Brief Definitive Report

Assessment of In Vivo Frequency of Mutated T Cells in Patients with Systemic Lupus Erythematosus

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Summary

The frequency of mutant T cells (FMC) in blood lymphocytes from patients with systemic lupus erythematosus (SLE) was measured by growing cells in the presence and in the absence of 6-thioguanine. Patients with SLE had a spectrum of FMC ranging from normal to about 100 times normal. This high FMC among cells from SLE patients appears to reflect excessive in vivo activation and proliferation during the course of the disease. This represents the first demonstration of such a T cell abnormality in SLE; it supports the hypothesis that SLE T cells demonstrate increased in vivo division and/or survival.

Human systemic lupus erythematosus (SLE) is an autoimmune disease that exhibits a characteristic spectrum of autoantibodies reactive with DNA, histones, various smaller nucleoproteins, cytoplasmic proteins, and cell surface components of lymphocytes and red blood cells (1). This intensive autoantibody production is associated with both polyclonal B cell activity and oligoclonal expansion of selective populations of antibody-forming cells (2). It is thought that T lymphocytes play a role in both processes, but are critical in the latter (2–5).

The *hprt* gene encodes the salvage pathway enzyme, hypoxanthine-guanine phosphoribosyl transferase. If a T cell sustains a mutation in *hprt* that reduces or abolishes *hprt* activity, the cell can be clonally selected in vitro (6, 7), since *hprt*$^-$ cells are able to grow in the presence of the purine analogue 6-thioguanine (6-TG). By comparing the growth of T cells in the presence and in the absence of 6-TG, it is possible to measure in vivo mutant frequencies of T cells and to isolate cells reactive with putative self-antigens from the mutant fraction (8).

The rationale for using 6-TG to detect in vivo T cell mutations in autoimmune disease is based upon certain assumptions. (*a*) During the course of disease, a fraction of the T lymphocytes (either oligoclonal or polyclonal) will be activated to proliferate; (*b*) random spontaneous mutations occur more frequently in proliferating than resting T cells; and (*c*) measurement of mutations in the *hprt* gene allows an estimate of the in vivo frequency of mutant T cells (FMC) as a crude indicator of past T cell proliferation.

Others have optimized and characterized this analysis of FMC (9, 10), and it has been used to demonstrate myelin basic protein–reactive T cells in multiple sclerosis (8). We chose to use it in the assessment of self-antigen–reactive T cells in SLE. However, before studying responses to self-antigens, we observed that many more lupus than normal T cell clones grew in the presence of 6-TG. That led to a formal analysis of the phenomenon and the present report.

Materials and Methods

Subjects. We studied 18 patients with SLE (11), nine normal individuals, and three patients with arthritis without lupus (two with rheumatoid arthritis and one with psoriatic arthritis). Normal individuals were blood bank donors at the Clinical Center (NIH). As a positive assay control, we studied two patients with partial *hprt* deficiency.

Cell Culture. Mononuclear cells (MNC) from the blood of patients and normals were isolated by Ficoll-Hypaque centrifugation and T cells were cultured to establish the fraction of *hprt*$^-$ mutants essentially as described (9). Medium consisted, by volume, of 55% RPMI 1640 (Biofluids, Rockville, MD) containing 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine, 5% FCS (Biofluids), 20% HL-1 medium (Ventrex, Portland, ME), and 20% lymphokine-activated killer (LAK) cell supernatant (12) as a source of IL-2, and other lymphokines (kindly provided by J. R. Yannelli, NCI, NIH). The culture medium contained 0.25 μg/ml PHA (Burroughs-Wellcome, Greenville, NC). As feeder cells, irradiated (90 Gy, from a ^{137}Cs source) Tk 6 cells were added (obtained from Dr. J. P. O'Neill, Genetics Laboratory, University of Vermont, Burlington, VT). For determination of the total lymphocyte cloning efficiency (CE), cells were seeded in round-bottomed microtiter plates (Costar, Cambridge, MA) at 0.25, 0.5, 1, 2, 4, and 8 lymphocytes/well. Each well also contained 5×10^3 irradiated Tk 6 cells. Selection of *hprt*$^-$ mutants was performed by seeding 2×10^3 and 2×10^4 lymphocytes per well in the presence of 10^4 irradiated Tk 6 cells and 10^{-5} M 6-thioguanine (2-amino-6-mercapto-purine; Sigma Chemical Co., St. Louis, MO). In some patients with high FMC, limiting dilution experiments

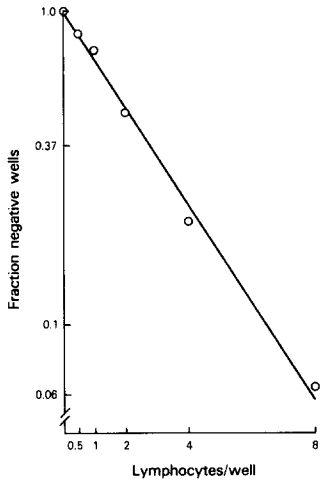

Figure 1. Representative limiting dilution experiment of a patient's T cells grown in the absence of 6-TG. The cloning efficiency for this patient was 35%.

