Is the long-term use of systemic corticosteroids beneficial in the management of Behçet's syndrome?

Original article Mat C *et al.* (2006) A double-blind trial of depot corticosteroids in Behçet's syndrome. *Rheumatology* 45: 348–352

SYNOPSIS

KEYWORDS Behçet's syndrome, depot corticosteroids, erythema nodosum, genital ulcer, oral ulcer

BACKGROUND

Corticosteroids are often used in the short term for the treatment of Behçet's syndrome; however, there are no controlled clinical trial data to support their use. The long-term effects of treatment with corticosteroids are of major concern.

OBJECTIVE

The objective of this study was to assess the effects of intramuscular depot corticosteroids on genital ulcers, arthritis, and other mucocutaneous manifestations in patients with Behçet's syndrome.

DESIGN AND INTERVENTION

This was a double-blind, randomized, placebo-controlled trial. Patients were included in the study if they fulfilled the International Study Group for Behçet's Disease's diagnostic criteria, had active disease, and were 18–45 years old. Patients were excluded from the study if they had received immunosuppressives or corticosteroids of >5 mg/day in the preceding month, if they had severe organ involvement or eye disease, or if they had diabetes mellitus, active infection, hypertension, peptic ulcer or pregnancy. Over a period of 27 weeks, participants received either methylprednisolone acetate 40 mg or placebo once every 3 weeks, and were also allowed to continue their existing therapies, apart from treatment with systemic immunosuppressives. The numbers of mucocutaneous lesions and joints with arthritis were counted by a dermatologist and a rheumatologist, respectively, at baseline, then every 3 weeks, and at 4 and 8 weeks after trial completion. Side effects were determined by questioning the patients.

OUTCOME MEASURES

The primary outcome measure of this study was the mean number of genital ulcers. Secondary outcome measures were the mean number of other mucocutaneous lesions and the mean number of joints with arthritis.

RESULTS

In total, 72 patients were included in this study: 34 in the treatment group and 38 in the placebo group. Overall, clinical characteristics were similar in the two groups at baseline, except that there were fewer patients with a history of erythema nodosum in the treatment group ($P = 0.004$). Analysis of the results showed that there were no significant differences in the mean number of genital ulcers, number of oral ulcerations, cases of folliculitis, or number of joints with arthritis between the treatment and placebo groups during treatment and post-treatment periods. Compared with the placebo group, the occurrence of erythema nodosum was significantly decreased in the treatment arm ($P = 0.0046$). This finding was more robust among the women in this group, although this enhanced effect of corticosteroids on erythema nodosum in women was not maintained in the post-treatment period. There were no significant differences in side effects between the treatment and placebo groups.

CONCLUSION

The authors conclude that long-term administration of low-dose depot intramuscular corticosteroids is not effective in treating genital ulcers, oral ulceration, folliculitis or arthritis in patients with Behçet's syndrome.

COMMENTARY

Shunsei Hirohata

Behçet's syndrome (or Behçet's disease) is characterized by recurrent aphthous stomatitis, genital ulcers, uveitis, and skin lesions, including folliculitis, erythema nodosum, and thrombophlebitis. The clinical characteristics of Behçet's syndrome include recurrent episodes of remission and exacerbation of various symptoms; however, chronic sustained inflammation in certain tissues is rare.[1] Although it has been suggested that short-term treatment with systemic corticosteroids might be beneficial, as they markedly reduce acute inflammation,[2] there have been no controlled studies exploring the efficacy of long-term administration of systemic corticosteroids in Behçet's syndrome. Despite this, systemic corticosteroids have been used to treat intractable mucocutaneous lesions and uveitis.

This study by Mat and colleagues makes an important and novel contribution to the literature. Although the authors recognize that significantly fewer patients had erythema nodosum in the treatment arm compared with the placebo, evidence is provided indicating that long-term administration of low-dose systemic corticosteroids did not have any beneficial effects on genital ulcers, oral ulcers and folliculitis in patients with Behçet's syndrome. The significant effect on erythema nodosum lesions was recognized as a beta error.

This study is the first in the literature to provide evidence that long-term administration of systemic corticosteroids does not have beneficial effects in the management of Behçet's syndrome. Although there have not been any previous controlled studies, a number of reports have suggested that short-term use of systemic corticosteroids is beneficial in the treatment of various manifestations of Behçet's syndrome.[2] The lack of any beneficial effect from long-term treatment with systemic corticosteroids might, therefore, be the result of their inability to prevent the recurrence of attacks. In this regard, colchicine has been demonstrated to significantly reduce the occurrence of some symptoms, including arthritis (in men and women) and genital ulcers and erythema nodosum (in women).[3] In addition, ciclosporin has been shown to be more effective than colchicine in suppressing an attack of uveitis.[4] Further controlled trials using methotrexate deserve attention, since there have been several reports on its efficacy in preventing the progression of central nervous system lesions and the occurrence of ocular attacks.[1]

Although Mat and colleagues report that there were no serious adverse effects related to 27-week treatment with depot corticosteroids, the effects of this treatment on osteoporosis have not been explored. Further to evidence showing that treatment with oral corticosteroids and more than 5 mg of prednisolone daily leads to a reduction in bone mineral density and a rapid increase in the risk of fracture during the treatment period (within 3–6 months),[5] it is necessary to further assess the effect of depot corticosteroids on bone mineral density. Calcium and vitamin D supplements should have been added to the treatment regimen to reduce bone mineral loss. Moreover, the study should have assessed other adverse effects of systemic corticosteroids, such as ocular hypertension and accelerated cataractogenesis.

It is recommended, therefore, that systemic corticosteroids should not be used in the long term to prevent the recurrence of various manifestations in Behçet's syndrome. Short-term use of systemic corticosteroids appears to be effective in improving symptoms of disease; however, further controlled trials will be required to confirm this point.

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Acknowledgments
The synopsis was written by Rachel Murphy, Assistant Editor, Nature Clinical Practice.

Competing interests
The author declared he has no competing interests.

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Received 1 May 2006
Accepted 17 May 2006
www.nature.com/clinicalpractice
doi:10.1038/ncprheum0224

PRACTICE POINT
Long-term use of systemic corticosteroids is not beneficial in the management of Behçet's syndrome

Research article

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Enhanced expression of mRNA for nuclear factor κB1 (p50) in CD34+ cells of the bone marrow in rheumatoid arthritis

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Received: 1 Oct 2005 Revisions requested: 8 Nov 2005 Revisions received: 27 Jan 2005 Accepted: 9 Feb 2006 Published: 6 Mar 2006

Arthritis Research & Therapy 2006, **8**:R54 (doi:10.1186/ar1915)
This article is online at: http://arthritis-research.com/content/8/2/R54

Abstract

Bone marrow CD34+ cells from rheumatoid arthritis (RA) patients have abnormal capacities to respond to tumor necrosis factor (TNF)-α and to differentiate into fibroblast-like cells producing matrix metalloproteinase (MMP)-1. We explored the expression of mRNA for nuclear factor (NF)κB in RA bone marrow CD34+ cells to delineate the mechanism for their abnormal responses to TNF-α. CD34+ cells were purified from bone marrow samples obtained from 49 RA patients and 31 osteoarthritis (OA) patients during joint operations via aspiration from the iliac crest. The mRNAs for NFκB1 (p50), NFκB2 (p52) and RelA (p65) were examined by quantitative RT-PCR. The expression of NFκB1 mRNA in bone marrow CD34+ cells was significantly higher in RA than in OA, whereas there was no significant difference in the expression of mRNA for NFκB2 and RelA. The expression of NFκB1 mRNA was not correlated with serum C-reactive protein or with the treatment with methotrexate or oral steroid. Silencing of NFκB1 by small interfering RNA abrogated the capacity of RA bone marrow CD34+ cells to differentiate into fibroblast-like cells and to produce MMP-1 and vascular endothelial growth factor upon stimulation with stem cell factor, granulocyte-macrophage colony stimulating factor and TNF-α without influencing their viability and capacity to produce β2-microglobulin. These results indicate that the enhanced expression of NFκB1 mRNA in bone marrow CD34+ cells plays a pivotal role in their abnormal responses to TNF-α and, thus, in the pathogenesis of RA.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by hyperplasia of synovial lining cells, consisting of macrophage-like type A synoviocytes and fibroblast-like type B synoviocytes [1]. It has been appreciated that type A synoviocytes, which are also called intimal macrophages, are derived from monocyte precursors in the bone marrow [1]. On the other hand, type B synoviocytes, which are also called fibroblast-like synoviocytes, have the morphological appearance of fibroblasts as well as the capacity to produce and secrete a variety of factors, including proteoglycans, cytokines, arachidonic acid metabolites, and matrix metalloproteinases (MMPs), that lead to the destruction of joints [1]. Apart from type A synoviocytes, the origin of type B synoviocytes has been unclear [1]. Of note, we have recently demonstrated that bone marrow CD34+ cells from RA patients have abnormal capacities to respond to tumor necrosis factor (TNF)-α and to differentiate into fibroblast-like cells producing MMP-1, suggesting that bone marrow CD34+ progenitor cells might generate type B synoviocytes and thus could play an important role in the pathogenesis of RA [2].

TNF-α is one of the first triggers to be found effective for the activation of nuclear factor (NF)κB in RA synovium [3]. This

β_2MG = β_2-microglobulin; ELISA = enzyme-kinked immunosorbent assay; GM-CSF = granulocyte-macrophage colony stimulating factor; HSCT = hematopoietic stem cell transplantation; MFI = mean fluorescence intensity; MMP = matrix metalloproteinase; MTX = methotrexate; NFκB = nuclear factor kappa B; OA = osteoarthritis; PCR = polymerase chain reaction; PE = phychoerythrin; RA = rheumatoid arthritis; SCF = stem cell factor; siRNA = small interfering RNA; TNF-α = tumor necrosis factor-alpha; VEGF = vascular endothelial growth factor.

mechanism of activation was followed by up-regulation of several inflammatory genes usually found in active RA. Accordingly, a number of studies have shown that TNF-α blockade has beneficial effects in the treatment of RA [4]. Moreover, inhibition of NFκB by the antioxidant N-acetylcysteine significantly reduced TNF-α- and NFκB-dependent gene expression and synovial proliferation [3]. We thus hypothesized that abnormal responses of RA bone marrow CD34+ cells to TNF-α might result from abnormal expression of NFκB genes. The current studies were undertaken, therefore, to explore the expression of mRNA for various components of NFκB in bone marrow CD34+ cells in RA.

