Interleukin 6 activates heat-shock protein 90β gene expression

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The levels of the cytokine interleukin-6 (IL-6) and the heat-shock protein hsp90 have both been reported to be elevated in patients with active systemic lupus erythematosus (SLE). We show that hsp90 protein accumulates to increased levels in both HuH7 hepatoma cells and peripheral blood mononuclear cells (PBMCs) treated with IL-6. In PBMCs this effect occurs without induction of the other hsps, paralleling the specific elevation of hsp90 in SLE. IL-6 is able to activate the hsp90 gene promoter directly; this activation can also be achieved by overexpressing either of the transcription factors NF-IL-6 or NF-IL-6β whose synthesis is induced by IL-6 treatment. Hence the induction of hsp90 protein accumulation by IL-6 is likely to be dependent on the enhanced activity of the hsp90β gene promoter produced by increased levels of NF-IL-6 and/or NF-IL-6β. These effects are discussed in terms of the role of hsp90 in the normal immune system and the mechanism of its activation in patients with SLE.

INTRODUCTION

Interleukin 6 (IL-6) is a multifunctional cytokine with many different effects on specific cell types, including the stimulation of B-cell differentiation and immunoglobulin secretion, T-cell differentiation and/or activation, and production of acute-phase proteins by the liver (reviewed in [1]). These effects are produced via the IL-6-induced activation of specific transcription factors such as NF-IL-6, NF-IL-6β and STAT-3 (APRF), which then bind to specific binding sites in the regulatory regions of target genes and activate their expression [2,3].

Interestingly, elevated levels of circulating IL-6 have been reported in a number of different autoimmune diseases such as rheumatoid arthritis [4], juvenile chronic arthritis [5] and systemic lupus erythematosis (SLE) [6], and the levels have been shown to be correlated with disease activity, being highest in patients with active disease. Moreover, spontaneous production of IgG by normal and SLE-derived B lymphocytes in culture can be enhanced by the addition of exogenous IL-6 and inhibited by antibody to IL-6 [6,7]. These findings therefore suggest that IL-6 might play a role in the pathogenesis of autoimmune diseases. In agreement with this idea, infusion of antibody to IL-6 can relieve disease symptoms in lupus-prone NZB/NZW F1 mice [8].

A role for IL-6 in disease pathogenesis is likely to involve the induction of the expression of specific genes within its target cells. We have shown previously that the 90 kDa heat-shock protein (hsp90) is expressed at elevated levels in peripheral-blood mononuclear cells (PBMCs) from a specific subset of SLE patients and that such overexpression correlates with disease activity in some organs or systems [9–11]. Overexpression of hsp90 has been shown to be dependent on the enhanced transcription of the hsp90β gene in these patients, whereas transcription of the hsp90α gene is unchanged [12]. Moreover, overexpression is also observed in lupus-prone MRL/1pr mice, where it precedes the onset of disease [13].

To elucidate the role of IL-6 in the activation of hsp90 in SLE patients we have studied the effect of IL-6 on hsp90 protein levels and on the hsp90β gene promoter.

MATERIALS AND METHODS

Patient samples and cell cultures

PBMCs were isolated by Ficol-paque (Pharmacia). Briefly, PBMCs were cultured at 10^6 per well in RPMI-1640 plus 5% (v/v) fetal calf serum in six-well plates (Nunc, Uxbridge, U.K.) at 37 °C in a 5% CO_2 incubator. PBMCs were treated with human IL-6 (Sigma, Poole, Dorset, U.K.) at the concentrations and time intervals indicated in the Figure legends. After recombinant (r)IL-6 treatment, cells were harvested for Western blot analysis. The human hepatoma cell line HuH7 (American Type Culture Collection, Rockville, MD, U.S.A.) was maintained in monolayer cultures in RPMI-1640 plus 10% (v/v) fetal calf serum. Before each experiment, cells were treated with trypsin and replated at a density of 10^6 per well in RPMI-1640 plus 5% (v/v) fetal calf serum in six-well plates with or without rIL-6 treatment. After treatment, cells were harvested for Western blot analysis, which was performed as described previously [10,11] with the AC88 antibody to hsp90 [14] and commercially available antibodies to the heat-inducible proteins hsp70 and hsp27 (Stressgen Biotechnologies, York, U.K.).

