INTERFERON-INDUCED 2'-5' ADENYLATE SYNTHETASE IN VIVO AND INTERFERON PRODUCTION IN VITRO BY LYMPHOCYTES FROM SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS WITH AND WITHOUT CIRCULATING INTERFERON*‡

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The recognition that interferons (IFN)[1] markedly affect a wide variety of immune responses has led to speculation that they may be involved in development of autoimmune diseases. Many patients with systemic lupus erythematosus (SLE) have high levels of circulating IFN (1–4). Since the IFN was inactivated at pH 2, it was originally assumed to be "immune" or gamma IFN (1). However, we recently demonstrated that the IFN in patients with SLE is not gamma IFN, but rather is an unusual acid-labile form of human leukocyte, or alpha, IFN (2, 3). A similar IFN was also found in patients with rheumatoid arthritis (3), autoimmune vasculitis (4), and the acquired immunodeficiency syndrome (5). In contrast, no IFN was detected in patients with drug-induced lupus (3), suggesting that production of IFN may be related to idiopathic immune disorders.

Others found that SLE lymphocytes were poor producers of IFN in culture (6), but they neither characterized the IFN made in vitro nor measured serum IFN levels in their patients. We therefore re-evaluated IFN production by lymphocytes from SLE patients with and without endogenous IFN. We also quantitated the IFN-induced enzyme 2'-5'-oligoadenylate (2–5A) synthetase in cells from SLE patients. This enzyme is induced in cultured cells treated with IFN (7) and in lymphocytes from patients undergoing IFN therapy (8) or viral infections (9). The results suggest that most, if not all, SLE patients produce IFN that has biological significance, as demonstrated by elevated 2–5A synthetase in circulating lymphocytes.

Materials and Methods

Sera and Patient Specimens. Serum and heparinized blood were obtained with informed consent from patients with SLE attending the Clinical Center at the National Institutes of Health for regularly scheduled appointments.

IFN Assay. IFN was quantitated using a semi-micromethod on human fibroblasts (GM2504) trisomic for chromosome 21, as described previously (2, 3, 5). Results were standardized to National Institutes of Health no. 023-901-527 reference human alpha IFN.

Characterization of IFN. Preliminary identification of alpha IFNs was made by assaying samples on bovine (MDBK) cells, as described previously (2, 3, 5). Human alpha IFN has full antiviral activity on MDBK cells, whereas human beta and gamma IFNs are inactive on these cells. These results were confirmed using highly specific rabbit anti-IFN antibodies (2, 3, 5).

To determine the stability of an IFN at pH 2, 100-μl aliquots were adjusted to pH 2 with 1 N HCl (6–10 μl per sample) as previously described (2, 3, 5); controls received equivalent volumes of sterile distilled H_2O. Reference human alpha, beta, and gamma IFNs diluted to 100 IU/ml in normal human serum (Flow Laboratories, McLean, VA) were treated in an identical manner. Residual IFN was assayed on GM2504 cells as described above. Reference human alpha and beta IFNs were completely stable during this procedure, whereas human gamma IFN was inactivated >30-fold (2, 3, 5). Control experiments with both mouse and human IFNs have shown that this method gives results equivalent to dialysis against buffer at pH 2, and is more convenient with small samples.

Induction of IFN In Vitro. Mononuclear cells were isolated from heparinized peripheral blood on Ficoll-hypaque (LSM Solution, Litton Bionetics, Kensington, MD) gradients, resuspended to 1–2 × 10^6 cells/ml in RPMI 1640 medium supplemented with 2–5% normal human AB serum (Flow Labs), and distributed in 0.2-ml aliquots to wells of microtiter plates. IFN inducers (UV-inactivated Newcastle disease virus (UV-NDV), pokeweed mitogen (PWM), and phyto-hemagglutinin (PHA) (P-L Biochemicals, Inc., Milwaukee, WI), poly rI:poly rC (poly I:C), and heat-killed *Corynebacterium parvum* strain CN6134 (Burroughs Wellcome, Greenville, NC) were prepared as 10x concentrates, and 20 μl was added to appropriate wells. Control cells received 20 μl of medium. Final concentrations of each inducer were: UV-NDV, 5.0 plaque-forming unit equivalents/cell; PWM, 5.0 and 50 μg/ml; PHA, 0.5 and 5.0 μg/ml; poly I:C, 20 μg/ml; and *C. parvum*, 100 μg/ml. Culture supernatants harvested after various times at 37°C were clarified by centrifugation and assayed for IFN on GM2504 cells.