Materials and methods

Patients and samples

Bone marrow samples were obtained from 49 patients with RA (8 males and 41 females: mean age, 58.6 years; age range, 35 to 78 years) who satisfied the American College of Rheumatology 1987 revised criteria for RA [5] and gave informed consent in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. The samples were taken during joint operations via aspiration from the iliac crest under anesthesia. As a control, bone marrow samples were similarly obtained from 31 patients with osteoarthritis (OA; 3 males and 28 females; mean age, 71.2 years; age range, 49 to 81 years) who gave informed consent. Most patients with RA and OA were taking non-steroidal anti-inflammatory drugs. Of the 45 patients with RA, 23 were treated with low dose methotrexate (MTX) and 33 were taking oral steroids when bone marrow samples were obtained. No OA patients were taking MTX or oral steroid.

Preparation of bone marrow CD34+ cells

Mononuclear cells were isolated by centrifugation of heparinized bone marrow aspirates over sodium diatrizoate-Ficoll gradients. CD34+ cells were purified from the mononuclear cells by positive selection with magnetic beads (CD34 progenitor cell selection system; Dynal, Oslo, Norway). The cells thus prepared were >95% CD34+ cells and <0.5% CD19+ B cells, as previously described [2]. In addition, CD34+ cells derived from bone marrow aspirates from the iliac crests of healthy individuals (purity >95%) were purchased from BioWhittaker (Walkersville, MD, USA).

RNA isolation and real-time quantitative PCR

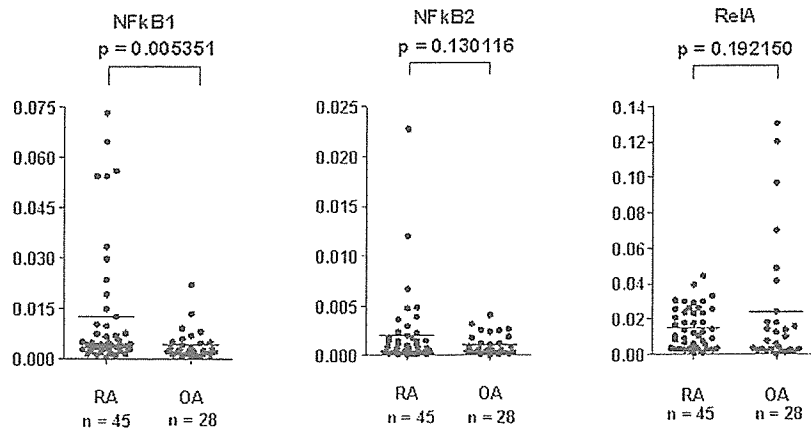
Total RNA was isolated from purified bone marrow CD34+ cells using the Trizol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. cDNA samples were prepared from 1 μg of total RNA using the SuperScript reverse transcriptase preamplification system (Life Technologies) with oligo (dT) primer and subjected to PCR. Real-time quantitative PCR was performed using the LightCycler rapid thermal cycler system (Roche Diagnostics, Lewes, UK) with primer sets for NFκB1, NFκB2, RelA or β-actin and Light Cycler-Fast Start DNA master SYBR Green I (Roche Diagnostics). The primers were designed using Oligo Primer Analysis Software version 5.0 (Takara Bio Inc., Ohtsu, Japan). The detail of primer sequences is shown in Table 1. Quantitative analysis was performed using LightCycler Software v.3.5. PCR reaction conditions composed of denaturing at 95°C for 10 minutes for 1 cycle, followed by 40 cycles of denaturing (10 seconds at 95°C), annealing (10 seconds at 60°C (NFκB2, RelA) or 62°C (NFκB1, β-actin)), and extension (5 seconds (NFκB1), 6 seconds (NFκB2, RelA), or 10 seconds (β-actin) at 72°C).

Table 1

Primer sequences used in real-time quantitative PCR for analysis of mRNA for various nuclear factor κB components

Gene product (GenBank accession no.)	Primer sequences	Nucleotides
NFκB1 [M58603]	Forward: 5'-TCC ACA AGG CAG CAA ATA GA-3'	3,125–3,144
	Reverse: 5'-GGG GCA TTT TGT TGA GAG TT-3'	3,244–3,263
NFκB2 [NM_002502]	Forward: 5'-TTC TGA AGG CTG GTG CTG AC-3'	2,220–2,239
	Reverse: 5'-AGT GAG GTC AAG AGG CGT GT-3'	2,352–2,371
RelA [NM_021925]	Forward: 5'-GAA GAA GAG TCC TTT CAG CG-3'	1,011–1,030
	Reverse: 5'-GGG AGG ACG TAA AGG GAT AG-3'	1,116–1,135
β-actin [X00351]	Forward: 5'-GCA AAG ACC TGT ACG CCA AC-3'	910–929
	Reverse: 5'-CTA GAA GCA TTT GCG GTG GA-3'	1,150–1,169

Figure 1

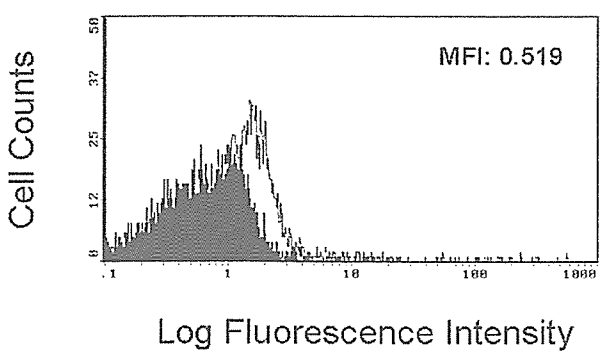


The expression of mRNAs for nuclear factor (NF)κB1 (p50), NFκB2 (p52) and RelA (p65) in bone marrow CD34+ cells. Total RNA was isolated from purified bone marrow CD34+ cells. The expression of mRNAs for NFκB1, NFκB2, RelA and β-actin was evaluated by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers to those of β-actin. Horizontal lines indicate the mean values. Statistical significance was evaluated by Welch's *t* test. OA, osteoarthritis; RA, rheumatoid arthritis.

Immunofluorescence staining and analysis

Purified bone marrow CD34+ cells (obtained from three RA patients and three OA patients) were treated with IntraPrep™ Permeabilization Reagent (Immunotech, Marseille, France), followed by staining with phycoerythrin (PE)-conjugated anti-NFκB p50 (E-10; a mouse IgG1 monoclonal antibody against amino acids 120 to 239 mapping at the amino terminus of human NFκB p50; Santa Cruz Biotech, Santa Cruz, CA, USA) or PE-conjugated normal mouse IgG1 (Santa Cruz). The cells were analyzed using an EPICS XL flow cytometer (Coulter, Hialeah, FL, USA) equipped with an argon-ion laser at 488 nm. A combination of low-angle and 90° light scatter measurements (forward scatter versus side scatter) was used to generate a bit map gating to identify bone marrow cells using Cyto-Trol™ Control Cells (Coulter) and Immuno-Trol™ Cells (Coulter) as standards. Specific mean fluorescence intensity (MFI) for NFκB1 (p50) was calculated by subtracting the non-specific MFI of staining with the isotype-matched control mouse IgG1.

Figure 2



Expression of nuclear factor (NF)κB1 (p50) protein in bone marrow CD34+ cells. Purified bone marrow CD34+ cells from a rheumatoid arthritis patient were permeabilized and then stained with phychoerythrin-conjugated anti-NFκB p50 monoclonal antibody or phychoerythrin-conjugated normal mouse IgG1, followed by analysis with flow cytometry. The level of NFκB1 protein was expressed by mean fluorescence intensity as described in Materials and methods.

Culture medium and cytokines

RPMI 1640 medium (Life Technologies) supplemented with L-glutamine (0.3 mg/ml) and 10% fetal bovine serum (Life Technologies) was used for all cultures. Recombinant human stem cell factor (SCF), granulocyte-macrophage colony stimulating factor (GM-CSF), and TNF-α were purchased from Pepro Tech EC (London, UK).

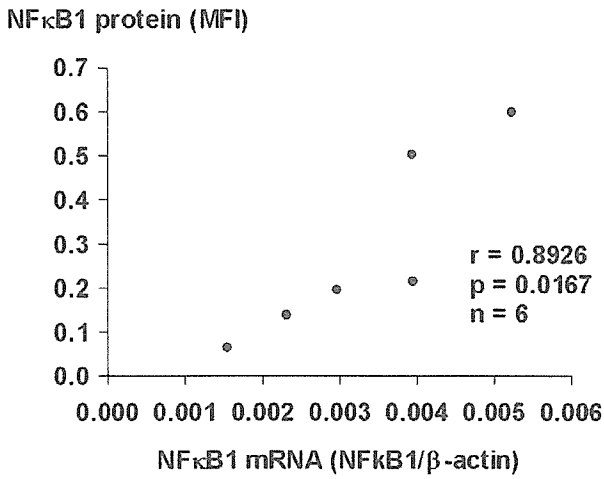
Silencing of NFκB1 in bone marrow CD34+ cells by small interfering RNA

SMARTpool® small interfering RNA (siRNA) for NFκB1 (p50) gene and nonsense scrambled control siRNA were purchased from Dharmacon (Lafayette, CO, USA). Chemical transfection of siRNAs into bone marrow CD34+ cells was performed using siPORT™ Amine Transfection Agent (Ambion, Austin, TX, USA) according to the manufacturer's directions. Briefly, purified bone marrow CD34+ cells were cultured in a 24-well microtiter plate (N0. 3524; Costar, Cambridge, MA, USA) at 2 × 10^5 cells per well in 0.2 ml culture medium in the presence of SCF (10 ng/ml) and GM-CSF (1 ng/ml). After 24 hours of incubation, chemical transfection of siRNAs was performed, and incubated for 4 hours.

Cell cultures and measurement of MMP-1 and vascular endothelial growth factor

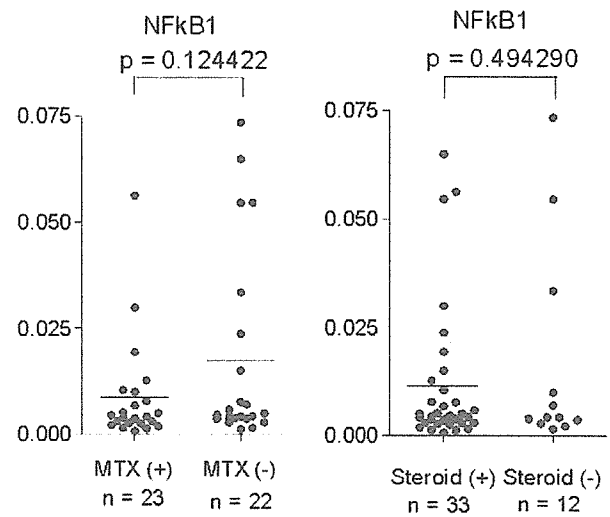
After transfection of siRNAs, the cells were cultured with SCF (10 ng/ml) and GM-CSF (1 ng/ml) in 1.0 ml culture medium for

Figure 3



Comparison of the expression of nuclear factor (NF)κB1 (p50) protein with that of NFκB1 mRNA in bone marrow CD34+ cells. Purified bone marrow CD34+ cells were permeabilized and then stained with phychoerythrin-conjugated anti-NFκB p50 monoclonal antibody or phychoerythrin-conjugated normal mouse IgG1, followed by analysis with flow cytometry. The NFκB1 protein levels as expressed by mean fluorescence intensity were compared with NFκB1 mRNA levels (expressed as the ratio of the mRNA copy numbers to those of β-actin) in bone marrow CD34+ cells from six patients (three rheumatoid arthritis patients and three osteoarthritis patients). Statistical significance was evaluated by linear regression test.