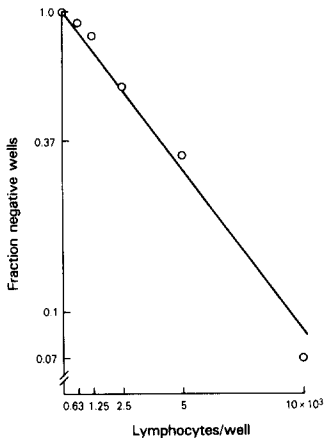

Figure 2. Growth of T cells in the presence of 6-TG. Far fewer cells grew in the presence of 6-TG. Nevertheless, a comparable log-linear relationship was found in limiting dilution studies.

were also carried out under 6-TG-selective pressure. For each cell concentration, at least two microtiter plates were set up for cultures without 6-TG and at least four for cultures with 6-TG. The cultures were incubated in 5–6% CO_2 in air, at 37°C for 11–14 d, and subsequently inspected using an invertoscope. 65 consecutive clones were studied for cell surface phenotypes; all were T lymphocytes.

Evaluation of Lymphocyte Outgrowth. Under the assumption of a Poisson distribution of clonable cells in limiting dilution studies, the FMC was established by dividing the apparent frequency of 6-TG-resistant cells by the CE, as follows: FMC = [−ln (fraction of negative wells in the presence of 6-TG)/no. lymphocytes per well]/CE. CE = [−ln (fraction of negative wells in the absence of 6-TG)/no. lymphocytes per well].

Results

Cells from patients and controls were cultured at limiting dilution in the presence and absence of 6-TG. It was determined that there was a log-linear relationship between cell input and fraction of negative wells: all showed a good linear

Table 1. *Age, Gender, and Therapy of SLE Patients Studied*

Patient	Age	Gender	Race	Current Rx	Past Rx	FMC
1	42	F	W	PR, PL, AZ	PR, PL, AZ	10,753
2	49	F	W	MED, CY, PL	PR	1,650
3	21	F	W	MED, CY	PR, AZ (3)	1,379
4	37	F	W	PR	PR, PL	1,200
5	41	F	W	PR, PL	PR, PL	1,090
6	60	M	W	PR, AZ	PR, AZ, CY	1,086
7	32	F	W	MED, CY	PR	655
8	24	F	W	PR	PR, PL	647
9	26	F	B	PR, CY	PR, PL	124
10	33	F	B	PR, CY	PR	92
11	46	F	W	MED	PR	81
12	33	F	W	PR	MED	69
13	30	F	W	PR	PR, AZ (3)	42
14	41	F	W	MED, CY	PR, PL	41
15	40	M	B	PR	PR	31
16	39	F	W	PR	PR, AZ (11)	25
17	31	F	B	MED	PR	24
18	16	F	W	PR	PR	19

PR, prednisone; MED, medrol; CY, Cyclophosphamide; PL, hydroxychloroquine; AZ, Azathioprine. Numbers in parentheses indicate years since last dose. Controls: 6 F and 3 M, with age range of 23–57 yr and mean age the same as that of the patients.

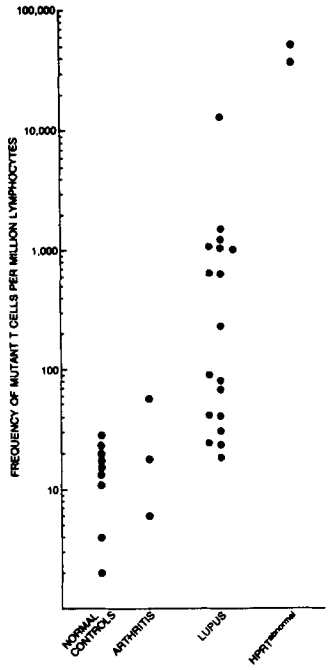

Figure 3. The apparent frequencies of mutant T cells are shown for nine normal controls, three patients with arthritis (two with rheumatoid arthritis and one with psoriatic arthritis), and 18 patients with systemic lupus. More than half of the lupus patients were >3 SD above the highest normal control. Also shown are two males with defects in *hprt* as positive controls for the ability of T cells to grow in 6-TG.

relationship comparable with that depicted in Fig. 1. The range of calculated cloning efficiencies was 6–90% for normal controls and 5–60% for the patients (data not shown).

