYC treated group, $p < 0.01$) as manifested by nephritis, neurologic disorder, and pulmonary arterial hypertension and more frequent end organ damage.

CYC upregulates aPL response while it downregulates SLE disease activity. The probability of being seroconversion-free was significantly lower in CYC-treated compared to non-CYC-treated patients (Figure 1). Treatment with CYC was effective in inducing disease remission as documented by a significant decrease in anti-dsDNA antibody titers (86.94 ± 178.87 vs 19.69 ± 27.26, $p < 0.01$) and ECLAM score (5.81 ± 1.68 vs 2.31 ± 1.14, $p < 0.01$), as well as an increase of C3 (61.81 ± 28.37 vs 87.31 ± 24.82, $p < 0.01$) and C4 (15.70 ± 9.53 vs 23.52 ± 12.51, $p < 0.01$) complement levels after treatment. The same improvement in ECLAM ($p < 0.01$), dsDNA ($p < 0.01$), C3($p < 0.01$), and C4($p < 0.01$) after treatment was noted for the 22 seroconverted patients in the CYC treated group. Seroconversion involving both aPL IgG ($p < 0.01$) and IgM ($p < 0.01$) antibodies occurred in these patients despite and irrespectively of effective control of disease activity (Figure 2).

Markers of disease severity do not accurately predict seroconversion. The fact that the patients in the CYC treated group had more severe disease at presentation prompted us to further consider the hypothesis that there was an association between the severity of the initial disease and the probability of seroconversion. To address this issue we chose 2 markers of disease severity, the ECLAM score and prednisone dosage, and attempted to characterize each of these variables with respect to their accuracy to detect seroconversion. Sensitivity and specificity for ECLAM and prednisone were calculated from 2 × 2 contingency tables for each possible cutoff value (of ECLAM score and prednisone dose, respectively) and were plotted as ROC curves (Figure 3). The area under the curve (AUC) for ECLAM was 0.66 with 95% CI (0.57-0.76) whereas the AUC for prednisone was 0.63 with 95% CI (0.56-0.69).

Thus, although both ECLAM and prednisone dosage can discriminate between patients who will and will not seroconvert, the diagnostic accuracy for both variables is moderate at best. On the other hand, as shown by a simple logistic model, the CYC treatment was better correlated with seroconversion than the ECLAM score, the prednisone treatment, or the disease duration (Table 2).

Clinical features of APS can develop despite intensive immunotherapy with CYC. Six patients from the seroconverted CYC treated group developed clinical features consistent with APS versus none in the non-CYC treated group

Table 1. Differences in clinical and serological characteristics between CYC treated and non-CYC treated patients.

Patient Characteristics	CYC Treated (n = 117)	Non-CYC Treated (n = 203)	p <
Clinical and pathologic findings	N (%)	N (%)	
Women	105 (89.7)	181 (89.1)	0.99
Men	12 (10.2)	22 (10.83)	0.99
Arthritis	57 (48.7)	95 (46.7)	0.82
Photosensitivity	64 (54.7)	119 (58.6)	0.42
Raynaud's phenomenon	61 (52.1)	126 (62.0)	0.10
Alopecia	58 (49.5)	56 (27.5)	0.01
Oral ulcers	56 (47.8)	73 (39.9)	0.04
Secondary Sjögren's syndrome	22 (18.8)	69 (33.9)	0.01
Nephritis	78 (66.6)	35 (17.2)	0.01
Vasculitis	31 (26.4)	36 (17.7)	0.09
Serositis	35 (17.2)	43 (21.1)	0.10
Venous thrombosis	10 (8.5)	19 (9.3)	0.99
Arterial thrombosis	9 (7.6)	10 (4.9)	0.33
Abortions/N of women	8/112 (7.1)	21/181 (11.6)	0.24
Strokes	7 (5.9)	10 (4.9)	0.80
Neurologic disorder	32 (27.3)	27 (13.3)	0.01
Livedo reticularis	44 (37.6)	76 (37.4)	0.99
Pulmonary hypertension	8 (6.8)	1 (0.4)	0.01
Fever	53 (45.2)	71 (34.9)	0.08
Laboratory tests			
Autoimmune hemolytic anemia	18 (15.3)	19 (9.3)	0.15
Leukopenia	58 (49.5)	106 (52.2)	0.73
Thrombocytopenia	30 (25.6)	51 (25.1)	0.99
Anti-Ro/SSA positive	42 (35.8)	72 (35.4)	0.99
Anti-La/SSB positive	22 (18.8)	35 (17.2)	0.76
Anti-Sm positive	13 (11.1)	26 (12.8)	0.73
Anti-U1RNP positive	14 (11.9)	44 (21.6)	0.04
Antiphospholipid positive[†]	25 (21.4)	6 (3.0)	0.01
Outcome			
End stage disease (renal, CNS, lung)	25 (21.3)	2 (0.9)	0.01
Death	4 (3.4)	2 (0.9)	0.20