Figure 4



The relevance of treatment with the expression of mRNAs for nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells. Total RNA was isolated from purified bone marrow CD34+ cells from 45 rheumatoid arthritis patients. The expression of mRNAs for NFκB1 and β-actin was evaluated by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers to those of β-actin. Effect of treatment with methotrexate (MTX) or oral steroids (Steroid) was evaluated by Welch's *t* test. Horizontal lines indicate the mean values.

48 hours and then harvested for RNA extraction. Alternatively, the cells were cultured in a 24-well microtiter plate at 2×10^5 cells per well in 1.0 ml culture medium for 4 weeks in the presence of SCF (10 ng/ml), GM-CSF (1 ng/ml) and TNF-α (10 ng/ml) without medium change, as previously described [2]. The differentiation of fibroblast-like cells was observed under the phase-contrast light microscopy. The concentrations of MMP-1 and vascular endothelial growth factor (VEGF) in the culture supernatants were measured using the Biotrak human MMP-1 ELISA system (Amarsham Pharmacia Biotech, Buckinghamshire, UK) and human VEGF immunoassay kit (BioSource International, Camarillo, CA, USA), respectively. The concentrations of β_2-microglobulin (β_2MG) were determined by an ELISA as previously described [6].

Statistics

Comparison between RA and OA patients and between RA patients with MTX or steroid and those without MTX or steroid was carried out using Welch's *t* test. Significance of the effects of siRNA transfection on the generation of fibroblast-like cells and on the production of MMP-1 and VEGF was evaluated by Wilcoxon's signed rank test. Correlation between serum C-reactive protein and NFκB1 mRNA in bone marrow CD34+ cells and that between NFκB1 mRNA and protein were evaluated using a liner regression test. Correlation between NFκB1 mRNA in bone marrow CD34+ cells and the generation of fibroblast-like cells was analyzed using a Spearman's rank correlation test.

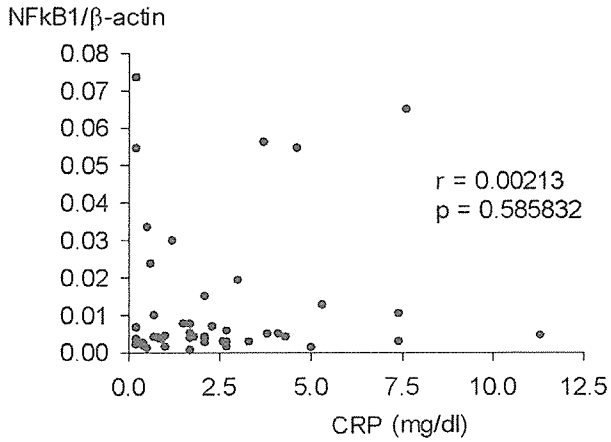
Results

Expression of mRNAs for various components of NFκB in bone narrow CD34+ cells

The expression of mRNA for NFκB1 (p50), NFκB2 (p52), and RelA (p65) in bone marrow CD34+ cells is shown as the ratio of the copy numbers to those of β-actin mRNA in Figure 1. The expression of NFκB1 mRNA was significantly higher in RA bone marrow CD34+ cells than in OA bone marrow CD34+ cells ($p = 0.005351$), whereas there were no significant differences in the expression of NFκB2 mRNA ($p = 0.130116$). Although the expression of RelA mRNA appeared to be lower in RA bone marrow CD34+ cells than in OA bone marrow CD34+ cells, it did not reach statistical significance ($p = 0.192150$). These results indicate that the expression of mRNA for components of NFκB1 is exclusively enhanced in bone marrow CD34+ cells from patients with RA.

Next, experiments were carried out to examine whether the elevation of NFκB1 mRNA expression parallels the elevation of NFκB1 protein expression in bone marrow CD34+ cells. The protein expression of NFκB1 was evaluated by staining of permeabilized bone marrow CD34+ cells from three RA patients and three OA patients with anti-NFκB p50 monoclonal antibody, followed by analysis with flow cytometry. As can be seen

Figure 5



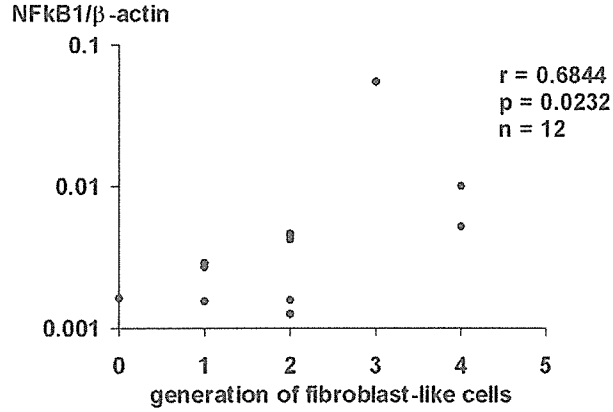
The correlation of the expression of mRNAs for nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells with serum C-reactive protein (CRP). Total RNA was isolated from purified bone marrow CD34+ cells from 45 rheumatoid arthritis patients. The expression of mRNAs for NFκB1 and β-actin was evaluated by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers to those of β-actin. Statistical significance was evaluated by linear regression test.

in Figure 2, bone marrow CD34+ cells express NFκB1 (p50) protein, the quantity of which can be expressed as MFI. Moreover, there is significant correlation between MFI for NFκB1 and NFκB1 mRNA in the six bone marrow CD34+ cells (Figure 3). The results indicate that the elevation of NFκB1 mRNA leads to the increase in NFκB1 protein expression.

Relevance of expression of NFκB1 mRNA in bone marrow CD34+ cells from RA patients to treatment and clinical parameters

Of note, 22 and 33 of the 45 RA patients were treated with MTX and oral steroids, respectively, whereas no OA patients were taking either MTX or oral steroids. It is therefore possible that MTX and oral steroids might have affected the expression of NFκB1 mRNA in bone marrow CD34+ cells. As shown in Figure 4, however, there were no significant differences in the expression of NFκB1 mRNA in bone marrow CD34+ cells between RA patients taking MTX or oral steroids and those who were not, although the expression of NFκB1 mRNA appeared to be lower in RA patients taking MTX or oral steroids. It is unlikely, therefore, that the medication the RA patients were taking would have resulted in the upregulation of NFκB1 mRNA expression in bone marrow CD34+ cells. It should be also noted that the expression of NFκB1 mRNA in bone marrow CD34+ cells was not significantly correlated with serum C-reactive protein (CRP) levels in RA patients (Figure 5). The data thus indicate that the upregulation of NFκB1 mRNA in bone marrow CD34+ cells is independent of the activity of the systemic inflammation, as reflected by serum CRP.

Figure 6



Comparison of the expression of nuclear factor (NF)κB1 (p50) mRNA in bone marrow CD34+ cells with their capacity to differentiate into fibroblast-like cells. The expression of NFκB1 mRNA in bone marrow CD34+ cells from 12 rheumatoid arthritis patients was evaluated by real-time quantitative PCR prior to the culture. The bone marrow CD34+ cells were incubated in culture medium with stem cell factor (10 ng/ml), granulocyte-macrophage colony stimulating factor (1 ng/ml) and tumor necrosis factor-α (10 ng/ml) for 4 weeks with no medium changes. Morphological changes were evaluated under light microscopy. The percentages of fibroblast-like cells were calculated from two view fields at ×20 magnifications. The degree of the generation of fibroblast-like cells were scored as follows: 0, fibroblast-like cells <5%; 1, fibroblast-like cells 5% to 25%; 2, fibroblast-like cells 25% to 50%; 3, fibroblast-like cells >50%; 4, formation of a pile or a cluster in at least one view field. Statistical significance was evaluated by Spearman's rank correlation test.

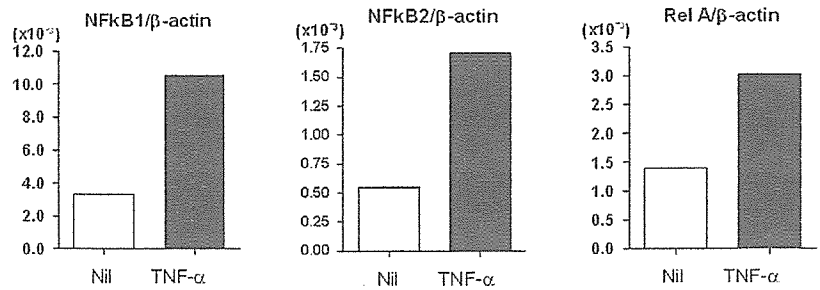
Relevance of the expression of NFκB1 mRNA to the generation of fibroblast-like cells

There was a variation in the expression of NFκB1 mRNA among the RA patients. We next examined the relationship of the initial levels of NFκB1 mRNA in RA bone marrow CD34+ cells with their capacity to differentiate into fibroblast-like cells. As shown in Figure 6, there was a significant correlation between the NFκB1 mRNA expression and the generation of fibroblast-like cells from bone marrow CD34+ cells upon stimulation with SCF, GM-CSF and TNF-α for 4 weeks in 12 RA patients. The data indicate that the enhanced expression of NFκB1 mRNA is important for the enhanced generation of fibroblast-like cells.