Plasmid constructs

The 5′ HSP90β promoter chloramphenicol acetyltransferase (CAT) constructs A and C were kindly provided by Neil Rebbe (Washington University School of Medicine, St. Louis, MO, U.S.A.) [15]. Construct A-HSP90β consisted of a fragment from -1044 to $+36$ relative to the transcriptional start site coupled to a CAT vector. Construct C-HSP90β consisted of a -299 to $+36$ fragment coupled to the CAT vector.

DNA transfection and CAT assay

Transfection of DNA was performed by the method of Gorman [16]. Transfection of HuH7 cells plated at a density of 10^6 per well in six-well plates was performed with the calcium phosphate method. Briefly, 10 μg of reporter plasmid and the indicated

Abbreviations used: CAT, chloramphenicol acetyltransferase; hsp, heat-shock protein; (r)IL-6, (recombinant) interleukin 6; PBMCs, peripheral-blood mononuclear cells; SLE, systemic lupus erythematosus.

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amounts of NF-IL-6 expression vectors were transfected for 4–6 h and the cells were osmotically shocked with 15% (v/v) glycerol in PBS for 2 min and washed in PBS.

The cells were re-fed with complete medium and harvested 48 h later. The cells were then lysed by three cycles of repeated freeze–thawing. To normalize for transfection efficiency, CAT activities were corrected for β-galactosidase activities. Assays of CAT activity were performed by the method of Gorman [16] on samples that had been equalized for protein content as determined by the method of Bradford [17].

RESULTS

To test whether hsp90 levels could be stimulated by IL-6, the IL-6-responsive HuH7 hepatoma cell line was treated with IL-6 and the levels of hsp90 were measured by Western blotting. As illustrated in Figure 1, a clear increase in hsp90 levels was observed in the IL-6-treated cells that was comparable with that observed in heat-shocked cells (results not shown). Interestingly, however, other heat-shock proteins, hsp70 and hsp27, were also induced in the IL-6-treated cells, suggesting that IL-6 might induce all the hsps.

Although these results indicated that IL-6 did indeed induce hsp90, the results differed from our observations in SLE, where hsp90 overexpression in patient PBMCs is not accompanied by the overexpression of other hsps [10,11]. To test whether this difference represented a cell-type-specific effect of IL-6, we added IL-6 to PBMC cultures from normal individuals. As shown in Figure 2 (upper panel), clear up-regulation of hsp90 expression was observed in these experiments. In contrast, however, no IL-6-induced up-regulation of hsp70 was observed in PBMCs, and the levels of hsp27 seemed to fall dramatically (Figure 2, lower panel). Hence in PBMCs, IL-6 does indeed specifically induce hsp90, but not the other hsps.

In our previous experiments [12], up-regulation of the hsp90 protein in SLE, patient PMBCs was dependent on enhanced transcription of the hsp90β gene. We therefore transfected HuH7 with a construct in which the hsp90β gene promoter drives expression of the readily assayable gene for CAT. In these experiments (Figure 3) we observed a dose-dependent activation of the hsp90β gene promoter by IL-6, indicating that the IL-6-induced up-regulation of hsp90 protein levels is likely to be mediated by activation of the corresponding gene promoter.

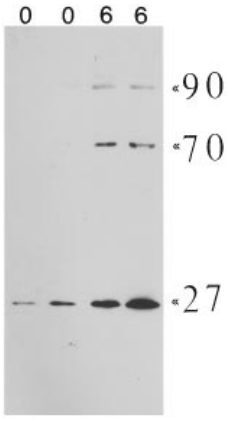

Figure 1 Western blot with the antibodies to hsp90, hsp70 and hsp27 and protein extracts prepared from either untreated (0) or HuH7 cells treated with 50 ng of IL-6 for 24 h (6)

The positions of hsp90, hsp70 and hsp27 are indicated.