Simultaneously, similar cultures were set up with 6-TG in the medium. As expected, a much lower frequency of clones was found. As a result, it was possible to construct formal limiting dilution curves only for individuals with a very high frequency. In those cases, however, we again observed a log-linear relationship, as shown in Fig. 2. In this individual, T cell clone growth for 6-TG-resistant (i.e. *hprt*-negative) cells was 1/4,000 or $250/10^6$.

For all individuals, cultures were established and analyzed in medium with and without 6-TG. The results of both cultures were used to derive an apparent FMC for each individual (see Materials and Methods). The data are summarized in Fig. 3. As a group, patients with systemic lupus had substantially higher FMC than did controls (Fig. 3). Several SLE patients, especially a few with very high FMC, were reevaluated: the results of repeat studies were quite comparable; however, only the data from the original evaluation were used to construct Fig. 3.

It is appropriate to be concerned about the possible contribution of cytostatic and cytotoxic drugs to the results obtained. Table 1 gives the information on therapy in individual patients. The patient with the highest FMC was receiving azathioprine at the time of study. Patient 6 with a high FMC also was receiving that drug. Two other patients, nos. 3 and 13, had previously received azathioprine, and it had been stopped in both 3 yr before study. One had quite a high FMC but the FMC of the other was 20-fold less. Several patients had received cyclophosphamide; however, there was no obvious correlation with FMC. The two boys with a (partial) genetic defect in the *hprt* gene had, as expected, a very high frequency of cells able to grow in 6-TG; however, it did not approach 100%.

Discussion

Among 18 patients with SLE studied, several had increased FMC as measured by the *hprt*-mutant selection system. This was not a general feature of lupus patients in that some had normal FMC. Those with elevated FMC represented a spectrum from slightly increased to some 2 logs increased.

It is presumed that SLE patients with elevated FMC had increased numbers of T cells that had been driven to proliferate, thereby causing an increase in random mutations, as has been argued previously (8). Of the six patients studied, herein, with disease duration of 20 yr, five had very high FMC ($>1{,}000/10^6$ cells). It is possible that some product of severity and duration of lupus disease activity might ultimately correlate with FMC. However, we believe that a larger number of patients would have to be studied serially to determine whether or not this relationship is true.

In theory, the SLE patients with massive T cell growth in the presence of 6-TG could be carriers of a partial defect in *hprt*. We believe that such an explanation does not account for the observed growth of SLE T cells in 6-TG because: (*a*) such a carrier frequency would be far greater than expected, and (*b*) the broad range seen among SLE patients might better be explained by a variable history of in vivo T cell activation.

We found no obvious correlation between drug therapy and FMC. Although certain drugs potentially could increase the FMC, for most drugs, the increase would not be expected to be as great as that observed herein, and in some studies it has been quite modest (13). One drug, azathioprine, could potentially be responsible for some of the elevated FMC. This drug might both induce mutations and select in vivo for 6-TG-resistant clones. However, only two of the patients were receiving azathioprine at the time of study. Three additional patients had received azathioprine in the past, two with modest and one with a high FMC. Thus, in several patients, high FMC appears to be largely independent of drug therapy.

In this study, we used cells from two individuals with partial defects in *hprt* to provide a positive assay control for growth in 6-TG. Although these individuals demonstrated the greatest growth in 6-TG, their FMC was not quite 10%, whereas it might have been expected to be closer to 100% if their defect in *hprt* was uniform and severe. This result raises the possibility that our cloning efficiency in 6-TG was less than we assumed, and that the FMC in patients might actually be greater than we have calculated.

T cells of patients with SLE are subject to in vivo activation (14), which is thought to stimulate B cells to produce antibodies, including autoantibodies (2, 4, 5). Over time, such T cell activation would be expected to result in an accumulation of mutations. By chance, some occur in the *hprt* gene and are detectable in the assay system used herein. It would be expected that studies of other genes would also demonstrate mutations in SLE T cells. Unfortunately, biochemical selection strategies are not readily available for other genes.

Although it has been postulated that there might be excessive division and/or prolonged survival of SLE T cells (including self-reactive T cells), evidence supporting such an hypothesis has been lacking. The present report, for the first time, demonstrates an increased mutant T cell frequency in many individuals with lupus. Additional experiments will assess the mutations in the *hprt* gene as well as TCR usage among the panels of T cell clones. Such work will provide information as to whether the *hprt* mutant T cells represent oligoclonal expansion of only a few progenitors, or, alternatively, constitute a population that was substantially polyclonal in origin. Further studies will also analyze possible skewing of mutant T cell clones toward autoreactivity.

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