Effect of TNF-α on the expression of mRNAs for various components of NFκB in bone marrow CD34+ cells

Previous studies have demonstrated that TNF-α plays a critical role in the pathogenesis of RA [4]. It is possible, therefore, that the up-regulation of NFκB1 mRNA in bone marrow CD34+ cells might be secondary to the increased levels of TNF-α in the born marrow; experiments were carried out to test this possibility. Highly purified bone marrow CD34+ cells from healthy individuals were cultured in the presence of TNF-α (10 ng/ml) for 24 hours, after which the expression of mRNA for various components of NFκB was examined. As shown in Fig-

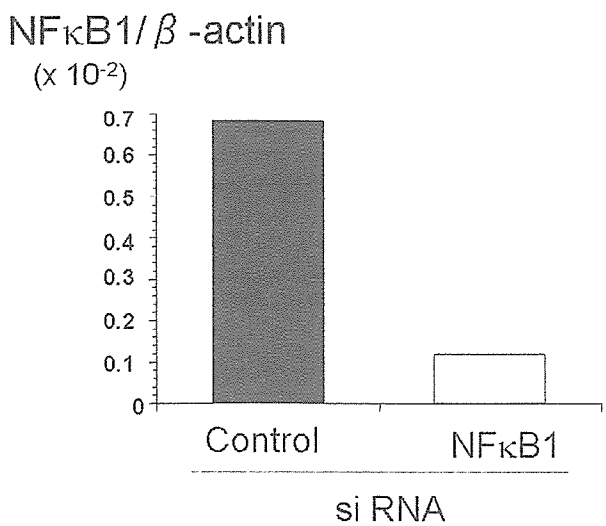
Figure 7



Effect of tumor necrosis factor (TNF)-α on the expression of mRNAs for nuclear factor (NF)κB1 (p50), NFκB2 (p52) and RelA (p65) in bone marrow CD34+ cells. Bone marrow CD34+ cells from healthy individuals were incubated in culture medium with or without TNF-α (10 ng/ml) for 24 hours. After the incubation, total RNA was isolated for evaluation of the expression of mRNAs for NFκB1, NFκB2, RelA and β-actin by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers to those of β-actin. The data are representative of two different experiments.

ure 7, treatment of bone marrow CD34+ cells with TNF-α upregulated not only the expression of NFκB1 (p50) mRNA, but that of NFκB2 (p52) mRNA and RelA (p65) mRNA. Since only the expression of NFκB1 mRNA, but not that of NFκB2 mRNA and RelA mRNA, was significantly upregulated in RA bone marrow CD34+ cells, the increased expression of NFκB1 mRNA in RA bone marrow CD34+ cells might not be accounted for simply by the increased levels of TNF-α in the bone marrow.

Figure 8

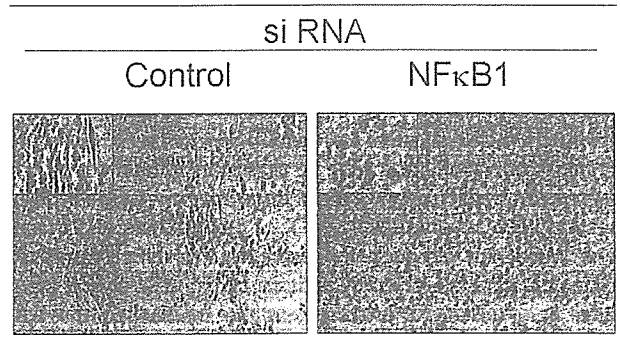


Silencing of nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells by small interfering RNA (siRNA) for NFκB1. Purified bone marrow CD34+ cells were transfected with siRNA for NFκB1 or a scrambled sequence control siRNA after a 24 hours incubation in culture medium with stem cell factor (10 ng/ml) and granulocyte-macrophage colony stimulating factor (1 ng/ml). After the transfection, the cells were further incubated for 48 hours in culture medium with stem cell factor and granulocyte-macrophage colony stimulating factor, and total RNA was isolated for evaluation of the expression of NFκB1 mRNA and β-actin mRNA by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers to those of β-actin.

Effect of silencing mRNA for NFκB1 on differentiation of RA bone marrow CD34+ cells into fibroblast-like cells upon stimulation with SCF, GM-SCF and TNF-α

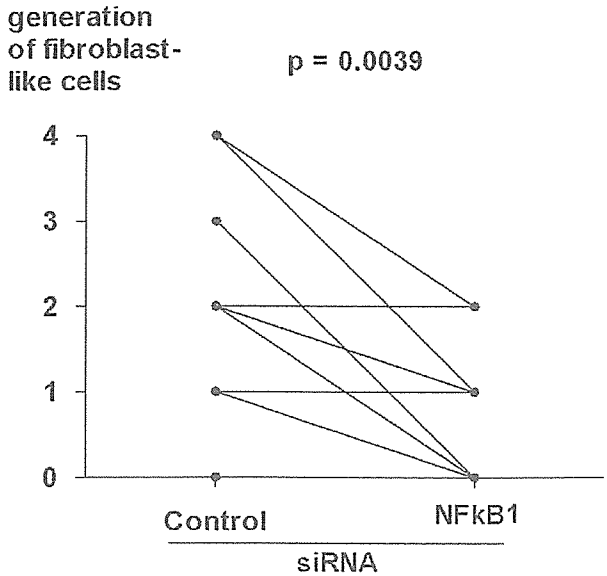
We next examined whether silencing of NFκB1 (p50) mRNA in RA bone marrow CD34+ cells might correct their abnormal responses to TNF-α. As shown in Figure 8, treatment of bone marrow CD34+ cells with siRNA for NFκB1 reduced the expression of NFκB1 mRNA by approximately 80%. More importantly, reduction of NFκB1 mRNA markedly suppressed the generation of fibroblast-like cells from RA bone marrow CD34+ cells upon stimulation with SCF, GM-CSF and TNF-α (Figures 9 and 10). Accordingly, silencing of NFκB1 by siRNA

Figure 9



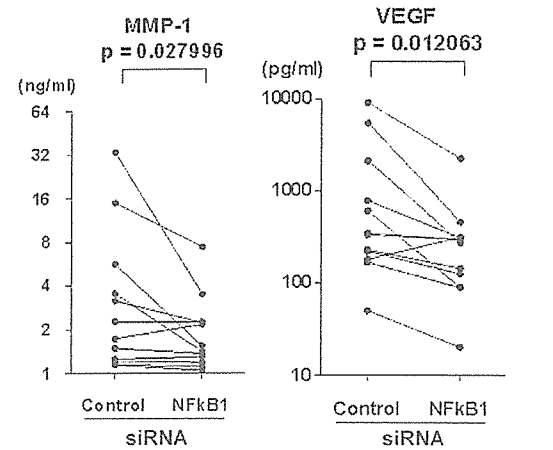
Inhibition of the generation of fibroblast-like cells by silencing nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells from patients with rheumatoid arthritis. Purified bone marrow CD34+ cells were transfected with small interfering RNA (siRNA) for NFκB1 or a scrambled sequence control, after which the cells were incubated in culture medium with stem cell factor (10 ng/ml), granulocyte-macrophage colony stimulating factor (1 ng/ml) and tumor necrosis factor-α (10 ng/ml) for 4 weeks with no medium changes. Morphological changes were observed under light microscopy (original magnification, ×20; inset, ×50 magnification). The data are representative of 12 different experiments.

Figure 10



Inhibition of the generation of fibroblast-like cells by silencing nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells from patients with rheumatoid arthritis. Purified bone marrow CD34+ cells were transfected with small interfering RNA (siRNA) for NFκB1 or a scrambled sequence control siRNA, after which the cells were incubated in culture medium with stem cell factor (10 ng/ml), granulocyte-macrophage colony stimulating factor (1 ng/ml) and tumor necrosis factor-α (10 ng/ml) for 4 weeks with no medium changes. Morphological changes were observed under light microscopy. The percentages of fibroblast-like cells were calculated from two view fields at ×20 magnifications. The degree of the generation of fibroblast-like cells were scored as follows: 0, fibroblast-like cells <5%; 1, fibroblast-like cells 5% to 25%; 2, fibroblast-like cells 25% to 50%; 3, fibroblast-like cells >50%; 4, formation of a pile or a cluster in at least one view field. Statistical significance was evaluated by Wilcoxon's signed rank test.

Figure 11



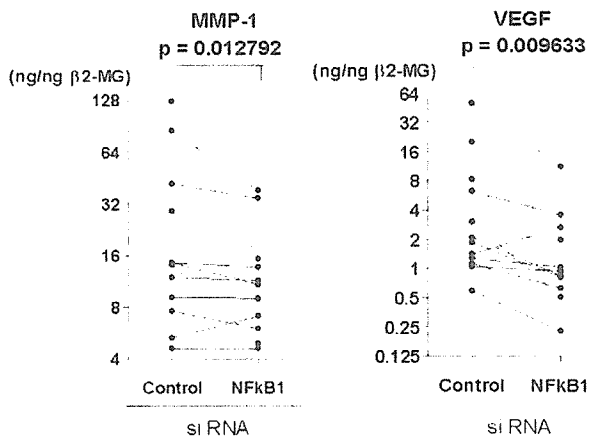
Suppression of the production of matrix metalloproteinase (MMP)-1 and vascular endothelial growth factor (VEGF) by silencing nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells from patients with rheumatoid arthritis. Purified bone marrow CD34+ cells from 12 patients with rheumatoid arthritis were transfected with small interfering RNA (siRNA) for NFκB1 or a scrambled sequence control siRNA, after which the cells were further incubated in culture medium with stem cell factor (10 ng/ml), granulocyte-macrophage colony stimulating factor (1 ng/ml) and tumor necrosis factor-α (10 ng/ml) for 4 weeks with no medium changes. After the incubation, the supernatants were harvested and assayed for MMP-1 and VEGF by ELISA. Statistical significance was evaluated by Wilcoxon's signed rank test.

significantly decreased the levels of MMP-1 and VEGF in culture supernatants of RA bone marrow CD34+ cells (Figure 11). Since bone marrow CD34+ cells proliferate in response to SCF, GM-CSF and TNF-α, it was possible that differences in MMP-1 and VEGF might be a result of alteration in cell proliferation by NFκB1 siRNA. Previous studies disclosed that β_2MG is produced by a number of cell types, including lymphocytes, myeloid cells, and tumor cells [7-9]. The production of β_2MG generally correlates with cell proliferation [6-9]. In fact, the levels of β_2MG in the culture supernatants paralleled the viable cell counts of bone marrow CD34+ cells stimulated with SCF, GM-CSF and TNF-α. Of note, silencing of NFκB1 also significantly decreased the ratios of MMP-1 and VEGF to β_2MG (MMP-1/β_2MG and VEGF/β_2MG) in culture supernatants of RA bone marrow CD34+ cells (Figure 12). Consistently, whereas siRNA for NFκB1 inhibited the differentiation of RA bone marrow CD34+ cells stimulated with SCF, GM-CSF and TNF-α into fibroblast-like cells (Figure 13), it significantly influenced neither the viable cell numbers nor the levels of β_2MG in the culture supernatants (Figure 14). These results confirm that the enhanced expression of NFκB1 mRNA in RA bone marrow CD34+ cells led to their abnormal capacity to differentiate into fibroblast-like cells producing MMP-1 upon stimulation with SCF, GM-CSF and TNF-α without affecting cell viability or proliferation. The data suggest, therefore, that the enhanced expression of NFκB1 mRNA in bone marrow hematopoietic stem cells might play a pivotal role in the pathogenesis of RA.