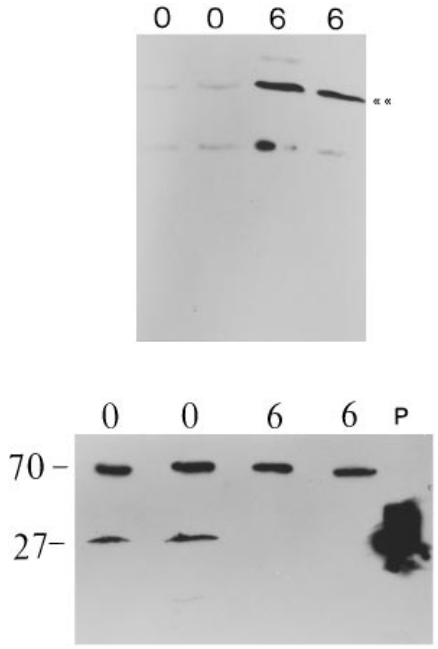

Figure 2 Western blots to measuere levels of hsp90, hsp70 and hsp27

Upper panel: Western blot to measure the levels of hsp90 in protein extracts from PBMCs either untreated (0) or treated with 50 ng/ml IL-6 (6) for 24 h. The arrows indicate the position of hsp90. Lower panel: Western blot to measure the levels of hsp70 and hsp27 in protein extracts from PBMCs either untreated (0) or treated with IL-6 (6). The positions of hsp70 and hsp27 are indicated. Lane P contained purified hsp27 protein.

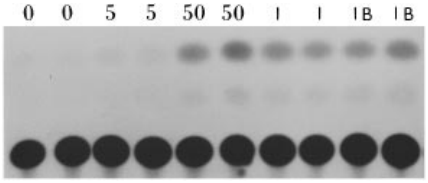

Figure 3 Assay of CAT activity in HuH7 cells transfected with a hsp90β gene reporter construct

CAT activity was measured in HuH7 cells transfected with a hsp90β gene reporter construct and either left untreated (0) or treated with 5 ng/ml (5) or 50 ng/ml (50) IL-6 for 24 h before harvesting, or co-transfected with expression vectors encoding NF-IL-6 (I) or NF-IL-6β (IB).

In many different cell types, IL-6-mediated gene activation is dependent on the increased activity of the transcription factors NF-IL-6 and NF-IL-6β [2,3]. We therefore determined whether overexpression of these factors in HuH7 cells could substitute for IL-6 in activating the hsp90β gene promoter. As illustrated in Figure 3, co-transfection of expression vectors encoding either of these factors with the hsp90β gene reporter construct (containing bases -1044 to $+36$ relative to the transcriptional start site) resulted in its activation to a level similar to that observed with IL-6. This finding therefore suggests that the activation of the hsp90β gene promoter by IL-6 is likely to be mediated by the increased levels of NF-IL-6 and NF-IL-6β in IL-6-treated cells.

Indeed this effect of NF-IL-6 and NF-IL-6β could even be observed in the ND7 neuronal cell line, which is not responsive to IL-6. Thus the hsp90β gene promoter was activated by co-transfection with NF-IL-6 and NF-IL-6β expression vectors in these cells (Figure 4, upper panel), and the degree of activation was greater than that observed on co-transfection with an

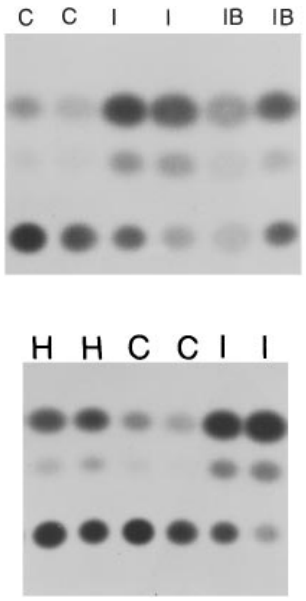

Figure 4 Assay of CAT activity in ND7 cells

Upper panel: assay of CAT activity in ND7 cells transfected with an hsp90β gene reporter construct together with either expression vector lacking any insert (C) or the same vector expressing NF-IL-6 (I) or NF-IL-6β (IB). Lower panel: assay of CAT activity in ND7 cells transfected with an hsp90β gene reporter construct together with either expression vector lacking any insert (C) or the same vector expressing NF-IL-6 (I) or HSF1 (H).

expression vector encoding the heat-shock factor HSF1, which mediates heat-inducibility of the hsp genes (Figure 4, lower panel).

All these experiments used a construct (A) containing the hsp90β gene promoter from -1044 to $+36$ bases relative to the transcriptional start site. To begin to map the region of the hsp90 promoter involved in these effects, we used a shorter reporter construct (C) containing only nucleotides from -299 to $+36$. This construct was not activated by co-transfection with NF-IL-6, although the full-length construct was activated as before (Figure 5). Hence the region from -1044 to -300 of the hsp90β gene promoter is required for its activation. In agreement with this idea, inspection of the DNA sequence in this region [15] revealed four potential binding sites for NF-IL-6 and NF-IL-6β.

