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Anti-Double-Stranded DNA Antibodies and Immunostimulatory Plasmid DNA in Combination Mimic the Endogenous IFN-α Inducer in Systemic Lupus Erythematosus[1]

Helena Vallin,* Anders Perers,* Gunnar V. Alm,[2]* and Lars Rönnblom[†]

Patients with systemic lupus erythematosus (SLE) have increased blood levels of IFN-α, which correlate to disease activity. We previously identified an IFN-α-inducing factor (IIF) in the blood of SLE patients that activated the natural IFN-α-producing cells in cultures of normal PBMC. The SLE-IIF contained DNA and IgG, possibly as small immune complexes. In our study, we demonstrated that SLE-IIF correlated to the presence of anti-dsDNA Abs in patients and contained anti-dsDNA Abs as an essential component. Purified anti-DNA Abs or SLE-IgG caused only a weak IFN-α production in cultures of normal PBMC in the presence of costimulatory IFN-α2b. However, they converted the plasmid pcDNA3, which itself induced no IFN-α production in PBMC, into an efficient IFN-α inducer. A human monoclonal anti-ss/dsDNA Ab had the same effect. This IFN-α-inducing activity of the plasmid was abolished by methylation, suggesting that unmethylated CpG DNA motifs were important. Like IIF in SLE serum, the combination of SLE-IgG and pcDNA3 appeared to stimulate IFN-α production in natural IFN-α-producing cells, a unique cell population resembling immature dendritic cells. The IFN-α production was greatly enhanced by IFN-α2b and IFN-β, and for SLE-IIF it was also enhanced by GM-CSF but inhibited by IL-10. We have therefore identified a new function of DNA-anti-DNA Ab complexes, IFN-α induction, that might be important in the pathogenesis of SLE. ***The Journal of Immunology,*** **1999, 163: 6306–6313.**

Systemic lupus erythematosus (SLE)[3] is a multisystem autoimmune disease characterized by production of autoantibodies against many different autoantigens, typically including nucleic acid and associated proteins (1). The pathogenesis is largely unknown, although several factors of importance in the development of the disease have been reported (1). They include type I IFN, products of a multigene family comprising 13 IFN-α subtypes, 1 IFN-β and 1 IFN-ω, all acting on the IFN-$\alpha\beta$ receptor (2–4). In particular during active disease, SLE patients have an activated type I IFN system with detectable IFN-α in blood and increased levels of type I IFN-inducible intracellular proteins (5–11). The type I IFNs could be involved in development and maintenance of SLE, because they have several immunoregulatory effects that could interfere with development of self tolerance and promote autoimmunity (3, 12–16). In support of this, IFN-α therapy of patients with nonautoimmune disorders results in frequent development of various autoimmune phenomena, including antinuclear Abs, Abs to native DNA, and occasionally SLE-like syndromes (17–21).

An ongoing IFN production in SLE suggests the presence of an IFN inducer, because type I IFN gene expression is usually triggered by microorganisms, especially viruses (3, 22, 23). In fact, we recently described that sera from SLE patients frequently induced production of IFN-α in normal PBMC in vitro (24). Our further work indicated that this was due to an IFN-α-inducing factor (SLE-IIF) and that the IFN-α production occurred in the natural IFN-α-producing cells (IPC), which resemble immature dendritic cells (25). Furthermore, the SLE-IIF activity was most prominent in patients with active disease and IFN-α in serum and was strongly enhanced by exposure of the PBMC in vitro to the combination of IFN-α and GM-CSF. The SLE-IIF had a molecular mass in the 300- to 1000-kDa range and appeared to contain Ig and DNA, suggesting that it could consist of DNA-anti-DNA Ab immune complexes.

The main purpose of our study was to identify the essential IFN-α-inducing components in SLE-IIF and to clarify to what extent the IFN-α production is regulated by different cytokines. We found that anti-DNA Abs, probably with specificity for dsDNA, were involved in triggering IFN-α production. Furthermore, the endogenous DNA in SLE-IIF could be substituted for by a plasmid with immunostimulatory DNA (isDNA) motifs containing unmethylated CpGs, and such DNA together with purified anti-DNA Abs became a potent IFN-α inducer. The IFN-α production induced in PBMC by plasmid and SLE-IgG, like that induced by serum SLE-IIF, appeared to occur in the natural IPC. We also found that the IFN-α production was enhanced by IFN-$\alpha\beta$ and in the case of serum SLE-IIF also by GM-CSF, but strongly inhibited by IL-10.

Materials and Methods

Patients and controls

Nine patients who fulfilled the ACR classification criteria for SLE (26) were included. The patients (seven female and two male) had a median age

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[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; DELFIA, dissociation-enhanced lanthanide fluoroimmunoassay; IIF, IFN-α inducing factor; IPC, IFN-α producing cells; isDNA, immunostimulatory DNA; UV-HSV, HSV inactivated by UV light; NS, normal serum.

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of 32 years (range, 16–74 years), a median duration of disease of 8 years (range, 2–32), and a median ACR index of 6 (range, 4–10). The median disease activity as assessed by a modified SLE disease activity index, where complement levels and anti-DNA Abs were excluded (27), was 4 (range, 0–22). One patient was untreated, 8 were treated with prednisolone (mean, 9.4 mg/day), 2 with cyclosporin A (mean, 125 mg/day), 2 with hydroxychloroquine sulfate (mean, 300 mg/day), 2 with azathioprine (mean, 75 mg/day), 1 with methotrexate (7.5 mg/week), and 1 with cyclophosphamide (100 mg/day). Four normal male blood donors served as controls [median age, 28 (range, 20–31)]. Citrated plasma samples were obtained, converted to serum by addition of 1 M $CaCl_2$ in 0.15 M NaCl to a final concentration of 10 mM $CaCl_2$ and stored at −80°C. The study protocol was approved by the Committee of Ethics, Faculty of Medicine, Uppsala University, Uppsala, Sweden.