Discussion

The importance of TNF-α in the pathogenesis of RA has been well appreciated. Thus, anti-TNF-α antibodies and soluble TNF receptors have been demonstrated to have beneficial effects in the treatment of RA [4]. On the other hand, increasing attention has been paid to the role of bone marrow abnormalities in the pathogenesis of RA. In this regard, we demonstrated that RA bone marrow CD34+ cells have abnormal capacities to respond to TNF-α and to differentiate into fibroblast-like cells producing MMP-1 [2]. It should be noted that NFκB plays an important role in signal transduction and expression of a variety of genes, including MMP-1, under the influence of TNF-α [3]. The results in the current study have demonstrated that the expression of mRNA for NFκB1 is increased in RA bone marrow CD34+ cells. Of note, the expression of NFκB1 mRNA was significantly correlated with that of NFκB1 protein. Moreover, the initial levels of NFκB1 mRNA in RA bone marrow CD34+ cells were correlated with

Figure 12



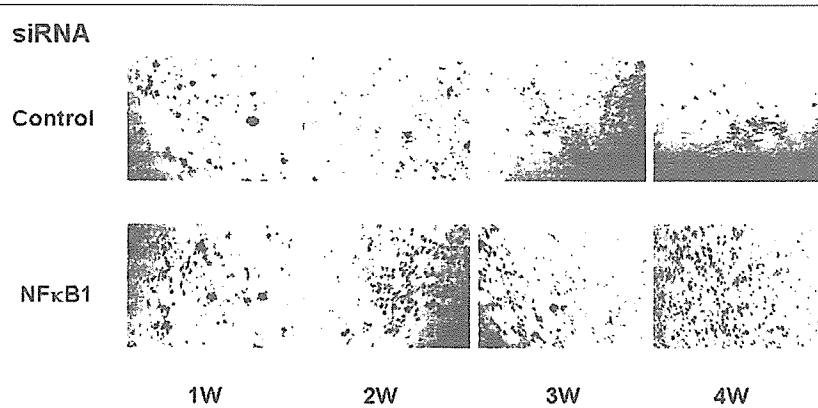
Suppression of the production of matrix metalloproteinase (MMP)-1 and vascular endothelial growth factor (VEGF) by silencing nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells from patients with rheumatoid arthritis. Purified bone marrow CD34+ cells from 12 patients with rheumatoid arthritis were transfected with small interfering RNA (siRNA) for NFκB1 or a scrambled sequence control siRNA, after which the cells were further incubated in culture medium with stem cell factor (10 ng/ml), granulocyte-macrophage colony stimulating factor (1 ng/ml) and tumor necrosis factor-α (10 ng/ml) for 4 weeks with no medium changes. After the incubation, the supernatants were harvested and assayed for MMP-1, VEGF and β_2-microglobulin (β_2MG) by ELISA. Statistical significance was evaluated by Wilcoxon's signed rank test.

their capacity to differentiate into fibroblast-like cells upon stimulation with TNF-α. The data suggest that the increased expression of NFκB1 mRNA might lead to constitutive overproduction of NFκB p50 molecules and thus result in abnormal responses to TNF-α of RA bone marrow CD34+ cells. Of note, bee venom and its major component melittin have been shown to display anti-arthritic effects through inactivation of NFκB [10]. Since bee venom and melittin delay and reduce nuclear translocation of the p50 subunit of NFκB but not p65 (RelA) [10], the importance of NFκB p50 rather than p65 in the pathogenesis of inflammatory arthritides has been underscored.

In the present study, significant numbers of RA patients were treated with MTX and oral steroids. However, there were no significant differences in the expression of NFκB1 mRNA in bone marrow CD34+ cells between RA patients receiving MTX or oral steroids and those who were not, although the expression of NFκB1 mRNA appeared to be lower in RA patients receiving these drugs. It is suggested, therefore, that administration of MTX and oral steroids might have made the differences in the expression of NFκB1 mRNA in bone marrow CD34+ cells between RA and OA less marked. On the other hand, the expression of NFκB1 mRNA in bone marrow CD34+ cells was not correlated with serum CRP levels in RA patients. The upregulation of NFκB1 mRNA in bone marrow CD34+ cells might not, therefore, be secondary to systemic inflammation, but may be a primary abnormality intrinsic to RA.

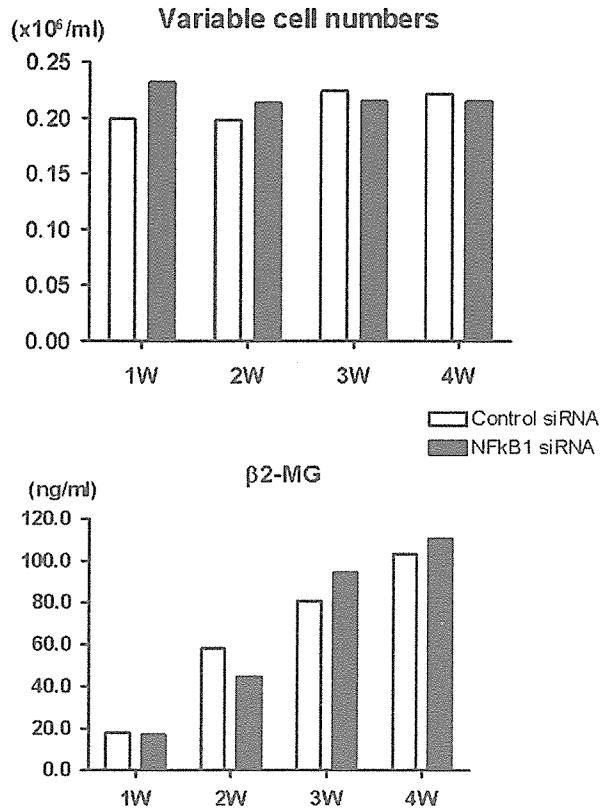
In the present study, the expression of mRNA for RelA (p65) appeared to be decreased in RA bone marrow CD34+ cells compared with that in OA bone marrow CD34+ cells, although this decrease did not reach statistical significance. Of note, a previous study demonstrated that embryonic fibroblasts from RelA-deficient mice are defective in the TNF-α mediated induction of mRNAs for IκBα [11]. Moreover, in RelA deficient fibroblasts, IκBβ protein was absent, presumably due

Figure 13



Time-kinetic effect of silencing nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells from patients with rheumatoid arthritis on the generation of fibroblast-like cells. Purified bone marrow CD34+ cells from patients with rheumatoid arthritis were transfected with small interfering RNA (siRNA) for NFκB1 or scrambled sequence control siRNA, after which the cells were further incubated in culture medium with stem cell factor (10 ng/ml), granulocyte-macrophage colony stimulating factor (1 ng/ml) and tumor necrosis factor-α (10 ng/ml) up to 4 weeks with no medium changes. After various periods of incubation (W, weeks), the morphological changes of the cells were observed under light microscopy. The data are representative of three different experiments.

Figure 14



Time-kinetic effect of silencing nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells from patients with rheumatoid arthritis on the viable cell counts and the production of β_2-microblobulin (β_2MG). Purified bone marrow CD34+ cells from patients with rheumatoid arthritis were transfected with small interfering RNA (siRNA) for NFκB1 or scrambled sequence control siRNA, after which the cells were further incubated in culture medium with stem cell factor (10 ng/ml), granulocyte-macrophage colony stimulating factor (1 ng/ml) and tumor necrosis factor-α (10 ng/ml) up to 4 weeks with no medium changes. After various periods of incubation (W, weeks), the cells were counted and the quantities of β_2MG in the culture supernatants were determined by ELISA. This is the same experment as shown in Figure 13. Data are representative of three different experiments.

to the decreased stability of IκBβ mRNA [11]. Since IκB plays an important role in inhibition of translocation of NFκB into the nucleus, the decrease in RelA mRNA might result in enhanced activation of NFκB related genes through upregulation of the translocation of NFκB. It is suggested, therefore, that the decreased expression of RelA mRNA in RA bone marrow CD34+ cells might also contribute to abnormal response to TNF-α.

It is possible that the upregulation of NFκB1 mRNA in bone marrow CD34+ cells might be secondary to the increased levels of TNF-α in the bone marrow. In fact, the treatment of bone marrow CD34+ cells from healthy individuals with TNF-α resulted in the increased expression of NFκB1 mRNA. However, TNF-α also enhanced the expression of mRNAs for NFκB2 and RelA in bone marrow CD34+ cells from healthy individuals. Of note, the expression of RelA mRNA appeared to be rather decreased in RA bone marrow CD34+ cells as mentioned above. Taken together, these data strongly suggest that the enhanced expression of NFκB1 mRNA might not be due simply to the increased levels of TNF-α in the bone marrow. Further studies to explore the mechanism of abnormal expression of NFκB1 mRNA in bone marrow CD34+ cells would be important for delineation of the pathogenesis of RA.

The role of the enhanced expression of NFκB1 mRNA in RA bone marrow CD34+ cells in their abnormal responses to TNF-α was further confirmed by the experiments of selective silencing of NFκB1 mRNA. Reduction of NFκB1 mRNA in RA bone marrow CD34+ cells by transfection of siRNA for NFκB1 markedly suppressed the generation of fibroblast-like cells as well as the production of MMP-1 and VEGF under the influence of TNF-α without affecting the viability or the capacity to produce β_2MG. These results indicate that upregulation of NFκB1 mRNA expression leads to the enhanced responses of RA bone marrow CD34+ cells to TNF-α. Thus, the enhanced NFκB1 mRNA expression might be a critical defect in RA bone marrow CD34+ cells.

Autologous hematopoietic stem cell transplantation (HSCT) has been used to treat severe RA in limited case reports [12,13]. However, a study with large numbers of patients has disclosed that recurrence of RA is frequent in patients who received autologous HSCT [14,15]. Frequent recurrence after autologous HSCT for RA suggests that abnormalities in bone marrow stem cells might persist after the treatment [16,17]. It is possible that the enhanced expression of NFκB1 mRNA might be closely related with such abnormalities in bone marrow stem cells, although further studies are required to confirm this point. It would also be important to explore whether there might be another transcription factor that could be inhibited without suppressing the differentiation of bone marrow CD34+ cells into fibroblast-like cells in order to confirm the importance of NFκB1 mRNA expression in the pathogenesis of RA.

Conclusion

The present study has revealed the enhanced expression of NFκB1 mRNA in RA bone marrow CD34+ cells as possible intrinsic abnormalities in bone marrow, resulting in abnormal responses to TNF-α. Further studies to delineate the mechanisms for the abnormal NFκB1 mRNA expression would be important for a complete understanding of the pathogenesis and etiology of RA.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SH designed the study, and participated in experimental procedures, collection, analysis, and interpretation of data, and manuscript preparation. YM and NC contributed to analysis and interpretation of data. TT, HY, and TO contributed to collection and analysis of data. All authors read and approved the final text before submission of the manuscript.

Acknowledgements

This work is supported by a grant-in-aidfromthe Health Science Research grant from theMinistry of Health and Welfare of Japan and grants from Aventis Pharma Co., Ltd, Tokyo, and from Eisai Co., Ltd, Tokyo, Japan. The authors wish to thank Tamiko Yanagida, PhD, for her technical assistance.