[†] aPL IgG and IgM antibodies documented at least twice, 12 wks apart, and in titers higher than the 99th percentile of 100 normal sera obtained from individuals younger than 55 yrs. CYC: cyclophosphamide; CNS: central nervous system.

($p < 0.01$). The characteristics of these patients and the cumulative dose of CYC at the time of APS diagnosis are shown in Table 3. In all cases the thrombotic lesions were documented either through biopsy (patients 3 and 5), or through the use of the appropriate imaging techniques (patients 2 and 6) and/or electrocardiographic and hemodynamic assessment (patients 1 and 4).

The mean dose of CYC at the time of clinical diagnosis of APS was 21.5 g (range 13-28 g) and the mean time interval from the documentation of seroconversion to the documentation of APS was 9 months (range 6 mos-1 yr). In all cases the acute events occurred despite intensive immunotherapy with CYC and while the underlying disease was in remission. A schematic representation of the gradual improvement in disease activity measures and the simultaneous increase in aPL as well as the timing of APS diagnosis is depicted in Figure 4, using relevant data for patient 1.

Regarding coexistent known risk factors for vascular thrombosis, patient 6 was an ex-smoker (5 pack/yrs) and patient 4 had well controlled arterial hypertension. The rest of the patients did not have known risk factors for vascular disease and did not carry a diagnosis of vasculitis.

$ß_2$-GPI antibody responses. $ß_2$-GPI ELISA became available in our institution in January 2000. Since that time we have been using the test to verify aPL positivity. Between January 2000 and December 2005 10 patients in the CYC treated group seroconverted based on the aPL titers. Of these patients, 7 demonstrated a concomitant positivity of $ß_2$-GPI IgG antibody titers. During the same time period one patient seroconverted in the non-CYC treated group and had concurrently positive $ß_2$-GPI IgG. We should note that 4 out of the 6 patients with APS in the CYC treated group were diagnosed between 2000 and 2005, and of these, 3 also had increased $ß_2$-GPI IgG antibody titers. Since we did not use $ß_2$-GPI antibodies as a screening test, we cannot comment on the prevalence of an isolated increase of these antibodies in either the treated or the control population in our study.