Preparation of IgG on protein G columns

The sera were 0.45 μm filtered, followed by 1 h of incubation at 37°C with equal volumes of 2000 U/ml DNase I (Boehringer Mannheim, Mannheim, Germany) in 100 mM Tris-HCl (pH 7.5) and 10 mM $MnCl_2$, to destroy endogenous DNA. They were separated on protein G-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) as recommended by the manufacturer. The pH of the IgG-containing eluates was adjusted to pH 7 by addition of 1 M Tris-HCl (pH 9.0), and the eluates were dialyzed against RPMI 1640 (ICN Biomedical, Costa Mesa, CA) with penicillin (60 μg/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 20 mM HEPES. The IgG fractions were used at a final concentration of 1 mg/ml and serum at 12.5% (v/v) in the IFN-α induction cultures (see below).

Separation on dsDNA-cellulose columns

The sera were 0.45 μm filtered and diluted with equal volumes of 0.1 M Tris-HCl (pH 7.5) with 0.15 M NaCl. They were separated on dsDNA cellulose or uncoupled cellulose columns (Worthington, Freehold, NJ), using elution with 3 M $MgCl_2$ (28). The eluates were stabilized with 1 mg/ml human serum albumin (HSA; Pharmacia and Upjohn, Stockholm, Sweden). Both effluents and eluates were desalted and transferred to RPMI 1640 medium supplemented as described above using PD-10 columns (Amersham Pharmacia Biotech). The approximate final IgG concentrations used in the IFN-α induction cultures were 1 mg/ml for effluents and 5 μg/ml for eluates.

Herpes simplex virus

HSV type 1 was propagated in human WISH cells, grown in DMEM (ICN Biomedicals, Aurora, OH) supplemented as above and with 5% FCS (Myoclone, Life Technologies, Paisley, U.K.). The HSV at 2×10^7 PFU/ml was UV inactivated by 1 J at 254 nm (UV-HSV) and used at optimal concentrations (10× dilution) for induction of IFN-α.

Preparation of plasmids

The plasmid pcDNA3 (Invitrogen, San Diego, CA) was propagated in *Escherichia coli* (Epicurian Coli XL-1-Blue Supercompetent Cells, Stratagene, La Jolla, CA) and purified using EndoFree Plasmid Maxi or Mega kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The content of endotoxin in the plasmid preparations was estimated by the *Limulus* amebocyte lysate test (QCL-1000 test, BioWhittaker, Walkersville, MD), and a Detoxi-Gel column (Pierce, Rockford, IL) was used according to the manufacturer's instructions when further endotoxin removal was necessary. Plasmid preparations contained <0.1 endotoxin units/ml. The CpG dinucleotides in pcDNA3 were methylated using *Sss*I methylase according to the manufacturer's instruction (New England Biolabs, Beverly, MA). The enzyme was removed by phenol extraction, followed by repeated ethanol precipitations and resuspension in endofree TE-buffer (Qiagen). The methylation of CpG dinucleotides was verified by resistance to *Hpa*II digestion, and the endotoxin content was estimated as described above. The pcDNA3 was used at a final concentration of 0.5 μg/ml in the IFN-α induction cultures.

Cytokines and anti-DNA mAb

When indicated, the cultures were supplemented with the recombinant cytokines IFN-α2b (500 U/ml; Intron-A, Schering-Plough, Bloomfield, NJ), IFN-β1a (500 U/ml; Avonex, Biogen, Cambridge, MA), IFN-γ (500 U/ml; Genzyme, Cambridge, MA), GM-CSF (1 ng/ml; Leucomax, Schering-Plough), IL-4 (10 ng/ml; Genzyme), IL-10 (10 ng/ml; Genzyme), IL-12 (10 ng/ml; R&D Systems, Abingdon, U.K.), IL-13 (10 ng/ml; Pepro Tech EC, London, U.K.), or IL-18 (100 ng/ml; Pepro Tech EC). The indicated figures represent the final concentrations in the cultures. For each cytokine, 10-fold lower and higher concentrations were also tested.

The human anti-ss/dsDNA mAb MER-3 (Serotec, Oxford, U.K.) was dialyzed against RPMI 1640, supplemented as described above, and used at 2.5 μg/ml in the PBMC cultures.

Preparation and culture of PBMC for IFN-α induction

The PBMC were prepared by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation of buffy coats from normal blood donors. The cells were washed in PBS and frozen as described (29). The cells were thawed immediately before use, washed twice, and resuspended in RPMI 1640 supplemented as described above and with heat-inactivated FCS at 5% final concentration. Cultures were in triplicates using final volumes of 0.1 ml/well and a cell concentration of 2×10^6/ml in 96-well flat-bottom microtiter plates (Nunclon, Nunc, Roskilde, Denmark). For flow cytometry, 1-ml medium volumes per well in 24-well plates (Nunc) were used, containing 8×10^6 cells. Serum fractions, Abs, HSV, plasmid, and combinations of them were incubated for 30 min at room temperature in the culture plates, before the PBMC and indicated cytokines were added. The cultures were incubated for 5 h (flow cytometry) or 24 h (all other experiments) at 37°C and 7% CO_2.

Immunoassays

The levels of IFN-α in culture supernatants were determined by a dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) as described (24), with modifications. In brief, the mAb LT27:293 to human IFN-α was used for capture. This mAb recognizes most IFN-α subtypes, except the IFN-α2b often used for costimulation. Samples and standard were then coincubated with the europium-labeled LT27:297 anti-IFN-α mAb for 1 h at 37°C in the LT27:293-coated immunoplates. The lower limit of detection of this assay was 2 U leukocyte IFN-α per ml. For determination of IFN-α in serum, a more sensitive DELFIA (detection limit, ≥0.5 U/ml) was used (24). In this assay, mAbs LT27:293 and LT27:273 were used for capture. The IFN-α immunoassay standard was calibrated against the National Institutes of Health reference leukocyte IFN-α GA-23-902-530.