To confirm that the region of the hsp90β promoter from -1044 to -300 also mediates its activation by IL-6 itself, we transfected the A and C constructs into HuH7 cells and determined their response to IL-6. As shown in Table 1, construct A was induced approx. 5-fold by IL-6, whereas construct C showed decreased activity after IL-6 treatment. Hence this region does indeed mediate the response to both IL-6 and NF-IL-6/NF-IL-6β. To investigate further the role of NF-IL-6/NF-IL-6β in this effect we synthesized an oligonucleotide containing the region of the hsp90β promoter from -643 to -623, which contains a binding site for these factors (TGCTGGAAAT). This oligonucleotide was cloned upstream of the thymidine kinase promoter in the vector pBLCat2 [18]. As shown in Table 1, this binding site rendered the thymidine kinase promoter inducible 4.5-fold by IL-6, whereas the parental promoter was unaffected. Hence a binding site in the hsp90 promoter for NF-IL-6 can indeed confer the observed response to IL-6.

DISCUSSION

The work presented here indicates that IL-6 can induce the accumulation of hsp90 in both the human hepatoma HuH7 cell line and in human PBMCs. At least in the HuH7 cell line this effect is mediated by IL-6 activation of the hsp90β gene promoter, which can also be produced in different cell types by the overexpression of the IL-6-induced transcription factors NF-IL-6 and NF-IL-6β. Moreover, the region of the promoter from -1044 to -300, which mediates the response to IL-6 itself, also mediates its activation by NF-IL-6, and a short region of the hsp90 promoter containing a binding site for these factors can confer responsiveness to IL-6 on a heterologous promoter. This strongly suggests that these factors play a critical role in the induction of the hsp90 promoter by IL-6. It should also be noted, however, that the region of the promoter from -1044 to -300 contains two potential binding sites for the STAT-3 transcription factor, which can also mediate IL-6-dependent gene activation [19]. It will thus be necessary to conduct further studies to determine whether STAT-3 also plays a role in hsp90 gene activation.

Although hsp90 was originally identified on the basis of its induction by elevated temperature and other stresses [20,21], its induction by other stimuli has also been reported. Thus for example the induction of hsp90 is observed when the U937 monocytic cell line is induced to differentiate to a more mature macrophage-like phenotype [22]. Similarly the activation of T cells by phorbol esters [23], mitogenic lectins [23,24], anti-CD3 [25] or interleukin 2 [26] also activates hsp90 synthesis. Moreover, at least for phorbol esters, such treatment results in preferential activation of the hsp90β gene rather than of the hsp90α gene, which is preferentially induced by heat shock [23].

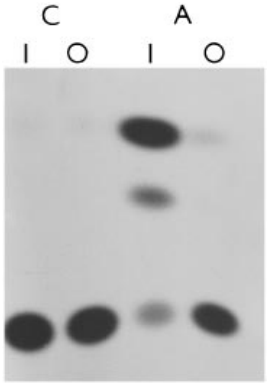

Figure 5 Assay of CAT activity in HuH7 cells transfected with the full-length hsp90β gene reporter construct A or the truncated construct C lacking the region from -1044 to -300 relative to the transcriptional start site

In each case the reporter construct was transfected with expression vector lacking any insert (O) or the same vector expressing NF-IL-6 (I).

Table 1 Effect of IL-6 on the activity of hsp promoter constructs

Values represent the CAT activity of each construct in HuH7 cells either untreated ($-$IL-6) or treated ($+$IL-6) with IL-6 for 24 h before harvesting and were obtained by scanning densitometry of assays of the type shown in Figures 3–5.

Construct	CAT activity (arbitrary units)	
	$-$IL-6	$+$IL-6
Hsp90A (-1044 to $+36$)	9.18	49.49
Hsp90C (-299 to $+36$)	3.39	1.80
pBLCat2 vector	1.00	1.00
pBLCat2 $+$hsp90 NF-IL-6 site (-643 to -623)	1.01	4.67

The induction of hsp90 by IL-6 is therefore likely to represent one aspect of its regulation by a number of agents, including cytokines, that modulate immune function. Hence, as well as their induction by stressful stimuli, which allow them to protect cells against stress, hsps would also play a role in normal immune functioning and would therefore be regulated by stimuli that control this system. Such a role would presumably involve the chaperoning function of the hsps in regulating the correct folding and transport of other proteins (reviewed in [26]). In agreement with this role, hsp90 or peptides derived from it have been found associated with class I MHC molecules on the cell surface [27,28].