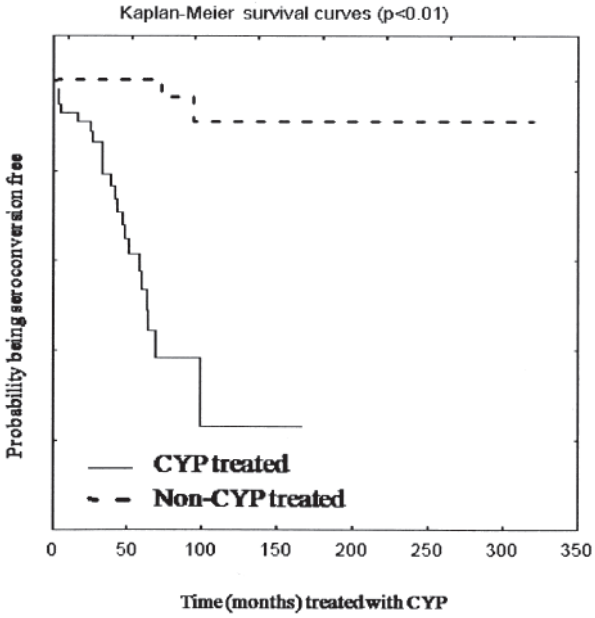

Figure 1. The probability that patients with SLE remain seroconversion free after initiation of treatment.

DISCUSSION

Our data suggest that aPL antibodies are induced in CYC treated SLE patients with a much higher frequency than in the non-CYC treated ones. This induction occurs despite successful treatment of the underlying disease as demonstrated by reduction of ECLAM score and anti-dsDNA and complement levels. Induction of aPL occurred in 22 patients in the CYC treated group versus only 2 in the larger non-CYC treated group. Although the former group had, by treatment criteria, more severe disease at the onset of treatment, the disease was in remission when the aPL antibodies were first detected.

Seroconversion occurred at variable timepoints from presentation and after variable doses of CYC had been administered, with the minimum dose being 4 g and the mean cumulative dose approximately 18 g. Further, a total of 6 patients in the seroconverted group developed full blown APS demonstrated by arterial or venous thrombosis affecting various organs. None of the non-CYC treated patients developed APS. The diagnosis of APS occurred within a few months to a year after first documentation of