Serum levels of anti-dsDNA Abs were determined by an anti-dsDNA ELISA kit (Dako, Glostrup, Denmark) as recommended by the manufacturer. The levels of total IgG in serum were determined by a conventional ELISA, using rabbit anti-human Ig (Dako) for capture, peroxidase-labeled rabbit anti-human IgG (Dako) for detection, and human IgG (Jackson ImmunoResearch, West Grove, PA) as standard.

Analysis of cells by flow cytometry

The PBMC were stimulated for 5 h either by UV-HSV or by the combination pcDNA3 and protein G-purified IgG in the presence of costimulatory IFN-α2b and GM-CSF. The cells were then fixed in paraformaldehyde and stained for intracellular IFN-α as described (30). Briefly, the fixed PBMC were permeabilized with Tween 20 and subsequently incubated with the biotinylated anti-IFN-α mAb LT27:295 and finally with PE-conjugated streptavidin (Jackson ImmunoResearch). The frequency, staining intensity, as well as light scatter characteristics of the IFN-α-containing cells were analyzed with a FACScan flow cytometer and Cellquest software (Becton Dickinson, San Jose, CA).

Statistical analysis

Data are expressed as means ± SD. The significance of differences between groups was determined by ANOVA in combination with Fischer's protected least significance difference. Correlation analysis was performed with the Spearman rank correlation coefficient. The StatView 5.0 program was used, taking $p < 0.05$ as minimum significant level.

Results

IFN-α production by PBMC caused by SLE sera correlates to anti-dsDNA Abs and is enhanced by plasmid DNA

Normal PBMC were cultured for 24 h with sera from nine SLE patients and four normal individuals, and the IFN-α produced was measured by immunoassay. As described before (25), the SLE sera but not control sera caused production of IFN-α (Table I). Furthermore, addition of recombinant IFN-α2b to the PBMC cultures markedly increased the IFN-α production induced by SLE sera and caused the appearance of low levels of IFN-α in cultures with control sera. The levels of IFN-α, anti-dsDNA Abs, and IgG were determined for the individual sera (Table I), and the results indicate that IFN-α- and anti-DNA Ab-positive sera were the most

Table I. *IFN-α-inducing capacity of sera from SLE patients in the presence or absence of pcDNA3, in relation to serum levels of IFN-α, anti-dsDNA Abs, and total IgG*

	Serum concentrations of[a]			Induced IFN-α (U/ml)[b]			
				Without pcDNA3		With pcDNA3	
Serum	IFN-α (U/ml)	Anti-dsDNA (U/ml)	Total IgG (mg/ml)	Without priming	With priming	Without priming	With priming
SLE 1	12	112	5	35 ± 12	444 ± 75	2454 ± 147	3802 ± 110
SLE 2	13	239	25	306 ± 143	1034 ± 82	5250 ± 592	6310 ± 327
SLE 3	7	2326	18	511 ± 54	1661 ± 57	6250 ± 318	7600 ± 607
SLE 4	19	35	8	11 ± 5	182 ± 14	548 ± 104	1074 ± 101
SLE 5	<1	7	2	13 ± 7	128 ± 21	<2	254 ± 54
SLE 6	<1	9	23	<2	126 ± 27	52 ± 43	378 ± 67
SLE 7	<1	12	10	3 ± 4	54 ± 24	18 ± 18	77 ± 19
SLE 8	<1	10	7	<2	27 ± 5	<2	35 ± 10
SLE 9	<1	11	6	6 ± 6	271 ± 53	<2	242 ± 41
NS 1	<1	7	6	<2	18 ± 11	<2	22 ± 16
NS 2	<1	5	7	<2	4 ± 4	<2	20 ± 10
NS 3	<1	3	6	<2	6 ± 6	<2	31 ± 30
NS 4	<1	5	8	<2	9 ± 12	<2	19 ± 12
Medium				<2	<2	<2	<2

[a] The concentrations of IFN-α, anti-dsDNA Abs, and total IgG were determined in nine SLE sera (SLE 1–9) and four normal sera (NS 1–4) by immunoassays.
[b] PBMC of a normal blood donor were cultured in triplicates for 24 h with indicated sera (12.5%), with and without IFN-α2b (500 U/ml) priming and with and without the immunostimulatory plasmid pcDNA3 (0.5 μg/ml). Concentrations of IFN-α in culture supernatants (mean ± SD) were determined by immunoassays. Data from one of two experiments with similar results are shown.

potent IFN-α inducers. In fact, the capacity to induce IFN-α production showed a positive correlation to the serum concentration of anti-dsDNA Abs ($r = 0.8$ both with and without IFN-α2b costimulation; $p = 0.006$ and $p = 0.005$, respectively), but not to the serum concentration of IgG ($r = 0.4$ with and $r = 0.1$ without costimulation).

An important part of the SLE-IIF in serum appears to be DNA (25), possibly in the form of isDNA. The addition of the plasmid pcDNA3, known to contain isDNA motifs (31), greatly enhanced the IFN-α production in those PBMC cultures containing SLE serum with a significant level of anti-DNA Abs (Table I). In contrast, little or no IFN-α production was seen with the SLE sera that had low concentrations of anti-DNA Abs and with the control sera. Costimulation with IFN-α2b further increased the IFN-α production caused by plasmid addition, although this increase was proportionally smaller with the anti-DNA Ab-containing SLE sera.