A similar role for other hsps such as hsp70 in the functioning of the immune system has also been postulated. Thus a peptide containing the major T-cell epitope of cytochrome *c* has been shown to bind to hsp70 [29], whereas the Bip member of the hsp70 family plays a critical role in immunoglobulin assembly [30]. Indeed hsp70 expression is activated by many of the stimuli that activate hsp90 expression in the immune system such as differentiation of U937 cells [22] or T-cell activation due to mitogenic lectins [23,24], anti-CD3 [24] or interleukin 2 [25]. Similarly the induction of hsp70 by IL-6 has previously been reported in human leucocytes [31].

In agreement with this induction of hsp70 by IL-6 in some cell types, we demonstrated that both hsp90 and hsp70 are induced by treatment of the human hepatoma HuH7 cell line with IL-6. Interestingly, hsp27 was also induced by IL-6 in HuH7 cells, but not in PBMCs. Indeed a considerably decreased signal was seen in IL-6-treated PBMCs with the hsp27 antibody. As hsp27 is known to be phosphorylated after IL-6 treatment [32], this effect might reflect a decreased affinity of the antibody for phosphorylated hsp27. Thus the specific induction of hsp90 by treatment of PBMCs with IL-6 indicates that cell-type-specific differences in the response of hsps to IL-6 exist and suggests that hsp90 might have a specific function in IL-6-treated PBMCs, whereas hsp70 is not required. In this regard it is also of interest that differentiation of U937 cells also results in increased levels of NF-IL-6 [33].

Such specific induction of hsp90 by IL-6 parallels the situation in SLE, where we have identified a group of patients with an HLA A1, B8, DR3-negative haplotype who show elevated levels of hsp90 correlated with disease activity [10,11]. Moreover, SLE patients do not show elevated levels of other hsps such as hsp60 or ubiquitin [10,34,35]. Similarly the patients with elevated hsp90 do not exhibit elevation of hsp70, although this is observed in a small number of other SLE patients lacking the A1, B8, DR3-negative haplotype and not showing elevated levels of hsp90 [10,36].

Hence both the induction of hsp90 in PBMCs by IL-6 and the elevation of hsp90 in the PBMCs of a subset of SLE patients are specific to hsp90 compared with other phenomena that induce all the hsps. It is therefore possible that the elevation in IL-6 levels that is known to occur in SLE patients with active disease [6] is involved in inducing the observed increase in hsp90 levels. In turn, because this elevation in hsp90 is accompanied by the appearance of autoantibodies to the protein in both A1, B8, DR3-negative patients [37] and MRL/1pr mice [38], such induction of hsp90 by IL-6 could be involved in the pathogenic effect of IL-6 in these autoimmune diseases.

Further studies monitoring the levels of IL-6 in patents with and without elevation in hsp90 and their fluctuation with disease activity will be required to test this possibility. It is, however, already clear that IL-6 can induce hsp90 expression by activating the hsp90β gene promoter and that in PBMCs this effect occurs without induction of the other hsps.