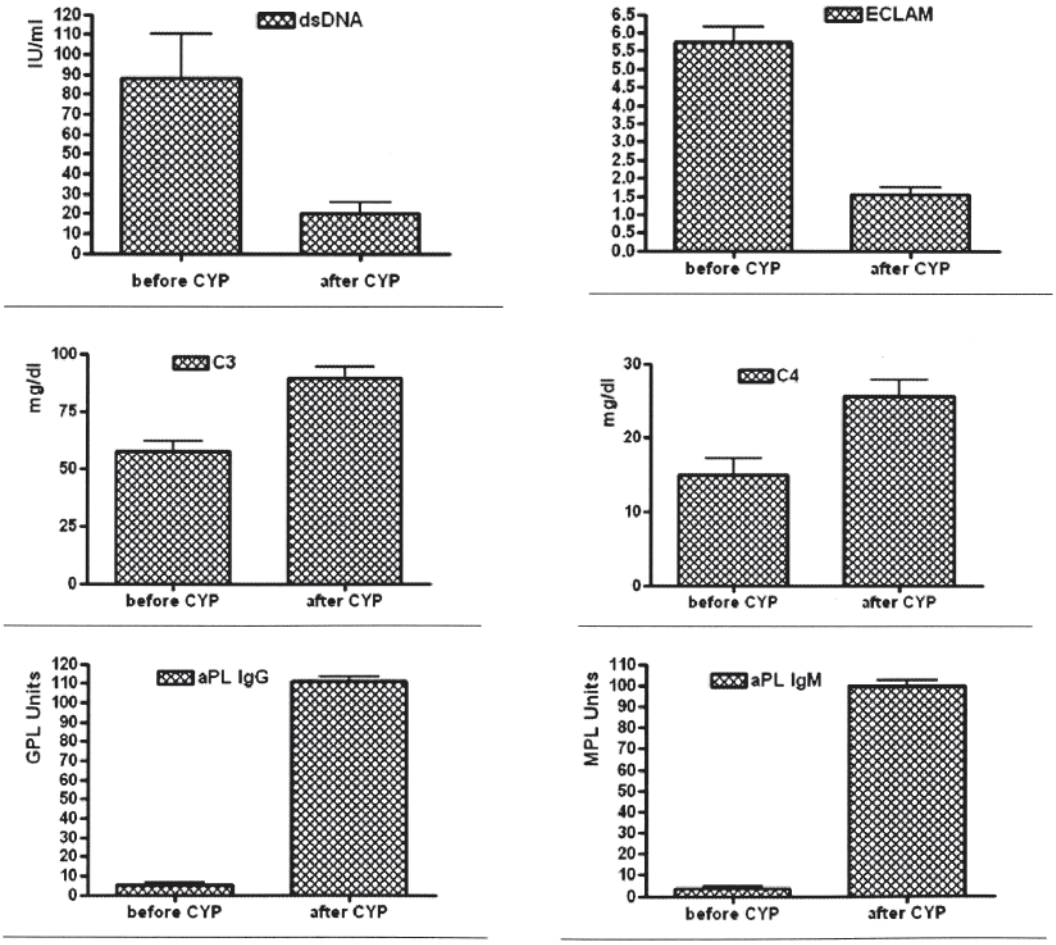

Figure 2. Changes in SLE activity measures and antiphospholipid antibodies (aPL) before and after cyclophosphamide (CYP) in 22 seroconverted patients.

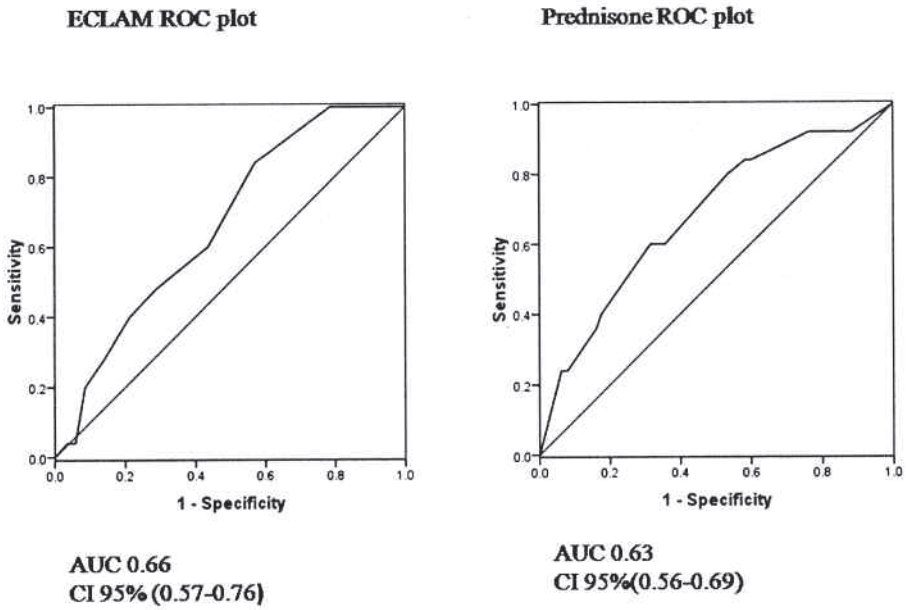

Figure 3. ROC analysis of diagnostic accuracy of ECLAM and prednisone dosage in regards to seroconversion.

Table 2. Adjusted odds ratios (OR) with 95% confidence intervals (CI) showing the association between seroconversion, therapy, disease activity, and disease duration.

Characteristic	Seroconverted (n = 24)	Non-seroconverted (n = 296)	OR (95% CI)
Treatment			13.39 (2.66–67.50)
CYC	22	95	
Non-CYC	2	201	
ECLAM score*			0.74 (0.20–2.76)
≤ 2	21	253	
> 2	3	42	
Disease duration, yrs, mean ± SD	11.08 ± 6.93	9.93 ± 6.50	1.02 (0.95–1.10)‡
Prednisone dose#			3.53 (0.38–33.13)
High	23	169	
Low	1	127	

* at time of seroconversion; ‡ comparison between 2 means; # determined within the first 6 mos from presentation. ECLAM: European Consensus Lupus Activity Measurement; SD: standard deviation.

seroconversion. Although the contribution of additional risk factors predisposing to arterial thrombosis cannot be completely excluded, known atherosclerotic factors were either not present or well controlled in all patients. It should also be noted that the clinical features of APS appeared despite the institution of aspirin treatment in all seroconverted patients.