Induction of IFN-α production in PBMC by SLE-IgG and pcDNA3

To further explore the nature of the SLE-IIF, we treated the sera from the nine SLE patients and four normal individuals with DNase I. As previously reported, this destroys their IFN-α-inducing activity (25). The IgG prepared from these treated sera by protein G separation completely lacked IFN-α-inducing capacity in PBMC cultures (Table II). However, in the presence of costimulatory IFN-α2b, significant levels of IFN-α were induced by especially the three SLE-IgG preparations with high levels of anti-DNA Abs, but not by the control IgG.

Table II. *Production of IFN-α by normal PBMC cultured with pcDNA3 in combination with IgG prepared from SLE or control serum*

	Induced IFN-α (U/ml)[b]					
	Without priming			With priming		
Serum IgG[a]	No pcDNA3	pcDNA3	Meth. pcDNA3	No pcDNA3	pcDNA3	Meth. pcDNA3
SLE 1	<2	1504 ± 527	<2	186 ± 16	5143 ± 687	111 ± 34
SLE 2	<2	220 ± 18	<2	255 ± 17	2322 ± 248	121 ± 10
SLE 3	<2	1296 ± 320	<2	171 ± 30	4792 ± 467	124 ± 62
SLE 4	<2	153 ± 27	<2	11 ± 5	2072 ± 145	35 ± 26
SLE 5	<2	<2	<2	11 ± 13	44 ± 26	15 ± 8
SLE 6	<2	3 ± 1	<2	2 ± 5	37 ± 14	11 ± 5
SLE 7	<2	3 ± 4	<2	15 ± 9	189 ± 102	19 ± 6
SLE 8	<2	6 ± 2	<2	5 ± 2	320 ± 27	14 ± 10
SLE 9	<2	3 ± 11	<2	18 ± 11	248 ± 77	18 ± 12
NS 1	<2	<2	<2	<2	95 ± 17	<2
NS 2	<2	3 ± 1	<2	<2	128 ± 25	<2
NS 3	<2	<2	<2	<2	<2	<2
NS 4	<2	<2	<2	<2	169 ± 15	<2
Medium	<2	<2	<2	<2	2 ± 4	<2

[a] The IgG was obtained by DNAse I treatment of serum obtained from nine SLE patients (SLE 1–9) and four normal individuals (NS 1–4), followed by separation on protein G-Sepharose columns.
[b] The PBMC were cultured for 24 h with serum IgG (1 mg/ml) combined or not with the normal or methylated (meth.) plasmid pcDNA3 (0.5 μg/ml), and with or without IFN-α priming (500 U/ml). The cultures were in triplicates and concentrations of induced IFN-α in culture supernatants (mean ± SD) were determined by immunoassay. Results from one of two experiments with similar results are shown.

Table III. *Importance of anti-DNA Abs in the IFN-α-inducing capacity of SLE serum*[a]

Expt.	Serum	Fraction	Concentration During Induction		Induced IFN-α (U/ml)[b]			
					Without priming		With priming	
			IgG (mg/ml)	Anti-dsDNA Ab (U/ml)	No pcDNA3	pcDNA3	No pcDNA3	pcDNA3
I	SLE 1	Unseparated serum	0.6	9	14 ± 7	997 ± 181	148 ± 8	1294 ± 38
	SLE 1	Protein G eluate	1	11	<2	333 ± 46	236 ± 84	1629 ± 446
	SLE 1	dsDNA-cellulose effluent	1	1.5	<2	18 ± 4	15 ± 9	90 ± 32
	SLE 1	dsDNA-cellulose eluate	0.066	44	<2	725 ± 37	419 ± 62	1959 ± 94
	NS 3	Unseparated serum	0.5	1	<2	<2	13 ± 1	9 ± 5
	NS 3	Protein G eluate	1	<0.5	<2	<2	<2	<2
	NS 3	dsDNA-cellulose effluent	1.5	<1	<2	<2	<2	<2
	NS 3	dsDNA-cellulose eluate	0.003	<3.5	<2	<2	<2	10 ± 10
		Medium			<2	<2	<2	<2
II	SLE 1	Unseparated serum	1	10	3 ± 3	657 ± 102	97 ± 17	724 ± 118
	SLE 1	Protein G eluate	1	10	<2	244 ± 15	95 ± 33	2036 ± 166
	SLE 1	Control cellulose effluent	1	7	10 ± 5	651 ± 81	116 ± 6	795 ± 95
	SLE 1	Control cellulose eluate	0.006	<3.5	<2	<2	3 ± 1	<2
		Medium			<2	<2	<2	<2
III	SLE 1	Protein G eluate	1	11	<2	1452 ± 289	111 ± 31	5930 ± 333
	NS 3	Protein G eluate	1		<2	<2	4 ± 1	20 ± 6
		mAb anti-ss/dsDNA	0.0025	1.3	<2	252 ± 62	5 ± 7	1156 ± 67
		Medium			<2	<2	<2	<2

[a] Sera obtained from one SLE patient (SLE 1) and one normal blood donor (NS 3) were separated on dsDNA cellulose columns. Separations on control (uncoupled) cellulose columns and DNase I treatment followed by separation on protein G-Sepharose columns were included as controls. The total IgG yields on the cellulose columns were ~95%, and the proportion retained and eluted <0.07%. Similar results were obtained with two more SLE sera and control sera.

[b] Normal PBMC were cultured with indicated concentrations of serum or serum fractions or a human anti-ss/dsDNA mAb, combined or not with pcDNA3 (0.5 μg/ml), with or without IFN-α2b (500 U/ml) priming. Cultures were established in triplicate, and concentrations of IFN-α in 24-h culture supernatants (mean ± SD) were determined by immunoassay. Data from one of two PBMC donors with similar results are shown.