2-5A Synthetase Assays. Mononuclear cell extracts were adsorbed to poly I:C-agarose for synthesis of 2-5A as described previously (10). After incubation for 4–6 h, the supernatants were treated with bacterial alkaline phosphatase and assayed for 2-5A by a competition immunoen-zymometric assay that employed rabbit antibodies to 2-5A and microtiter wells coated with conjugates of 2-5A and derivitized Ficoll (Johnston, M. I., P. F. Torrence, J. Imai, and O. T. Preble, manuscript in preparation). Characterization of the antibodies will appear elsewhere (Johnston, M. I., J. Imai, K. Lesiak, and P. F. Torrence, manuscript submitted for publication). Results were normalized to picomoles of 2-5A synthesized per hour per A_{260} unit of protein.

Results

Production of IFN by Lymphocytes from SLE Patients. Mononuclear cells from SLE patients were cultured without IFN inducers, or with classical inducers of human alpha IFN (UV-NDV), beta IFN (poly I:C), or gamma IFN (PHA or PWM). Culture fluids were harvested from mitogen-stimulated cells after 1, 3, 5, and 7 d. However, since IFN levels from normal lymphocytes and SLE lymphocytes incubated with PWM were greatest at 3 d, Table I shows only the data for 3 d. Lymphocytes from IFN-positive patients did not spontaneously secrete IFN in vitro at any time. With the exception of the 3-d sample for patient no. 8, no SLE lymphocytes produced IFN in response to the T cell mitogen PHA even though microscopic evaluation showed that cell proliferation was fair to good in all cultures. PHA did induce IFN in cultures of lymphocytes from two normal individuals (Table I). In contrast, PWM induced

TABLE I

Interferon Production by Mitogen-stimulated Lymphocytes In Vitro

Serum IFN	Mononuclear Cell Donor	IFN (IU/ml) after stimulation with:*				
		None	PHA (low)	PHA (high)	PWM (low)	PWM (high)
IU/ml						
≤4	normal 1	<4	100	400	800	1,600
	normal 2	<4	25	100	800	1,600
13–50	SLE 1	<4	<4	<4	50	50
	SLE 2	<4	<4	<4	13	50
	SLE 3	<4	<4	<4	400	1,600
	SLE 4	<4	<4	<4	50	50
≤4	SLE 5	<4	<4	<4	50	100
	SLE 6	<4	<4	<4	100	100
	SLE 7	<4	<4	<4	400	400
	SLE 8	<4	50	<4	100	200

* Mononuclear cells (1–2 × 10⁶ cells/ml) were incubated without mitogens, with low (0.5 μg/ml) or high (5.0 μg/ml) doses of PHA, or with low (5.0 μg/ml) or high (50.0 μg/ml) doses of PWM. Culture fluids harvested after 3 d were clarified and assayed for IFN on human GM2504 cells.

TABLE II

Interferon Production by UV-NDV-stimulated SLE Lymphocytes

# Patients	Serum IFN		IFN produced in response to UV-NDV		
	IU/ml*	Inact. at pH 2‡	IU/ml*	GM2504§ / BK	Inact. at pH 2‡
4	25–100	4–8	800–1,600	2–4	0
5	≤4	—	800–1,600	0.5–2.0	0

* IFN titers (IU/ml) assayed on human GM2504 fibroblasts.

‡ Fold-reduction in titer after incubation at pH 2. The values for reference human alpha, beta, and gamma IFNs were 0, 0, and >32 respectively (2, 3, 5). The values for serum IFN samples from the IFN + SLE patients were all 4–8.

§ Ratio of IFN titers in duplicate assays on human GM2504 fibroblasts and bovine MDBK cells. Ratios for reference human alpha, beta, and gamma IFNs were 0.5–2.0, >32, and >32, respectively.

