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

By Frits Gmelig-Meyling, Sahar Dawisha, and Alfred D. Steinberg

From the Cellular Immunology Section, Arthritis and Rheumatism Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland 20892

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.

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% RPM1 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, Green ville, NC). As feeder cells, irradiated (90 Gy, from a 137Cs 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 x 10^3 irradiated Tk 6 cells. Selection of hprt- mutants was performed by seeding 2 x 10^5 and 2 x 10^4 lymphocytes per well in the presence of 10^4 irradiated Tk 6 cells and 10^-3 M 6-thioguanine (2-amino-6-mercaptopurine; Sigma Chemical Co., St. Louis, MO). In some patients with high FMC, limiting dilution experiments...
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% CO₂ 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: $FMC = \frac{-\ln \left( \text{fraction of negative wells in the presence of 6-TG} \right)}{\text{no. lymphocytes per well}} / \CE = \frac{-\ln \left( \text{fraction of negative wells in the absence of 6-TG} \right)}{\text{no. lymphocytes per well}}$.

**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 (yr) | 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.
but the FMC of the other was 20-fold less. Several patients. The patient with the highest FMC was receiving azathioprine at the time of study. Patient 6 with a high FMC had received cyclophosphamide 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.

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 (>1,000/10⁶ 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.

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%.

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 250/10⁶.

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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 \textit{hprt} gene as well as TCR usage among the panels of T cell clones. Such work will provide information as to whether the \textit{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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Address correspondence to Alfred D. Steinberg, Cellular Immunology Section, Building 10, Room 9N-218, National Institutes of Health, Bethesda, MD 20892. F. Gmelig-Meyling’s present address is the Department of Immunology, University Hospital, Room F03.821, P.O. Box 85500, 3508GA Utrecht, The Netherlands.

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