Addition of the plasmid pcDNA3 alone to the PBMC cultures did not induce IFN-α production, even in the presence of costimulatory IFN-α2b (Table II). In contrast, pcDNA3 caused high levels of IFN-α in the presence of the four SLE-IgG preparations with significant levels of anti-DNA Abs (>20 U/ml). In the presence of costimulatory IFN-α2b, these four SLE-Ig preparations, together with pcDNA3, caused a very high IFN-α production, in the order of 2000–5000 U/ml. The remaining SLE-IgG and the control IgG preparations gave IFN-α levels that were much lower. Finally, methylation of cytosines in the CpG motifs of pcDNA3 completely inhibited the IFN-α induction by the plasmid.

Consequently, we have indications that immunostimulatory plasmid DNA can replace the DNA believed to be part of SLE-IIF in serum. Furthermore, IgG appears to convert such plasmid DNA into a potent IFN-α inducer in PBMC, especially when these cells are costimulated by IFN-α2b.

Anti-DNA Abs are essential in the IFN-α induction

The finding that pcDNA3 could reconstitute DNase I treated SLE-IIF, as well as the correlation between SLE-IIF activity and serum anti-dsDNA Ab levels, suggested that anti-dsDNA Abs could be part of SLE-IIF. This was tested by separating serum from SLE patients with anti-dsDNA Abs on dsDNA-cellulose columns and on control uncoupled cellulose columns. Normal serum was used as control in the dsDNA cellulose separations. Using the dsDNA cellulose column, >95% of applied IgG was recovered in the effluent and <0.07% in the eluate, the latter containing most of the anti-DNA Abs (Table III).

The various fractions, as well as unseparated sera and protein G-purified IgG, were tested for IFN-α-inducing capacity in cultures of normal PBMC. It was found that passage of SLE serum over dsDNA-cellulose, but not control uncoupled cellulose, markedly reduced the IFN-α induction caused by pcDNA3, regardless of whether costimulatory IFN-α2b was present or not (Table III). When tested alone, the anti-dsDNA Ab-containing eluate from SLE-serum separated on dsDNA cellulose induced no IFN-α, but relatively high levels of IFN-α were seen in the presence of costimulatory IFN-α2b (Table III, 419 U/ml). The further addition of pcDNA3 to this eluate caused high IFN-α production in the PBMC, which was further increased in the presence of costimulatory IFN-α2b. In general, the IFN-α-inducing capacity of purified anti-DNA Abs appeared similar to or higher than that of serum or purified IgG (Table III). Importantly, pcDNA3 combined with a human mAb reactive against ss/dsDNA was able to induce IFN-α production, which was further enhanced by costimulatory IFN-α2b.

Direct comparison between the concentration (units per milliliter) of anti-dsDNA Abs in the dsDNA-cellulose eluate or in the protein G-purified IgG and their IFN-α-inducing capacity indicated that the dsDNA-cellulose-purified preparation was generally more active (Fig. 1). This could at least in part be due to a decline of IFN-α-inducing capacity at higher concentrations of the protein G-purified IgG. In particular, the purified anti-DNA Abs induced considerable IFN-α production in a dose-dependent manner when costimulated by IFN-α2b, and this was greatly increased in the presence of pcDNA3 (Fig. 1). Finally, the dsDNA-cellulose itself did not appear to contribute to the results because the eluates or effluents from normal serum separated on dsDNA-cellulose induced no IFN-α production when combined with pcDNA3 and costimulatory IFN-α2b (Table III).

Effects of cytokines on the IFN-α-inducing capacity of SLE serum or the combination SLE-IgG and pcDNA3

Because recombinant IFN-α2b in most cases had a marked stimulatory effect on the IFN-α production induced in PBMC cultures by SLE sera and by SLE-IgG alone or in combination with pcDNA3, it was of interest to examine the effects of other cytokines. Normal PBMC were therefore stimulated by SLE serum (SLE 1), the combination of SLE-IgG (SLE 1) and pcDNA3, or

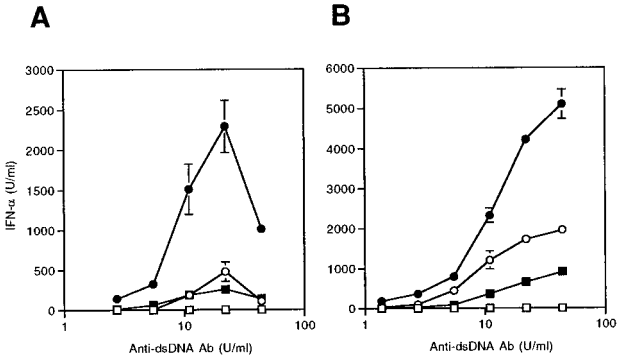

FIGURE 1. Production of IFN-α by normal PBMC in relation to the concentration of anti-dsDNA Abs (units per milliliter) in cultures receiving either IgG purified on protein G (*A*) or anti-DNA Abs purified on dsDNA cellulose (*B*), both from donor SLE1. The cultures further received the plasmid pcDNA3 (0.5 μg/ml; ●, ○) or not (□, ■), with costimulatory IFN-α2b (500 U/ml; ●, ■) or not (○, □). Cultures were done in triplicate, and the IFN-α concentrations (units per milliliter; mean ± SD) in 24-h culture media were measured by DELFIA.

UV-inactivated HSV. As a control, PBMC were cultured with normal serum (NS), medium with or without pcDNA3, or NS-IgG with or without pcDNA3. The PBMC cultures further contained costimulatory IFN-α2b or not, as well as one of the cytokines IFN-β, IFN-γ, GM-CSF, IL-4, IL-10, IL-12, IL-13, or IL-18.