IFN production by cells from both normal and SLE patients. However, lower levels of IFN were produced by cells from most SLE patients (50–400 IU/ml) than by cells from control subjects (1,600 IU/ml). These results are similar to previously reported data (9). Cells from IFN-positive patients tended to produce less IFN than cells from IFN-negative patients. The IFN in these cultures at 1, 3, 5, and 7 d was also assayed on MDBK cells, and like human gamma IFN, was completely inactive on bovine cells. Synthesis of gamma IFN was expected under these conditions (11).

SLE lymphocytes cultured with poly I:C produced very low levels (8–50 IU/ml) of IFN with little activity on MDBK cells (data not shown). Others found that normal lymphocytes produced 10–150 U of IFN in response to poly I:C (6). Synthesis of beta IFN might be expected in response to poly I:C (11). Lymphocytes from all SLE patients, cultured for 24 h with UV-NDV, produced 800–1,600 IU/ml of alpha IFN that had similar antiviral activity on bovine and human cells (Table II). However, unlike the serum IFN in these patients, the alpha IFN produced in vitro was completely stable at pH 2 (Table II), and appears to be like previously studied virus-induced alpha IFNs (11). Neighbor and Grayzell (6) found that patients with acute SLE did not produce IFN in response to NDV; none of our patients had acute SLE.

Normal human leukocytes stimulated in vitro with *C. parvum*-produced IFN that was neutralized by antiserum to human alpha IFN and active on bovine cells (12).

TABLE III

Interferon Production by SLE Lymphocytes in Response to C. parvum

Mononuclear cell donors	Number of donors	Serum IFN (IU/ml)	IFN produced in response to C. parvum*		
			IU/ml (range)	Ratio $\frac{GM2504}{DBK}$	Inactivation at pH 2
Normal	6	≤4	1,000–2,000	1–2	4–8
SLE	5	8–32	100–640	1–2	4–8
SLE	6	≤4	500–2,000	1–2	4–8

* Mononuclear cells were incubated for 24 h at 37°C with 100 μg/ml of heat-killed *C. parvum.* Cell culture supernatants were then assayed for IFN on human GM2504 and bovine MDBK cells, and tested for stability at pH 2 as described in Materials and Methods.

TABLE IV

2'5' Oligoadenylate Synthetase in Lymphocytes from SLE Patients

Mononuclear	Serum IFN	# Samples	2-5A Synthetase (pmol 2-5A/h/A$_{260}$)*		
			Normal (≤200)	Slightly high (230–250)	Very high (304–1,900)
	IU/ml				
SLE	8–25	9	1	1	7
SLE	≤4	10	4	1	5
Normal controls	≤4	3	3	0	0

* Cytoplasmic extracts of mononuclear cells were assayed for 2-5A synthetase activity as described in Materials and Methods. The data presented are the average of at least two separate determinations for each sample.

However, the IFN was partially inactivated at pH 2, and therefore appears similar to serum IFN in patients with SLE. We confirmed these results using mononuclear cells from six normal volunteers (Table III). As in experiments with PWM (Table I), cells from IFN-positive SLE patients produced less IFN after stimulation with *C. parvum* than cells from IFN-negative patients or normal subjects (Table III). However, all patients produced IFN with the biological properties expected for *C. parvum*-induced alpha IFN (i.e., active on bovine cells and partially inactivated at pH 2).

2-5A Synthetase in Serum IFN-Positive and Serum IFN-Negative Patients with SLE. IFN production by continuous cell lines can often be inhibited by prior exposure to high doses of IFN, a phenomenon known as "blocking." Although purified IFN did not have blocking activity on some mouse cell lines (13), the effects of acid-labile alpha IFN, which predominates in patients with SLE, are unknown. Since cells from serum IFN-negative SLE patients produced abnormally low levels of IFN in vitro, we investigated the possibility that cells from these patients had also been exposed to IFN in vivo. To do this, we quantitated 2-5A synthetase activity in extracts of mononuclear cells. Since mice infected with virus may have elevated 2-5A synthetase before, during, and after the peak of circulating inferferon, Revel and co-workers (14) postulated that (*a*) cells exposed to IFN may contain synthetase for some time after IFN itself disappears; (*b*) lymphocytes may be exposed to IFN produced locally but with no access to the circulation and therefore not detected in the peripheral blood; and (*c*) serum IFN levels below those detectable with assays currently available may contribute to elevation of 2-5A synthetase.