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ARTHRITIS & RHEUMATISM
Vol. 54, No. 1, January 2006, pp 312–324
DOI 10.1002/art.21539

Accuracy of Anti–Ribosomal P Protein Antibody Testing for the Diagnosis of Neuropsychiatric Systemic Lupus Erythematosus

An International Meta-Analysis

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Objective. **To quantitatively evaluate the diagnostic accuracy of antibodies to ribosomal P proteins (anti-P) for neuropsychiatric systemic lupus erythematosus (NPSLE) in general, for psychosis, mood disorder, or both, and for other diffuse manifestations.**

Methods. **This international meta-analysis combined standardized data from 1,537 lupus patients contributed by 14 research teams. Weighted estimation of sensitivity and specificity with fixed-effects and random-effects models, as well as summary receiver operating characteristic (SROC) curve analysis, was used to summarize test performance. The robustness of the overall estimates was examined in sensitivity analyses that included additional studies published up to November 1, 2004 in the Medline, EMBase, and Cochrane databases.**

Results. **Combining the data from the 14 teams, the weighted sensitivity and specificity estimates for the diagnosis of NPSLE were 26% (95% confidence interval [95% CI] 15–42%) and 80% (95% CI 74–85%), respectively. For psychosis, mood disorder, or both, the sensitivity and specificity were 27% (95% CI 14–47%) and 80% (95% CI 74–85%), respectively. For other diffuse manifestations, the sensitivity was 24% (95% CI 12–42%), and the specificity was 80% (95% CI 73–85%). The proportion of patients with anti-P antibodies did not vary markedly across different presentations of NPSLE. Between-study heterogeneity was substantial, but the SROC curves were consistent with the weighted estimates. In further analyses that included another 24**

Supported by a grant from the Joseph and Esther Gani Foundation (Ioannina, Greece). The Foundation had no role in the collection, analysis, or interpretation of the data or in the decision to submit the study for publication. Dr. Karassa's work was also supported by the Joseph and Esther Gani Foundation (grant 62/1516/-0001).

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Submitted for publication April 25, 2005; accepted in revised form September 29, 2005.

published studies, only the sensitivity for psychosis and/or mood disorder was slightly improved, but it was still suboptimal (42% [95% CI 30–53%]); the specificity remained essentially the same (81% [95% CI 76–85%]).

Conclusion. **Anti-P antibody testing has limited diagnostic value for NPSLE, and it is not helpful in differentiating among various disease phenotypes.**

Neuropsychiatric manifestations occur in approximately one-half of patients with systemic lupus erythematosus (SLE) and may cause substantial impairment of quality of life as well as disability (1–3). Moreover, multiple neuropsychiatric events during the disease course are associated with adverse long-term prognosis (4,5) and may lead to death, with a mortality rate of 7–19% (2,5,6). Neuropsychiatric SLE (NPSLE) encompasses a multitude of symptoms involving the central, peripheral, and autonomic nervous systems as well as psychiatric disorders (7). Recently, an ad hoc committee of the American College of Rheumatology (ACR) proposed a standard nomenclature for 19 neuropsychiatric syndromes associated with SLE (7), yet NPSLE is difficult to diagnose and is challenging to treat. Secondary factors, such as drugs, metabolic abnormalities, or infections, can also cause neuropsychiatric disturbances in lupus patients (3,7). Manifestations reflecting diffuse cerebral involvement pose the foremost difficulty in differentiating their exact origin, since psychiatric disorders may merely be reactive psychological disturbances (2,3,7).

During the last 2 decades, several studies have explored the utility of antibodies to ribosomal P proteins (anti-P) in detecting NPSLE (6,8–35). These antibodies are directed toward 3 large-subunit ribosomal phosphoproteins, called P0 (38 kd), P1 (19 kd), and P2 (17 kd), which share a common linear determinant in the carboxyl-terminal 22–amino acid sequence (36). Early studies claimed that serum anti-P antibodies were highly accurate for the diagnosis of SLE-mediated psychosis and depression (9,26), but subsequent reports were less optimistic (11–13,18,20,25,27,31). Other studies expanded the spectrum of neuropsychiatric features that could be correlated with anti-P to include active disease, diffuse manifestations, or NPSLE overall (6,25,28,30), making even more unclear their clinical value for this entity. Methodologic shortcomings, including the criteria used to define NPSLE, the approaches adopted for detecting anti-P antibodies, and the small sample size of isolated studies, may have contributed to the uncertainty.

Because SLE is a relatively uncommon disease and NPSLE is even more uncommon, no single study can reliably assess the operating characteristics of anti-P antibodies. Yet, a rigorous appraisal of a diagnostic test may reduce the number of unwanted clinical consequences related to misleading estimates of the accuracy of that test. Ideally, one would like to assess the diagnostic accuracy of a test across a large study population and use similar, standardized, and reproducible methods. In the absence of a single very large study that could do this, an attractive alternative is to standardize data across existing cohorts of lupus patients. Therefore, the aim of this study was to evaluate the diagnostic performance of anti-P antibodies for NPSLE in general, for diffuse NPSLE manifestations, and for particular psychiatric syndromes (psychosis, mood disorder, or both) in the context of an international collaborative meta-analysis, with standardization of the data contributed by a large number of investigators.

PATIENTS AND METHODS

Eligibility criteria. The meta-analysis included lupus patients with and without NPSLE who had undergone serum anti-P antibody testing by immunoblotting, a standard enzyme-linked immunosorbent assay (ELISA), or both (37–39).

To ensure consistency, participating investigators were asked to comply with the following rules. Patients had to fulfill the ACR criteria for the classification of SLE (40) and had to be evaluated for the presence or absence of neuropsychiatric lupus syndromes according to the ACR nomenclature and case definitions (7). Patients with a neuropsychiatric syndrome during any time in the course of SLE were classified into 3 subgroups: those with psychosis, mood disorders, or both; those with other diffuse (2,6) manifestations (including acute confusional state, generalized seizures, cognitive dysfunction, anxiety disorder, and headache other than migraine or cluster headache), and those with focal (2,6) neurologic events (including cerebrovascular disease, partial seizures, migraine or cluster headache, myelopathy, demyelinating syndrome, movement disorder, aseptic meningitis, and syndromes of the peripheral nervous system) (7). When both diffuse and focal events occurred in the same patient, the designation was made according to the predominant manifestation. Severe, sustained, or progressive presentations requiring more-aggressive

treatment with cytotoxic immunosuppressive agents were considered to be predominant.

Collaborating investigators provided a clear description of the immunoassay(s) used for anti-P determination, with sufficient detail to permit replication (41). When both immunoblotting and ELISA had been used, data were reported separately for each method. Patients who had undergone testing for anti-P multiple times were considered to have this autoantibody specificity if at least 1 of the determinations yielded positive results. Investigators were also asked to specify whether immunoassays were performed without knowledge of the clinical condition of the patients and whether the diagnosis of NPSLE, as well as the assignment of neuropsychiatric syndromes, was accomplished without knowledge of the anti-P status of the participants.

Organization of the international database. Research teams who have previously published data on cohorts of SLE patients were invited to participate in this meta-analysis, provided that the study patients met the eligibility criteria defined above. Collaborating teams were identified through searches of the Medline, EMBase, and Cochrane databases conducted in January 2003, using combinations of index terms (systemic lupus erythematosus, rheumatic diseases, connective tissue disease, or autoimmune disease, as well as ribosomal, antiribosomal, anti-P, or antineuronal), cited references of eligible studies and review articles, abstracts of major rheumatology conferences, and consultation with experts in the field. We e-mailed invitations to investigators working on SLE. The meta-analysis was also announced at an autoimmune disease–related scientific meeting (42). Pertinent data were contributed on a standard reporting form. The database remained open until July 2004.

Research teams from 14 centers (8 European, 4 Asian, and 2 South American) agreed to participate. We accepted data that were already available as well as data that were prospectively generated specifically by some of the participating teams for the purposes of the collaborative project. The effort was coordinated by the Clinical and Molecular Epidemiology Unit of the Department of Hygiene and Epidemiology at the University of Ioannina School of Medicine. The coordinating center was responsible for giving instructions to participating investigators on how to standardize and summarize their individual-level databases. The contributed data sets were assessed for potential errors or inconsistencies and then assembled at the coordinating center, which was also responsible for conducting the analyses. Queries were clarified through communications with the participating investigators.

Data synthesis and statistical analysis. Measures of diagnostic performance included sensitivity and specificity of anti-P antibodies for various forms of NPSLE. The main analysis involved the following 4 comparisons: NPSLE overall and each subgroup of NPSLE (psychosis and/or mood disorder, other diffuse manifestations, and focal events) versus the non-NPSLE group; all diffuse manifestations versus focal events; and psychosis and/or mood disorder versus other diffuse manifestations. These analyses address the discriminatory ability of the test for NPSLE in general, for each disease subtype, and for different neuropsychiatric presentations. To further pursue the possibility that anti-P may be specifically associated with particular psychiatric disorders (8,9,16,22,26), we evaluated the diagnostic accuracy of anti-P antibody for patients with psychosis and/or mood disorder versus all other lupus patients.

Test performance was estimated separately from studies that used immunoblotting for the detection of anti-P antibodies and from studies that used ELISA. In the overall analysis, when both immunoblotting and ELISA data were available from the same study, the results from the ELISA were used for the calculations. Diagnostic accuracy was also evaluated for subgroups defined by race.

Summary estimates were obtained with 2 meta-analytic methods: weighted independent estimation of sensitivity and specificity, and summary receiver operating characteristic (SROC) curve analysis.

Sensitivity and specificity estimates for each comparison were independently combined across studies, using both fixed-effects (Mantel-Haenszel) and random-effects (DerSimonian-Laird) models (43,44). Fixed-effects models weigh each study by the inverse of its variance. Random-effects models also incorporate between-study variation. The random-effects approach tends to provide wider confidence intervals (CIs) and is preferable in the presence of between-study heterogeneity. Except where indicated otherwise, random-effects estimates are provided below. Between-study heterogeneity was examined with Fisher's exact test.

Because sensitivity and specificity are interdependent, independent weighting may sometimes underestimate both measures. Hence, we used SROC curve analysis to account for this mutual dependence (45,46). The method fits a curve describing the tradeoff between sensitivity and specificity across studies, with different characteristics and thresholds for an abnormal test result. The regression is calculated as follows: $D = \alpha + \beta S$, where D is the difference in the logits of the true-positive rate (sensitivity) and the false-positive rate (1 − specificity), and S is the sum of these logits. When β is not significantly different from 0, the SROC curve is symmetric around the diagonal that runs from the top left corner to the bottom right corner of the diagram. Conversely, when β is significantly different from 0, the SROC curve is not symmetric, and the overall diagnostic performance varies in different parts of the curve, with an uneven tradeoff between sensitivity and specificity across studies. This may indicate significant between-study variation in the selected test threshold, study population, or other parameters. SROC curves should not be extrapolated outside the range of observed values. Both nonweighted and weighted SROC curves were estimated (46,47); nonweighted curves consider all studies equally in the calculations, whereas weighted curves weigh each study by the variance of D.