With SLE serum alone as inducer, IFN-α2b, IFN-β, and GM-CSF had significant ($p < 0.001$) stimulatory effects on the IFN-α production (Fig. 2*A*). The apparent inhibitory effect of IL-10 (Fig. 2*A*) was not statistically significant. With the combination of SLE serum and costimulatory IFN-α2b, IFN-β had no additional stimulatory effects, whereas GM-CSF further increased the IFN-α production in at least an additive manner ($p < 0.001$). The IL-10 here markedly inhibited the IFN-α production ($p < 0.001$).

When the combination of SLE-IgG and pcDNA was used as inducer, IFN-α2b ($p = 0.003$) and IFN-β ($p < 0.001$) enhanced the IFN-α production, whereas IL-10 was inhibitory ($p < 0.001$), both with and without IFN-α2b (Fig. 2*B*). The GM-CSF caused some increase in the IFN-α production in the presence of IFN-α2b ($p < 0.003$). When UV-HSV was used as inducer, a small stimulatory effect was obtained only with the combination of GM-CSF and IFN-α2b ($p < 0.001$), but a clear inhibition of the IFN-α production ($p < 0.001$) was seen with IL-10 in both the presence and the absence of costimulatory IFN-α2b (Fig. 2*C*). The cytokines IFN-γ, IL-4, IL-12, IL-13, and IL-18 had no significant effects on any of the three inducers (Fig. 2). Normal serum or pcDNA3 alone, as well as NS-IgG with or without pcDNA3, induced no IFN-α production in the presence or absence of the tested cytokines (results not shown).

Characterization of the IFN-α-producing cells induced by SLE-IgG combined with pcDNA3

A previous study demonstrated that the IFN-α-producing cells induced by SLE-IIF or by HSV had apparently identical antigenic phenotypes and a characteristic frequency and light scatter (25). We examined whether also the IFN-α-producing cells induced by the combination SLE-IgG and pcDNA3 resembled NIPC, by stimulating normal PBMC with SLE-IgG (SLE 1) and pcDNA3 or with UV-HSV in the presence of costimulatory IFN-α2b and GM-CSF. After 5 h, the cells were stained for intracellular IFN-α and analyzed by flow cytometry. The IFN-α-containing cells were gated and with both inducers consisted of a brightly fluorescent population (Fig. 3*A*), with a frequency among all PBMC of 0.04% for the IgG-pcDNA3 and 0.12% for the HSV inducer. These frequencies were proportional to the IFN-α concentrations in culture medium at 5 h that were 190 and 556 U/ml. The scatter characteristics of the IFN-α-positive cells were the same for the two inducers (Fig. 3*B*), corresponding to a cell population in between lymphocytes and monocytes (Fig. 3*C*).

Discussion

We have recently identified a factor in serum of SLE patients, consisting of IgG and DNA, that induces IFN-α production in normal PBMC in vitro (25). Several findings in the present study

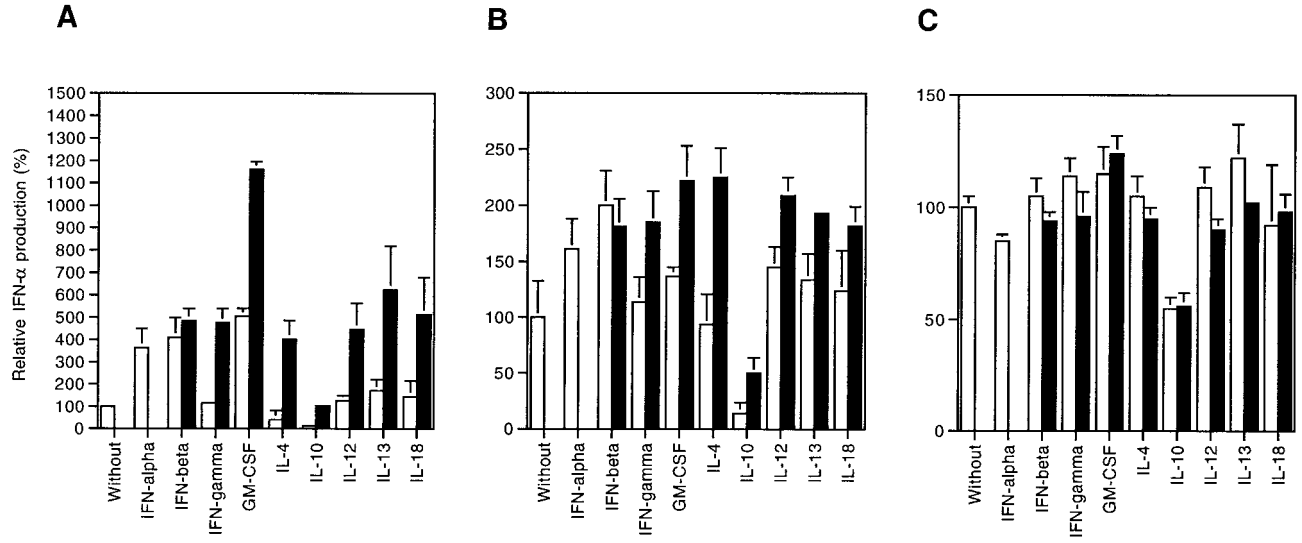

FIGURE 2. Effect of different cytokines on the production of IFN-α in cultures of normal PBMC induced by SLE serum (*A*), SLE-IgG combined with pcDNA3 (*B*), or UV-HSV (*C*). Cultures established in triplicate were supplemented with 500 U/ml IFN-α2b (■) or not (□), and one of the cytokines IFN-β (500 U/ml), IFN-γ (500 U/ml), GM-CSF (1 ng/ml), IL-4 (10 ng/ml), IL-10 (10 ng/ml), IL-12 (10 ng/ml), IL-13 (10 ng/ml), or IL-18 (100 ng/ml). The IFN-α concentrations in 24-h culture media were measured by DELFIA and expressed as percent of the IFN-α concentrations in the control cultures without added cytokines (mean ± SD). The actual control values were 92 U/ml (*A*), 441 U/ml (*B*), and 6724 U/ml (*C*). The serum donor was patient SLE1. Representative results from one of two PBMC donors.