Previous work has questioned the efficacy of CYC in the treatment of established APS and specifically its lack of effectiveness in halting the progression of the catastrophic form of the syndrome when compared with anticoagulants, corticosteroids, and plasmapheresis[14,15]. In isolated reports of beneficial effects of its use[16,17], CYC is combined with the above therapeutic modalities, making difficult any extrapolation as to its actual utility. Regarding the effect of CYC on aPL titers in particular, a retrospective study comparing the antibody titers in 9 patients with SLE before and after CYC found a decrease after small doses of CYC[18].

In our patient cohort we found 25 patients positive for aPL at the beginning of followup who were offered CYC due to severe SLE. CYC effected a decrease in the aPL titers in only 2 of them, as opposed to 3 patients in the non-CYC treated group with initially positive aPL. Although our data are insufficient to draw any conclusions regarding the effectiveness of CYC in patients with positive aPL or established APS, they do provide evidence of a possible association between treatment with CYC and the appearance of such antibodies in patients with SLE.

The alternative hypothesis, i.e., that the appearance of aPL is a consequence, not of the treatment with CYC, but of

Table 3. Clinical and serologic data of 6 seroconverted patients with documented APS.

Patient	Sex	Age, yrs	Findings	CYC Dose at APS Diagnosis, g	Time to APS[a], mo	IgG aPL before CYC, UGPL/ml	IgG aPL at APS[b], UGPL/ml	IgM aPL before CYC, UMPL/ml	IgM aPL at APS[b], UMPL/ml	B_2 GPI at APS[b] U/ml
1	F	35	Non Q myocardial infarction	23	7	0	111	0	116	NA
2	F	35	CNS micro-infarction	28	6	0	104	0	107	NA
3	M	20	Necrotic skin ulcer	24	12	4	119	5	96	118
4	F	50	Myocardial infarction	13	9	8	98	0	91	94
5	M	22	APS nephropathy	26	6	0	103	0	112	74
6	F	30	Venous thrombosis	15	8	10	120	8	98	119

[a] Time from seroconversion to the APS diagnosis; [b] At time of APS diagnosis. APS: antiphospholipid syndrome; CYC: cyclophosphamide; UGPL: GPL units; UMPL: MPL units.

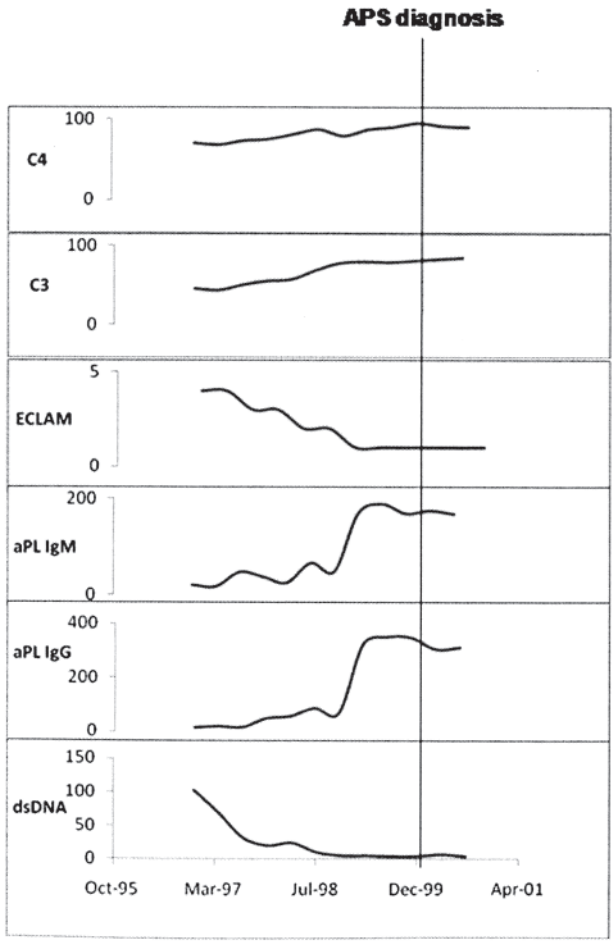

Figure 4. Changes in SLE activity measures and aPL after initiation of treatment with CYC, and timing of APS diagnosis (data from patient 1, Table 3).