Inclusion of other published data. Sensitivity analyses were conducted to examine whether the addition of further relevant published studies affected our summary estimates of the operating characteristics of anti-P antibodies. Only the following 2 comparisons were examined, since articles focused on these patient groups: the entire group of NPSLE

patients versus the non-NPSLE patient group, and patients with psychosis and/or mood disorder versus either the non-NPSLE patients or all other lupus patients. Finally, we evaluated the available data to compare active NPSLE versus non-NPSLE.

Eligible studies published in any language were retrieved during the stage of identification of pertinent articles and collaborating investigators, as described above. We updated the literature search of the 3 computerized databases in November 2004 to identify additional relevant studies published up to November 1, 2004. Meeting abstracts were not included because the results may not be final and may not have been subjected to formal peer review. Duplicate or overlapping data were counted only once. The inclusion criteria were similar to those of the collaborative meta-analysis, with no restriction on patient age or study location. Nevertheless, in these analyses, we did not use the stringent criteria regarding the method of antibody determination and classification of neuropsychiatric disease; studies were combined regardless of the assay used to detect anti-P antibodies and regardless of the criteria used to diagnose NPSLE.

Other sensitivity analyses. We also performed sensitivity analyses to assess the robustness of the quantitative estimates derived from the collaborative meta-analysis. These analyses were limited to studies that used the ACR criteria for NPSLE syndromes and limited to studies that specified blinding.

Software. Analyses were conducted with the use of the following software: SPSS, version 12.0 (SPSS, Chicago, IL), Meta-Test, version 0.6, New England Medical Center, Boston, MA, 1997 (Joseph Lau, Tufts–New England Medical Center, Boston, MA) and Meta-Analyst, version 0.991 (Joseph Lau, Boston, MA).

RESULTS

General characteristics. We sent inquiries to 104 investigators working on SLE. Of those 104 investigators, 65 did not reply, 18 did not have any data and could not produce such data for the project, and 4 declined to participate. Of the last group, 2 investigators had published studies that were included in the sensitivity analysis.

The collaborative meta-analysis considered 1,537 lupus patients from 14 teams of investigators. Of these, 1,295 patients underwent both anti-P antibody testing by immunoblotting or standard ELISA and evaluation for NPSLE according to the ACR case definitions. The median sample size per study was 91 patients (interquartile range [IQR] 48–162). Women accounted for 80–97% of each study population. Although more than one-half of the participants were of European descent, patients of other ancestries were also included (Table 1). The mean age of the patients at study entry ranged from 29.8 years to 41.6 years, and the median of the mean disease durations across study cohorts was 7.3 years (IQR 6.2–7.8).

Most studies used a solid-phase ELISA, with highly purified synthetic peptides of the carboxyl-terminal 22–amino acid sequence (n = 4), a multiple-antigen peptide format (n = 3), and purified native (n = 2) or recombinant (n = 3) proteins as coating antigen to detect anti-P antibodies. Seven studies designated a positive anti-P result as >2 SD (n = 1) or >3 SD (n = 6) above the mean value obtained in a normal population, whereas 5 studies reported results according to the suggested threshold for the commercial ELISA systems they used. Only 4 studies used Western blotting on cell extracts from various sources for the detection of this autoantibody specificity. A single study used a line immunoassay, which is an ELISA-based multianalyte assay (Table 1).

The median prevalence of anti-P antibodies was 18.2% (IQR 9.7–28.6%). These antibodies were more prevalent in lupus patients of Asian descent than among those of other racial ancestries. The study-specific frequencies of anti-P antibodies were 23.8–45.5% in 320 patients of Chinese, Japanese, Taiwanese, and Filipino ancestry and 6.4–25.4% in 1,212 patients of other ancestry.

Approximately one-third of the 1,537 lupus patients had NPSLE that manifested as syndromes described in the ACR case definitions (median prevalence 32% [IQR 12–42%]). In 1 study (Table 1), neuropsychiatric involvement was determined according to prespecified criteria other than the ACR case definitions. Eight research teams provided individual patient data; in these studies, 8% of patients had >1 neuropsychiatric disorder, but only 5% had both focal and diffuse presentations. The other 6 teams directly collected data on only the most prominent manifestation. More than one-half of the NPSLE patients presented with disorders reflecting diffuse cerebral involvement (median prevalence 54.5% [IQR 47.6–68.2%]). The median prevalence of psychosis, mood disorder, or both was 24.9% (IQR 17.1–38.4%). In most studies, NPSLE was diagnosed without knowledge of the anti-P antibody status, and test interpreters were blinded to the clinical condition of the patients (Table 1).

Diagnostic performance of anti-P antibody testing. Substantial heterogeneity was found in both the sensitivity and the specificity of anti-P antibody testing

Table 1. Characteristics of the studies and patient populations included in the collaborative meta-analysis*

Study ID	Investigator, country, year (ref.)	Study setting	No. of patients	% women	Ethnicity (%)†	Mean age, years	Mean disease duration, years	Anti-P antibody assay	Prevalence of NPSLE, %	NPSLE manifestation			Blinding‡
										Psychosis and/or mood disorder	Other diffuse manifestations	Focal events	
1	Doria A, Italy, 2004	University	101	88	Italian (98), African (2)	29.8	6.7	WB/ELISA	21	8	6	7	T, C
2	Morozzi G, Galeazzi M, Italy, 2004	University	20§	90	Italian (85), Chinese/Filipino (15)	35.7	7.6	ELISA	15	0	0	3	T, C
3	Afeltra A, Italy, 2004	University	43	88	Italian	41.6	8	ELISA	93	2	16	22	T, C
4	Mathieu A, Italy; Sanna G, UK, 2000 (24)	University	68¶	96	Italian	38.4	7.7	ELISA	49	7	9	17	T, C
5	Hoffman I, De Keyser F, Belgium, 2004 (14)	University	235#	88	Belgian, Dutch, Slovak, English	40	7.2	LIA	59	33	32	51	NS
6	Tzioufas A, Greece, 2000 (30)	University	185	96	Greek	34.7	4.3	ELISA	9	2	7	8	NS
7	Ambrozic A, Slovenia, 2003	University	150	91	Slovenian	38.1	7.8	WB	39	11	14	33	T, C
8	Inanc M, Turkey, 2004	University	218	89	Turkish	38.5	7.8	ELISA	23	20	5	26	T, C
9	Chang D-M, Taiwan, 2003	Community	80	91	Taiwanese	35	9.4	ELISA	6	1	3	1	NS
10	Mok CC, China, 2004	Community	33	97	Chinese	36.2	7	WB/ELISA	33	3	5	3	T, C
11	Hirohata S, Japan, 2003	University	50	80	Japanese	40.8	2.6	ELISA	32	5	7	4	T, C
12	Yoshio T, Japan, 2003 (35)	University	154**	90	Japanese	34.6	4.7	ELISA	40	14	24	24	T, C
13	Massardo L, Chile, 2002 (21)	University	141††	90	Chilean	33	7	WB/ELISA	9	5	1	6	T, C
14	Spindler AJ, Argentina, 2003	University	59	92	Argentinean	36	7.3	ELISA	44	11	4	11	T, C

* References and publication dates (when the contributed data were derived from published studies) are provided; otherwise, the year the data were collected and sent to the coordinating center are shown. See Patients and Methods for a full description of the 3 subgroups of neuropsychiatric systemic lupus erythematosus (NPSLE). Anti-P = anti–ribosomal P; WB = Western blotting; ELISA = enzyme-linked immunosorbent assay; LIA = line immunoassay.

† Percentages are given for studies that included patients of different ethnicities, when known.

‡ NPSLE was diagnosed without knowledge of the results of the anti-P antibody testing (T), and test interpreters were blinded to the clincial data (C). NS = not specified.

§ In this study, 3 patients had indeterminate results for anti-P antibodies and were not included in the quantitative synthesis.

¶ In this study, 5 patients who were not tested for anti-P antibodies were not included in the quantitative synthesis.

In this study, sufficient clinical information for NPSLE was available for 196 patients; the presence or absence of NPSLE was assessed using prespecified criteria other than the American College of Rheumatology case definitions (7); and data for disease duration were available for 197 patients.

** Only 44 patients were included in the published study.

†† In this study, 2 patients in addition to the ones listed under NPSLE manifestations had NPSLE, but the type of involvement was not known.

Table 2. Summary results of the collaborative meta-analysis*

Comparison	No. of studies	No. of subjects	Weighted sensitivity (95% CI)	Weighted specificity (95% CI)
NPSLE versus non-NPSLE	13	1,340	0.26 (0.15–0.42)	0.80 (0.74–0.85)
Psychosis and/or mood disorder versus non-NPSLE	12	1,024	0.27 (0.14–0.47)	0.80 (0.74–0.85)
Other diffuse neuropsychiatric manifestations versus non-NPSLE	12	1,034	0.24 (0.12–0.42)	0.80 (0.73–0.85)
Focal neurologic events versus non-NPSLE	13	1,110	0.29 (0.15–0.48)	0.80 (0.74–0.85)
All diffuse neuropsychiatric manifestations versus focal neurologic events	12	406	0.26 (0.14–0.43)	0.70 (0.50–0.84)
Psychosis and/or mood disorder versus other diffuse neuropsychiatric manifestations	12	228	0.28 (0.15–0.46)	0.75 (0.57–0.88)
Patients with psychosis and/or mood disorder versus all other lupus patients	12	1,322	0.27 (0.14–0.47)	0.80 (0.72–0.86)

* Weighted sensitivity and specificity were determined according to the random-effects model. Between-study heterogeneity was statistically significant for all comparisons ($P < 0.01$). 95% CI = 95% confidence interval; NPSLE = neuropsychiatric systemic lupus erythematosus.

using ELISA (Table 2). In the random-effects model, the overall weighted sensitivity and specificity estimates for the diagnosis of NPSLE were 26% (95% CI 15–42%) and 80% (95% CI 74–85%), respectively (Table 2).

Diagnostic performance for neuropsychiatric disease appeared to be somewhat better in studies that used Western blotting to detect anti-P antibodies (summary sensitivity 36% [95% CI 16–63%]; summary specificity 84% [95% CI 70–92%]), but significant between-study heterogeneity was still present ($P = 0.0001$ for heterogeneity in sensitivity estimates and $P = 0.0007$ for heterogeneity in specificity estimates), and data were too limited to be conclusive (4 studies; 424 patients). Test performance was poor for NPSLE in Asian patients (4 studies; 317 patients, yielding a summary sensitivity of 55% [95% CI 45–65%] and a summary specificity of 68% [95% CI 59–76%]). The weighted specificity tended to be higher in all other lupus patients, which were mostly of European descent, but there was low sensitivity (9 studies; 1,023 patients, yielding a summary sensitivity of 17% [95% CI 9–32%] and a summary specificity of 85% [95% CI 81–88%]).

