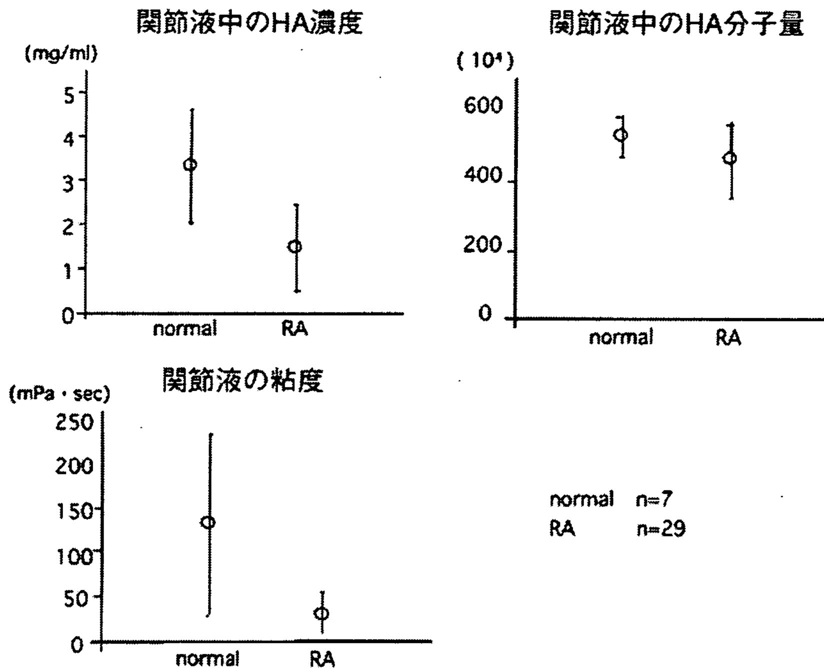

図6　関節液中のヒアルロン酸濃度と分子量，および関節液の粘度

正常コントロール群と比較してRA患者では関節液中のHAの濃度，分子量ともに低下しており，関節液の粘度も低下していた．

関節液の容量が増しているためであると思われる．関節の運動機能において，関節液の粘稠度は重要な因子であるが，HAは関節液に粘弾性を与える重要な成分の一つである．その中でも特に高分子，高濃度のHAが高い粘弾性を示すことが知られているが，コントロール群と比較してRA患者ではHAの濃度，分子量ともに低下しており(図6)，結果として粘弾性の低下を引き起こし，これが関節の潤滑液としての運動機能の低下に結びついていることは十分予測できる．

血中のHAの動態[3)4)5)16)17)]

HABPによるバインディングアッセイ法を用いて血中のHA濃度を測定した結果，RA患者ではコントロール群に比較して血中のHA濃度が有意に高値を示していた．他の種々の検査データと血中HA濃度との関係を検討した結果，CRPとは若干相関する傾向がみられたものの，赤沈や他の因子との相関はみられなかった．Steinbrockerのstage分類と比較してみると，血中のHA濃度はstageの高い症例で高値を示す傾向(stage Ⅰ＜Ⅱ＜Ⅲ＜Ⅳ)が認められ

図7　血中ヒアルロン酸濃度とSteinbrockerのstage分類

血中ヒアルロン酸濃度はstage Ⅲ，Ⅳの破壊が高度な群ではⅠ，Ⅱの破壊の軽度な群よりも有意に高値を示していたが，ⅢやⅣでも低値を示す症例も認められた(文献16より引用)．

る一方で，stageが高くてもHA濃度の低い症例もしばしば認められた(図7)．そこで，Steinbrocker分類よりも細かく関節の破壊について評価するLarsen分類を用いて，関節の破壊度と血中HA濃度との関係について解析した．Larsen分類による関節破壊のgradeが過去1年間で進行した症例では，非進行群に比較して血中のHA値が高値を示す傾向が認められた(図8)．さらに，4年間follow-upできた7症

例について，血中 HA 濃度の推移をみると，7例中1例で3年目から4年目にかけて血中 HA 値が急激に上昇していたが，これはこの時期に一致して，両膝関節の Larsen grade がIIIからIVに進行した症例であった．一方，他の6症例については，Larsen grade がIVやVと高度な関節破壊を示しているにも関わらず，血中 HA 濃度は高値を示さなかった．これらの6症例では Larsen grade は4年間不変であった．つまり，血中の HA 濃度は関節破壊の程度を表すのではなく，破壊が現在進行しつつあるかどうかという「状態」を表すものと思われ，血中の HA 濃度は関節破壊の活動性を示す適切なマーカーとなりうることが示唆された．

以上，今回は滑膜，軟骨，関節液，血中における HA の動態を検討したが，RA では HA の代謝が活発に行われていることが証明された．ここで滑膜から産生される HA の分子量について HAS-1, 2, 3 のそれぞれの陽性細胞との関係から注目すると，RA の炎症性の滑膜組織においては HAS-3 によると思われる低分子 HA の合成が亢進しているが，HAS-1，2によると思われる高分子 HA は滑膜組織，軟骨，関節液いずれにおいても消褪，低下が起こっていることが証明された（図9）．これは，RA 患者の関節における HA が低分子 HA 優位となって粘弾性が低下し，物理的に関節の運動障害をもたらすことを示唆するものである．したがって，治療としての高分子 HA の関節内投与は関節機能の改善をもたらす可能性を示唆している．そこで，次に変性軟骨のモデルを作製し，分子量の異なる HA を組織上で反応させることにより，その分子量間での反応性の違いを検討した．さらに RA 患者の関節液による培養軟骨細胞への傷害性を検討し，そこに分子量の異なる HA を添加して影響を比較した．