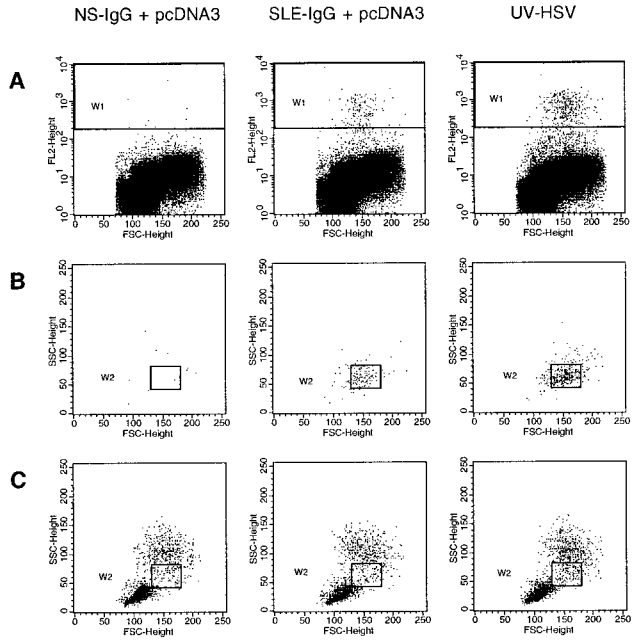

FIGURE 3. Light scatter characteristics of the IFN-α-producing cells induced by SLE-IgG combined with pcDNA3. Normal PBMC were cultured for 5 h with UV-HSV or with IgG prepared from either NS or SLE serum in combination with pcDNA3. All cultures were supplemented with costimulatory IFN-α2b (500 U/ml) and GM-CSF (1 ng/ml). The cells were then stained for intracellular IFN-α (PE) and analyzed by flow cytometry. The forward light scatter (FSC)/fluorescence (FL2) distribution of all PBMC are shown (*A*), the IFN-α containing cells being gated in window W1. The forward light scatter/side light scatter (SSC) of the cells in W1 is shown in *B,* with the small window W2 defining the typical scatter of natural IPC. *C,* position of W2 among all PBMC. Representative results from one of two PBMC donors.

indicate that anti-DNA Abs are an essential part of this IFN-α-inducing factor (SLE-IIF). Thus, in vitro SLE-IIF activity correlated to serum concentrations of anti-dsDNA Abs, and the SLE-IIF activity was retained by, and could partially be eluted from, a dsDNA-cellulose column. Furthermore, the IFN-α-inducing capacity of SLE serum, SLE-IgG, and eluted anti-dsDNA Abs was greatly increased when combined with plasmid pcDNA3. Finally, a human mAb against ss/dsDNA combined with pcDNA3 induced IFN-α production in PBMC. We therefore conclude that we have identified a new action of anti-DNA Abs, induction of IFN-α production when combined with the appropriate kind of DNA.

We found that unmethylated but not methylated pcDNA3, when combined with anti-dsDNA Abs, could induce IFN-α production in PBMC. Importantly, the methylation does not influence the binding of anti-dsDNA Abs to the plasmid (our unpublished results). This indicates the importance of immunostimulatory (is) DNA sequences with unmethylated CpGs in the plasmid, previously shown to have IFN-α-inducing ability (31–34). The fact that the SLE-IIF is sensitive to DNase I but not RNases (25) and our present finding that it could be replaced by unmethylated plasmid DNA suggest but do not prove the presence of isDNA in SLE-IIF. It is, however, interesting that isDNA sequences in the serum of SLE patients have been identified by molecular cloning (35–37), that the excess DNA present in immune complexes in SLE is hypomethylated, and that isDNA have been suggested to have a pathogenic role in SLE (38). Such DNA could be of either microbial or cellular origin, the latter possibility supported by observations of methylation defects and increased apoptosis in SLE (reviewed in Ref. 38). To our knowledge, biologically active unmethylated isDNA has, however, not been directly isolated and characterized in SLE patients. Such work is highly warranted, as is the identification of the origin of such DNA. We are now examining the hypothesis that it consists of small hypomethylated isDNA sequences derived from apoptotic cells, possibly in the form of nucleosomes.

It has previously been shown that isDNA can induce IFN-$\alpha\beta$ production in PBMC preferentially when combined with lipofectin (39). The mechanism whereby anti-DNA Abs convert plasmid isDNA to a potent IFN-α inducer could therefore also involve a lipofectin-like transfecting effect. In fact, certain anti-DNA Abs can penetrate the cell membrane and finally localize to the nucleus (40–42). Such Abs can also transport larger proteins into cells (43), conceivably also DNA or DNA-protein complexes such as nucleosomes. Indeed, enhanced internalization of nucleosomes in cells by Abs to DNA or histones has been reported (44). We therefore suggest that the SLE-IIF present in the serum of SLE patients at least consists of complexes between anti-dsDNA Abs and isDNA and that such complexes are able to traverse cell membranes and by means of isDNA trigger IFN-α production. It remains, however, to be determined whether additional important components are present in SLE-IIF, such as Abs with other specificities and complement factors. Furthermore, as discussed above, the DNA component must be directly identified.