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REFERENCES

1 Akira, S. and Kishimoto, T. (1992) Immunol. Rev. **127**, 25–45
2 Akira, S., Isshiki, H., Nakajima, T., Kinoshita, S., Nishio, Y., Natsuka, S. and Kishimoto, T. (1992) Ciba Found. Symp. **167**, 47–67
3 Taga, T. and Kishimoto, T. (1995) Curr. Opin. Immunol. **7**, 17–23
4 Eastgate, J. A., Symons, J. A., Wood, N. C., Grinlinton, F. M. O., Giovine, F. S. and Duff, G. W. (1988) Lancet **ii**, 706–709
5 De Benedetti, F., Massa, M., Robbion, R., Ravelli, A., Burgio, G. R. and Martini, A. (1991) Arthritis Rheum. **34**, 1158–1163
6 Linker-Israeli, M., Deans, R. J., Wallace, D. J., Prehn, J., Ozeri-Chen, T. and Klinenberg, J. R. (1991) J. Immunol. **147**, 117–123
7 Klashman, D. J., Martin, R. A., Martinex-Maza, O. and Stevens, R. H. (1991) Arthritis Rheum. **34**, 276–286
8 Finck, B. K., Chan, B. and Wofsy, D. (1994) J. Clin. Invest. **945**, 585–591
9 Norton, P. M., Isenberg, D. A. and Latchman, D. S. (1993) J. Autoimmun. **52**, 436–442
10 Dhillon, V. B., McCallum, S., Norton, P. M., Twomey, B. M., Erkeller-Yuksel, F., Lydyard, P., Isenberg, D. A. and Latchman, D. S. (1993) Ann. Rheum. Dis. **52**, 436–442
11 Dhillon, V. B., McCallum, S., Latchman, D. S. and Isenberg, D. A. (1994) Q. J. Med. **87**, 215–222
12 Twomey, B. M., Dhillon, V. B., McCallum, S., Isenberg, D. A. and Latchman, D. S. (1993) J. Autoimmun. **6**, 495–506
13 Faulds, G. B., Isenberg, D. A. and Latchman, D. S. (1994) J. Rheumatol. **21**, 234–238
14 Riehl, R. M., Sullivan, W. P., Vroman, B. T., Baver, V. J., Pearson, G. R. and Toft, D. O. (1985) Biochemistry **24**, 6586–6591
15 Rebbe, N. F., Ware, J., Bertina, R. M., Modrich, P. and Stafford, D. W. (1987) Gene **53**, 235–245
16 Gorman, C. M. (1985) in DNA Cloning: A Practical Approach (Glover, D. M., ed.), pp. 143–190, IRL Press, Oxford
17 Bradford, M. (1976) Anal. Biochem. **72**, 248–254
18 Luckow, B. and Schutz, G. (1987) Nucleic Acids Res. **15**, 5490
19 Wegenka, U. M., Latticken, C., Buschmann, J., Yuan, J., Lottspeich, F., Muller-Esterl, W., Schindler, C., Roeb, E., Heinrich, P. C. and Horn, F. (1994) Mol. Cell. Biol. **14**, 3186–3196
20 Lindquist, S. (1986) Annu. Rev. Biochem. **55**, 1151–1191
21 Lindquist, S. and Craig, E. A. (1988) Annu. Rev. Genet. **22**, 631–677
22 Twomey, B. M., McCallum, S., Isenberg, D. A. and Latchman, D. S. (1993) Clin. Exp. Immunol. **93**, 178–183
23 Hansen, L. K., Houchins, J. P. and O'Leary, J. J. (1991) Exp. Cell Res. **192**, 587–596
24 Have, R. N. and O'Leary, J. J. (1988) Exp. Cell Res. **179**, 65–78
25 Ferris, D. K., Harel-Bellan, A., Morimoto, R. I., Welch, W. J. and Farrar, W. L. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3850–3854
26 Ellis, R. J. (1990) Semin. Cell Biol. **1**, 1–72
27 Ferrarini, M., Heltai, S., Zocchi, M. F. and Rugarli, C. (1992) Int. J. Cancer **51**, 613–619
28 Jaradetzky, T. S., Lane, W. S., Robinson, R. A., Madden, D. R. and Wiley, D. C. (1991) Nature (London) **353**, 326–329
29 Vanbuskirk, A., Gump, B. L., Margoliash, E. P. and Pierce, S. K. (1989) J. Exp. Med. **170**, 1799–1809
30 Bole, D. G., Hendershot, L. M. and Kearney, J. F. (1986) J. Cell. Biol. **102**, 1558–1566
31 Koller, M., Hensler, T., Konig, B., Prevost, G., Aluuf, J. and Konig, W. (1993) Int. J. Med. Microbiol. Virol. Parisitol. Infect. Dis. **278**, 365–376
32 Belka, C., Ahlers, A., Sott, C., Gaestel, M., Herrman, F. and Bruch, M. A. (1995) Leukemia **9**, 288–294
33 Natsuka, S., Akira, S., Nishio, S., Hashimoto, S., Sugita, T., Isshiki, H. and Kishimoto, T. (1992) Blood **79**, 460–466
34 Twomey, B. M., Dhillon, V. B., McCallum, S., Isenberg, D. A. and Latchman, D. S. (1992) Autoimmunity **13**, 197–200
35 Dhillon, V. B., McCallum, S., Wilks, D., Twomey, B. M., Latchman, D. S. and Isenberg, D. A. (1993) Br. J. Rheumatol. **32**, 883–892
36 Twomey, B. M., Amin, V., Isenberg, D. A. and Latchman, D. S. (1993) Lupus **2**, 297–300
37 Conroy, S. E., Faulds, G. B., Williams, W., Latchman, D. S. and Isenberg, D. A. (1994) Br. J. Rheumatol. **33**, 923–926
38 Faulds, G. B., Conroy, S. E., Madaeo, M., Isenberg, D. A. and Latchman, D. S. (1995) Br. J. Rheumatol. **34**, 610–615