SROC analyses suggested similar performance for identifying SLE-induced neuropsychiatric disease. Weighted and nonweighted curves were practically coincident (Figure 1A). Anti-P antibodies had an almost equally meager discriminating ability for the diagnosis of either psychiatric syndromes or other forms of neuropsychiatric involvement in SLE (Table 2). Weighted random-effects independent estimates stand very close to the weighted SROC curves for these comparisons (Figures 1B–D), suggesting that they are appropriate approximations of the overall diagnostic performance. Statistically significant asymmetry was found in all these curves (Figure 1), indicating that an improvement in specificity was accompanied by a disproportionately large decrease in sensitivity.

Within the group with NPSLE (Table 2), anti-P antibody testing could not accurately discriminate patients presenting with diffuse manifestations from those presenting with focal events (summary sensitivity 26%; summary specificity 70%) (Figure 2A) or patients presenting with psychiatric disorders from those presenting with any other diffuse symptom (summary sensitivity 28%; summary specificity 75%) (Figure 2B). Test characteristics remained unchanged for the identification of patients with psychiatric disorders compared with all other lupus patients (with or without neuropsychiatric dysfunction) (Table 2). Significant asymmetry was found in the corresponding SROC curve (Figure 2C), implying that an improvement in specificity was accompanied by an uneven, large decrease in sensitivity.

Findings of additional analyses. Our search of the 3 databases identified a total of 306 potentially relevant articles, of which 243 studies were excluded upon reading the titles and abstracts. Another 39 studies were excluded after reviewing the complete reports: 8 were editorials, comments without original data, or review articles, 11 were case reports, 7 studies presented duplicate or overlapping data, 8 evaluated anti-P antibody testing for other SLE manifestations or other autoimmune diseases, 3 focused on isolated neuropsy-

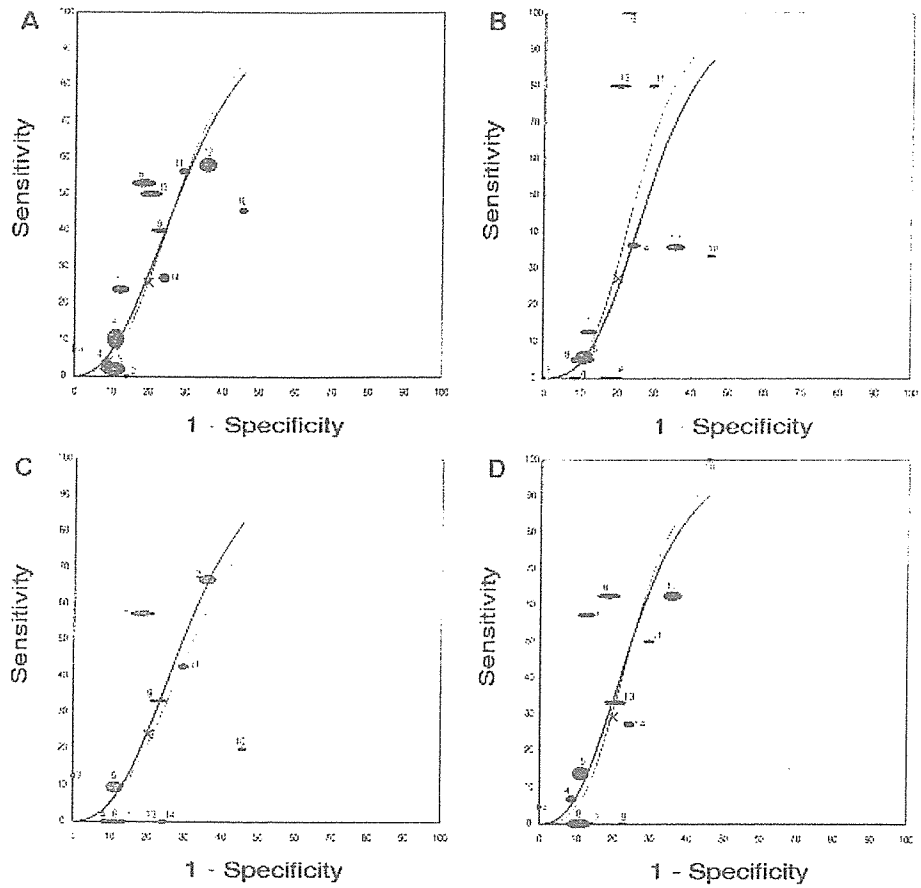

Figure 1. Summary receiver operating characteristic curves for the performance of antibodies to ribosomal P proteins in the diagnosis of various forms of neuropsychiatric systemic lupus erythematosus (NPSLE). Results are from the main analysis. Each ellipse corresponds to a study estimate of sensitivity and specificity; the area of each ellipse is proportional to the study size. Numbers beside the ellipses are study identification numbers and correspond to those shown in Table 1. Thin lines indicate nonweighted analyses; thick lines indicate weighted analyses. Shaded rectangles mark the 95% confidence intervals of the pooled sensitivity and pooled specificity obtained by random-effects calculations. × indicates exact estimates. A, NPSLE overall versus non-NPSLE. B, Psychosis and/or mood disorder versus non-NPSLE. C, Other diffuse neuropsychiatric manifestations versus non-NPSLE. D, Focal neurologic events versus non-NPSLE.

chiatric syndromes, and 2 provided insufficient data for calculating the sensitivity and specificity in any comparison considered.

Twenty-four additional publications (6,8–13,15–20,22,23,25–29,31–34) were retrieved from the database search, representing a total of 38 studies involving 3,713 lupus patients. Nevertheless, data for the comparison of NPSLE versus non-NPSLE groups were available in only 18 of the 24 additional studies; data for other comparisons were available in even fewer reports (Table 3). The results were consistent with those derived from the collaborative meta-analysis (Table 3 and Figure 3), but between-study heterogeneity was always considerable (Table 3). The overall weighted sensitivity and specificity estimates for identifying patients with NPSLE were 28% (95% CI 22–35%) and 80% (95% CI 75–85%), respectively. The SROC curve for this comparison was located very close to the diagonal, indicating poor diagnostic performance (Figure 3A). The overall sensitivity for psychosis, mood disorder, or both was slightly

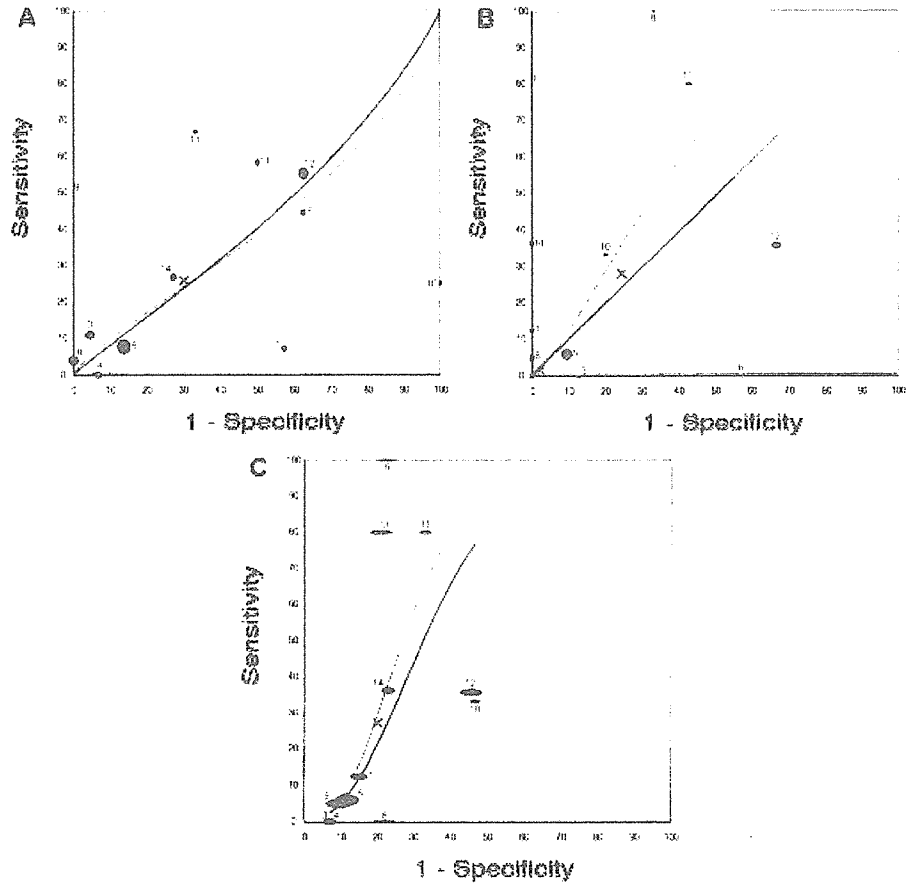

Figure 2. Summary receiver operating characteristic curves for the performance of antibodies to ribosomal P proteins in the diagnosis of various forms of neuropsychiatric systemic lupus erythematosus. Each ellipse corresponds to a study estimate of sensitivity and specificity; the area of each ellipse is proportional to the study size. Numbers beside the ellipses are study identification numbers and correspond to those shown in Table 1. Thin lines indicate nonweighted analyses; thick lines indicate weighted analyses. Shaded rectangles mark the 95% confidence intervals of the pooled sensitivity and pooled specificity obtained by random-effects calculations. × indicates exact estimates. **A**, All diffuse neuropsychiatric manifestations versus focal neurologic events. **B**, Psychosis and/or mood disorder versus other diffuse neuropsychiatric manifestations. **C**, Patients with psychosis and/or mood disorder versus all other lupus patients.

improved, but it was still suboptimal (42%), and the specificity remained essentially the same (81%). There was still significant asymmetry in the SROC curves for the diagnosis of psychiatric disorders (Figures 3B and C). Anti-P antibody testing was not more accurate when used to discriminate active NPSLE from non-NPSLE (Table 3 and Figure 3D). Weighted and nonweighted SROC curves were almost coincident in all these contrasts (Figure 3).

Findings of other sensitivity analyses. Analyses limited to studies that used the ACR criteria for NPSLE yielded similar results. The weighted sensitivity for NPSLE overall was 29% (95% CI 17–45%) and the weighted specificity was 79% (95% CI 73–84%). Analyses excluding studies that did not specify blinding yielded a sensitivity of 25% (95% CI 13–43%) for the diagnosis of NPSLE and a specificity of 79% (95% CI 70–86%). Likewise, the diagnostic performance of anti-