Acid-labile leukocyte IFN from SLE patients induced 2-5A synthetase in GM2504 and MDBK cells in vitro as expected (data not shown). Lymphocytes from eight of nine IFN-positive SLE patients had elevated levels of 2-5A synthetase compared with

normal lymphocytes (Table IV), suggesting that SLE patients do respond to their endogenous IFN. In addition, 6 of 10 serum IFN-negative patients had high levels of 2-5A synthetase, suggesting that these patients also produced endogenous IFN, but that IFN synthesis was either transient, local, or below detectable levels in serum. Previous estimates of the number of SLE patients actually producing IFN are therefore probably minimums.

Discussion

We confirmed that cells from SLE patients were deficient in their ability to synthesize IFN in vitro in response to some inducers. However, cells from SLE patients made conventional human alpha, beta, or gamma IFN in response to UC-NDV, poly I:C, and PWM, respectively. None of the lymphocyte cultures specifically produced acid-labile alpha IFN spontaneously or in response to any of the inducers tested although all lymphocytes produced acid-labile alpha IFN after stimulation with *C. parvum*. While we have not exhausted the list of potential IFN inducers in SLE patients, these results suggest that peripheral lymphocytes may not be the source of the circulating acid-labile alpha IFN in patients with SLE.

Previous results (8, 9, 14) suggested that measurement of the IFN-induced enzyme 2-5A synthetase may be a more sensitive indicator of endogenous IFN production than assay of serum IFN. We found that 14 of 19 patients with SLE (8 of 9 patients with circulating IFN, and 6 of 10 patients without detectable serum IFN) had elevated levels of 2-5A synthetase. Others also found 2-5A synthetase in mononuclear cells from patients with unspecified "collagen diseases" (9). These results suggest that most patients with SLE actually produce IFN. However, some patients may produce very low levels of IFN, and be recorded as false negatives, or produce IFN transiently or cyclically. Alternatively, IFN may be produced in lymphoid organs without enough "spillover" into serum to produce detectable IFN levels in all patients. If this is the case, local IFN titers could be high enough for potent immunoregulatory effects in both serum IFN-positive and serum IFN-negative patients.

Endogenous production of IFN may explain the abnormally low response of lymphocytes from patients with SLE and other immune disorders to mitogens and some IFN inducers in vitro. Analogous results were obtained using lymphocytes from IFN-treated mice (15). Our data suggest an inverse correlation between serum IFN and the amount of IFN produced in vitro. Patients with circulating IFN presumably have more endogenous IFN at that time than serum IFN-negative patients, so that more pronounced inhibition of cellular responses by their lymphocytes might be expected. Since serum IFN is more frequent in patients with active SLE (1–3), our data is consistent with previous results (6) showing a high proportion of nonresponders among patients with acute disease and reduced IFN production by cells from patients with active SLE compared with those with inactive SLE. Deficient activity of natural killer (NK) cells from SLE patients may also be partially due to continuous endogenous exposure to IFN; studies with both mice and humans have shown that long-term treatment with IFN can result in depressed NK activity (16).

Although mononuclear cells from normal individuals appear capable of producing an acid-labile alpha IFN in vitro in response to *C. parvum* (Table IV, and reference 14) and after influenza virus challenge (17), this form of alpha IFN is apparently a minor component of "conventional" human alpha IFN preparations. Why this particular

alpha IFN is produced out of proportion in patients with autoimmune disease is not yet known. There is, however, indirect evidence that IFN may contribute to autoimmune disease in mice (18, 19). The immunoregulatory effects of "conventional" human alpha IFN on normal human lymphocytes are well documented; investigation of immunomodulation by acid-labile alpha IFN is currently in progress.

Summary

The interferon (IFN)-induced enzyme 2-5A synthetase was elevated in mononuclear cells from both serum IFN-positive and -negative systemic lupus erythematosus (SLE) patients. This suggests that a much higher percentage of patients than previously thought produce endogenous IFN. These results may partly explain findings that mononuclear cells from SLE patients are deficient in IFN production in vitro in response to certain IFN inducers. Although normal lymphocytes can produce an acid-labile alpha IFN after stimulation with *C. parvum* in vitro, the reason for endogenous production of this unusual alpha IFN by SLE patients remains unknown.

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