the severity of the underlying disease, cannot be categorically excluded due to study design. However, our ROC analysis of the ECLAM score and prednisone dosage seems to support the hypothesis that the appearance of aPL is not closely related to disease severity, a fact that has already been observed by others[19]. It is possible for APS to be diagnosed at a time that SLE is quiescent; however, that would not explain the difference in prevalence between 2 patient groups with similar duration of disease. In addition, the observed differences should not be attributed to differences in the monitoring frequency of aPL between the 2 groups, as there is a policy of routine, at least 3 times yearly, monitoring of aPL in all patients with SLE in our institution.

How might CYC induce aPL? The pathogenic mechanism can only be speculated at this point. However, several observations suggest that CYC might demonstrate immune-enhancing properties at least under specific conditions; in a thymectomized mouse model, a single dose of CYC was associated with a 50% incidence of autoimmune gastritis associated with serum appearance of autoantibodies to the alpha and beta subunit of gastric H/K ATPase[20]. In addition, CYC induces Type 1 diabetes in the non-obese diabetic mouse[21,22] and enhances immune responses to allergens[23], tumors[24], as well as autoantigens[25]. Administration of CYC prevents oral tolerance to ovalbumin in mice and reverses the cellular immune response in the gut under these conditions[26].

On the basis of the available data, we assume that this immune enhancing effect of CYC is related to a reduction in regulatory T cells that normally suppress immune responses[27,28]. An alternative explanation might involve the release of large amounts of PL and PL-binding proteins due to CYC

induced cell apoptosis and/or necrosis[29,30], which then stimulate the immune system for the production of respective autoantibodies.

Our observation might be described as another manifestation of the phenomenon called "the kaleidoscope of autoimmunity," according to which a prior autoimmune disease and/or its treatment cause an immune disregulation that leads to a subsequent autoimmune disease. Several interventions might serve as triggers of autoimmunity including thymectomy[31], splenectomy[32], or bone marrow transplantation[33], among others. In a notable case the emergence of full blown APS followed thymectomy for myasthenia gravis[34]. In our case the successful treatment of SLE is followed by the appearance of new autoantibodies and, sometimes, a new autoimmune disease, i.e., APS. If this is the case, one might assume that the seroconversion is the result, not of CYC *per se*, but of the successful treatment of SLE that allows the appearance of new autoantibodies in genetically predisposed individuals. A comparison between CYC and other effective SLE regimens, such as mycophenolate, would be very interesting in this regard.

We should finally note that all our seroconverted patients received prednisone at some point during their disease course. In 96% of them (23/24) the initial prednisone dose exceeded 10 mg/day, although at the time of seroconversion they were maintained at much lower doses. The widespread use of prednisone in our patient cohort, including the patients who initially presented with positive aPL and became negative after treatment, prevents any conclusions as to a direct effect of prednisone on aPL antibodies. A literature search on the effect of prednisone in this regard yields few and contradictory data, with some studies reporting lowering of serum antibody titers[35] after use of prednisone and others showing no effect[36]. Our experience does not support use of prednisone in either primary or secondary APS, with the possible exception of the catastrophic form of the syndrome.