The combination of anti-DNA Abs and plasmid pcDNA3 was the most efficient IFN-α inducer in PBMC cultures. However, several DNase I-treated IgG preparations from normal donors and from patients with inactive SLE had a low but significant IFN-α-inducing activity together with pcDNA3, provided the PBMC were costimulated by IFN-α2b. It is possible that this is due to the presence of low concentrations of anti-DNA Abs that are masked in whole serum (45, 46). We also noted that whereas anti-DNA Abs purified on dsDNA-cellulose or SLE-IgG alone failed to cause IFN-α production in normal PBMC, costimulation of PBMC with IFN-α2b resulted in a significant production of IFN-α. Obviously, this could be due to the presence of residual isDNA bound to the anti-DNA Abs. Alternatively, some isDNA could actually emanate from dying cells in the PBMC cultures, an interesting possibility which is being explored.

The cytokine IFN-α2b markedly enhanced the IFN-α production by PBMC cocultured with serum, IgG, or separated anti-DNA Abs, combined or not with pcDNA3. In some cases, IFN-α2b costimulation actually appeared essential. When the effects of a selected number of cytokines on the IFN-α-inducing capacity of SLE serum were examined, only IFN-$\alpha\beta$ and GM-CSF were found to be clearly stimulatory, and their effects were also additive. The results agree with those previously obtained using glutaraldehyde-fixed HSV-infected WISH cells as IFN-α inducer (47), but not using the UV-HSV in the present study. Furthermore, the IFN-α production induced by SLE-IgG combined with pcDNA3 was only moderately enhanced by type I IFN, and not clearly by GM-CSF. The reason could be that the inducers UV-HSV and plasmid pcDNA3 themselves might initiate sufficient production of costimulatory cytokines in the PBMC cultures. The observed costimulatory effects of IFN-α and GM-CSF could be important also in vivo and in this way could be of pathogenic significance in SLE. For instance, a transient production of IFN-$\alpha\beta$ and GM-CSF caused by infections in patients with inactive SLE may precipitate a more prolonged and systemic production of IFN-α driven by the SLE-IIF and thereby activate the disease.

The costimulatory effect of type I IFN on the production of IFN-α induced by SLE-IIF is most likely related to a phenomenon termed priming, mainly studied with viral IFN inducers (3). In fact, such priming may under certain conditions be essential for IFN-$\alpha\beta$

gene expression (48). The priming is considered to be caused by up-regulated synthesis and/or activation of transcription factors such as STAT1, STAT2, p48 protein, IRF-1, and IRF-3 that are important in the activation of type I IFN gene expression (22, 23, 49–51). The GM-CSF may have a similar mode of action, because it can activate certain transcription factors in dendritic cells (52), that may enhance type I IFN gene expression.

The cytokines IFN-γ, IL-4, IL-12, IL-13, and IL-18 had no clear effects on any of the IFN-α inducers in the present study. In contrast, IL-10 strongly inhibited the IFN-α production caused by all studied inducers, especially that induced by SLE serum and the combination of SLE-IgG and pcDNA3. These results extend previous findings that IL-10 inhibit virus-induced IFN-α production in human PBMC (53). Because IL-10 also inhibits production of cytokines such as IL-1β, IL-6, IL-8, TNF-α, GM-CSF, and granulocyte-CSF (54–56), the strong inhibitory effects in our study are probably due to a general inhibition of production of cytokines, including costimulatory cytokines. Increased levels of IL-10 have been reported in SLE patients and suggested to have a pathogenic role (57–59), for instance by direct stimulation of autoimmune B cells (58) or induction of apoptosis in activated T cells (60). Because type I IFN can enhance production of IL-10 (61, 62), the continual IFN-α production in SLE could in fact be one cause of the overproduction of IL-10 in this disease. As indicated by the results of the present study, IL-10 could also perhaps have a paradoxical beneficial effect in SLE patients by inhibiting the IFN-α production.

The combination of SLE-IgG and pcDNA3 induced IFN-α production in infrequent cells among PBMC with the same light scatter characteristics and high IFN-α production per cell as the natural IPC previously shown to respond to SLE serum (25), bacteria (63), and several different types of virus (30, 64, 65). The phenotypes of these cells (25, 30) indicate that they are immature dendritic cells that for instance do not express the costimulatory molecules CD80 and CD86, or perhaps less likely represent a new type of leukocyte. The same cells are markedly decreased in number in the blood of SLE patients (24), but it remains to be clarified whether they are the source of the IFN-α also in vivo and whether they are localized to tissues, such as lymphoid organs.

We previously suggested that the prolonged production of IFN-α in vivo in SLE patients is the result of stimulation by SLE-IIF and could be of pathogenic significance and pivotal in breaking tolerance to nucleic acids and associated proteins (25). That possibility is suggested by the various immunomodulatory actions of type I IFN, including promotion of expression of the IL-12 receptor and survival of activated T cells, which would counteract maintenance of self tolerance (3, 12–16). It is more directly supported by the wide range of autoantibodies and autoimmune diseases appearing in patients treated with IFN-α (17–20). An increased rate of apoptosis caused in, for instance, T cells by IL-10 (60) and in virus-infected cells by type I IFN (66) would generate relevant nuclear autoantigens and perhaps endogenous isDNA, the latter forming IFN-α-inducing complexes with anti-DNA Abs. It is also interesting that type I IFN production and autoantigen presentation occur in similar if not identical dendritic cells, which should facilitate the autoimmunization process. Our results further suggest that costimulation with at least type I IFN and GM-CSF is important for the induction of IFN-α production. It is therefore possible that an initial period of type I IFN production, for instance caused by viral infections, is required for both autoimmunization and initiating the subsequent IFN-α production caused by IFN-α-inducing immune complexes. A mechanism resembling a vicious circle may subsequently sustain the disease process by means of continuous IFN-α production, occasionally boosted by infections or other processes that generate more autoantibodies, costimulatory cytokines, and isDNA.

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