The retrospective nature of our study represents its major limitation. Prospective controlled trials are needed to confirm and expand on our data. An important question that remains to be answered involves the presence and nature of predictive factors that will allow early recognition of this potentially fatal complication of CYC therapy. At the mechanistic level, the ability of CYC to induce aPL, if proven, raises important questions regarding the mechanism responsible for its salutary effects on SLE and the role of antibody independent mechanisms in the pathogenesis of the disease.

In our cohort of patients with SLE followed over 10 years, treatment with high doses of CYC was associated with high incidence of new development of aPL as well as APS, despite achievement of disease remission. Whether the observed association is a true cause and effect relationship needs to be further elucidated.

REFERENCES

1. Raitt JW. Wegener's granulomatosis: treatment with cytotoxic agents and adrenocorticoids. Ann Intern Med 1971;74:344-56.
2. Siang C, Seah E, Wong KH, Chew AG, Jayaratnam FJ. Cyclophosphamide in the treatment of systemic lupus erythematosus. Br Med J 1966;1:333-5.
3. McCune WJ, Golbus J, Zeldes W, Bohlke P, Dunne R, Fox DA. Clinical and immunologic effects of monthly administration of intravenous cyclophosphamide in severe systemic lupus erythematosus. N Engl J Med 1988;318:1423-31.
4. Varkila K, Hurme M. The effect of cyclophosphamide on cytotoxic T-lymphocyte responses: inhibition of helper T-cell induction in vitro. Immunology 1983;48:433-8.
5. Cass RM, Anderson BR, Vaughan JH. Waldenstrom's macroglobulinemia with increased serum IgG levels treated with low doses of cyclophosphamide. Ann Intern Med 1969;71:971-7.
6. Hill RS, Bakemeier RF, Eastcott JW, Hymes WF. Effects of cyclophosphamide on serum immunoglobulin levels in SJL/J mice. Nature 1971;31:558-60.
7. Gourley MF, Austin HA 3rd, Scott D, et al. Methylprednisolone and cyclophosphamide, alone or in combination, in patients with lupus nephritis. Ann Intern Med 1996;125:549-57.
8. Illei GG, Austin HA, Crane M, et al. Combination therapy with pulse cyclophosphamide plus pulse methylprednisolone improves long-term renal outcome without adding toxicity in patients with lupus nephritis. Ann Intern Med 2001;135:248-57.
9. Tan EM, Cohen AS, Fries JF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271-7.
10. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1997;40:1725.
11. Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006;4:295-306.
12. Tektonidou M, Sotsiou F, Nakopoulou L, Vlachoyiannopoulos PG, Moutsopoulos HM. Antiphospholipid syndrome nephropathy in patients with systemic lupus erythematosus and antiphospholipid antibodies. Arthritis Rheum 2004;50:2569-79.
13. Vitali C, Bencivelli W, Isenberg DA, et al. Disease activity in systemic lupus erythematosus: report of the Consensus Study Group of the European Workshop for Rheumatology Research. II. Identification of the variables indicative of disease activity and their use in the development of an activity score. The European Consensus Study Group for Disease Activity in SLE. Clin Exp Rheumatol 1992;10:541-7.
14. Bucciarelli S, Espinosa G, Cervera R, et al. Mortality in the catastrophic antiphospholipid syndrome: causes of death and prognostic factors in a series of 250 patients. Arthritis Rheum 2006;54:2568-76.
15. Vlachoyiannopoulos PG, Tsiakou E, Chalevelakis G, Raptis SA, Moutsopoulos HM. Antiphospholipid syndrome: Clinical and therapeutic aspects. Lupus 1994;3:91-6.
16. Koschmieder S, Miesbach W, Fauth F, Bojunga J, Scharrer I, Brodt HR. Combined plasmapheresis and immunosuppression as rescue treatment of a patient with catastrophic antiphospholipid syndrome occurring despite anticoagulation: a case report. Blood Coagul Fibrinolysis 2003;14:395-9.
17. Otsubo S, Nitta K, Yumura W, Nihei H, Mori N. Antiphospholipid syndrome treated with prednisolone, cyclophosphamide and double-filtration plasmapheresis. Intern Med 2002;41:725-9.
18. Lavalle-Graef A, Villegas-Acostab L, Lavallec C. Trends of anticardiolipin antibodies after low-dose methylprednisolone and cyclophosphamide treatment of systemic lupus erythematosus. Arch

Med Res 2004;35:421-7.
19. Amigo MC, Khamashta MA. Antiphospholipid (Hughes) syndrome in systemic lupus erythematosus. Rheum Dis Clin North Am 2000;26:331-48.
20. Barrett SP, Toh BH, Alderuccio F, van Driel IR, Gleeson PA. Organ specific autoimmunity by adult thymectomy and cyclophosphamide induced lymphopenia. Eur J Immunol 1995;25:238-44.
21. Brode S, Raine T, Zaccone P, Cooke A. Cyclophosphamide-induced type-1 diabetes in the NOD mouse is associated with a reduction of CD4+CD25+Foxp3+ regulatory T cells. J Immunol 2006;177:6603-12.
22. Harada M, Makino S. Promotion of spontaneous diabetes in non-obese diabetes-prone mice by cyclophosphamide. Diabetologia 1984;27:604-6.
23. Maguire HC Jr, Ettore VL. Enhancement of dinitrochlorobenzene (DNCB) contact sensitization by cyclophosphamide in the guinea pig. J Invest Dermatol 1967;48:39-43.
24. Machiels JP, Reilly RT, Emens LA, et al. Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. Cancer Res 2001;61:3689-97.
25. Kallen B, Dohlsten M, Klementsson H. Effect of cyclophosphamide pretreatment on autoimmune encephalomyelitis in rats. Acta Neurol Scand 1986;73:338-44.
26. Mowat AM, Strobel S, Drummond HE, Ferguson A. Immunological responses to fed protein antigens in mice. I. Reversal of oral tolerance to ovalbumin by cyclophosphamide. Immunology 1982;45:105-13.
27. Salomon B, Lenschow DJ, Rhee L, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity 2000;12:431-40.
28. Szanya V, Ermann J, Taylor C, Holness C, Fathman CG. The subpopulation of CD4+CD25+ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. J Immunol 2002;169:2461-5.
29. Mammon K, Savion S, Keshet R, et al. Expression of apoptosis-associated molecules in the fetoplacental unit of cyclophosphamide treated mice. Reprod Toxicol 2006;22:774-82.
30. Ohtani T, Nakamura T, Tode K, Furukawa F. Cyclophosphamide enhances TNF-a-induced apoptotic cell death in murine vascular endothelial cell. FEBS Lett 2006;580:1597-600.
31. Zonana MF, Reyes E, Weisman AK. Coexistence of four autoimmune diseases in one patient. The kaleidoscope of autoimmunity. J Clin Rheumatol 2002;8:322-5.
32. Levene NA, Varon D, Shtalrid M, Berrebi A. Chronic active hepatitis following splenectomy for autoimmune thrombocytopenia. Isr J Med Sci 1991;27:199-201.
33. Sherer Y, Shoenfeld Y. Autoimmune diseases and autoimmunity post-bone marrow transplantation. Bone Marrow Transplant 1998;22:873-81.
34. Shoenfeld Y, Lorber M, Yucel T, Yazici H. Primary antiphospholipid syndrome emerging following thymectomy for myasthenia gravis: additional evidence for the kaleidoscope of autoimmunity. Lupus 1997;6:474-6.
35. Silveira LH, Hubble CL, Jara LJ, et al. Prevention of anticardiolipin antibody-related pregnancy losses with prednisone and aspirin. Am J Med 1992;93:403-11.
36. Salazar-Paramo M, Jara LJ, Ramos A, Barile L, Machado G, Garcia-De La Torre I. Longitudinal study of antinuclear and anticardiolipin antibodies in pregnant women with systemic lupus erythematosus and antiphospholipid syndrome. Rheumatol Int 2002;22:142-7.