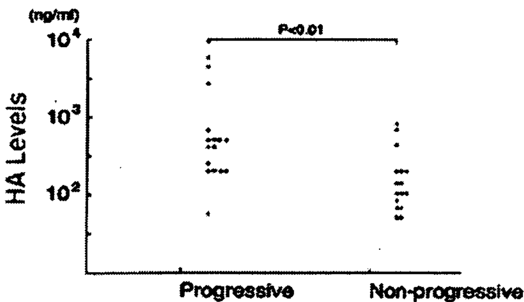

図8　関節破壊進行例と非進行例でのヒアルロン酸値の比較

1年間で Larsen grade が進行した群（Progressive）は非進行群（Non-progressive）に比較して有意に血中 HA 濃度が高値であった（P<0.01）

高分子ヒアルロン酸の投与効果を示す実験

1．変性軟骨に対する分子量の異なる HA の反応性の比較検討[8)14)]

手術で得られたヒト軟骨組織に放線菌由来ヒアルロニダーゼを反応させて切片上の既存の HA を完全に消化し，そのあとに分子量17万，64万，150万，190万の HA をそれぞれ反応させた．変性軟骨に反応した HA の反応性の証明は，それぞれの HA にあらかじめ FITC を標識して蛍光顕微鏡下で蛍光強度を観察する方法と，HA に特異的に結合する HABP にペルオキシダーゼ標識したものを用いるという，2通りで実験を行ったが，いずれにおいても分子量190万

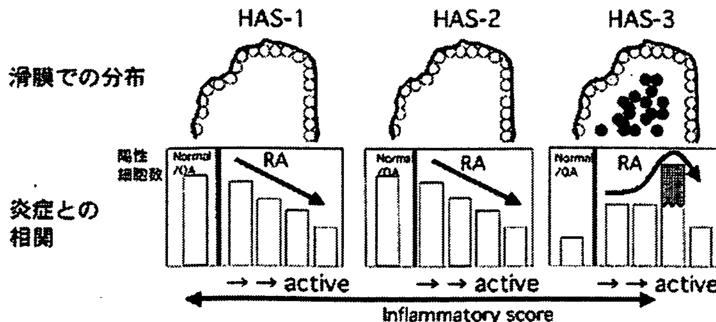

図9　滑膜組織での HAS-1, 2, 3 陽性細胞数と滑膜の炎症との関係

滑膜表層細胞については，HAS-1, HAS-2, HAS-3 ともに陽性となった（○印）．炎症が高度な時期の HAS-3 陽性細胞数の増加分（▦）は，炎症性細胞での陽性細胞の増加分を反映している（●印）と思われる．

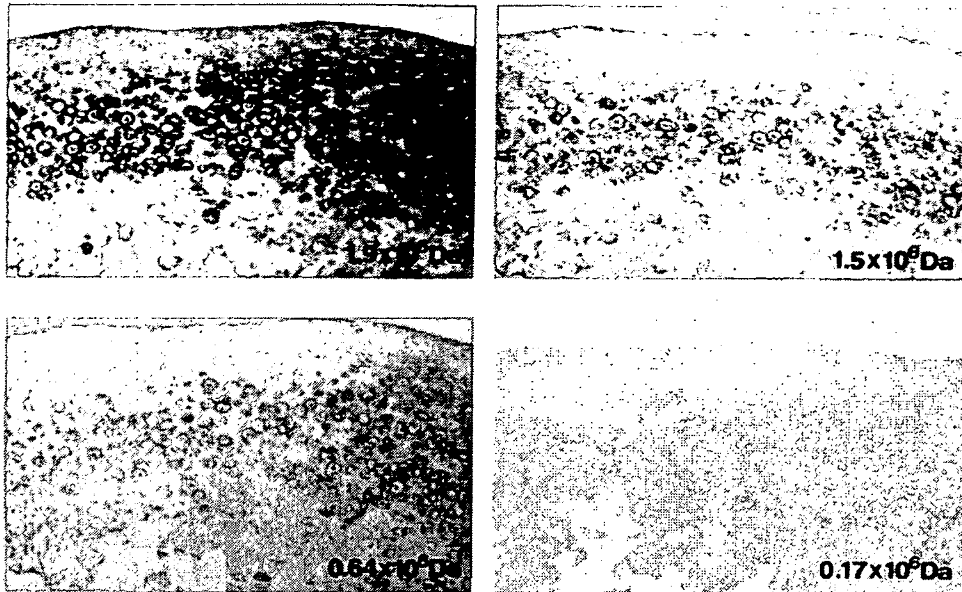

図10　分子量の異なるHAの変性軟骨への親和性の比較

軟骨組織に *Streptomyces* ヒアルロニダーゼを反応させて既存のHAを完全に消化したのちに，分子量17万，64万，150万，190万のHAをそれぞれ反応させた．変性軟骨に反応したHAの反応性の証明はビオチン化HABPにて行なったが，分子量190万のHAによる反応性が最も強く，変性軟骨組織と強固に反応していることが証明された（文献8より引用）．

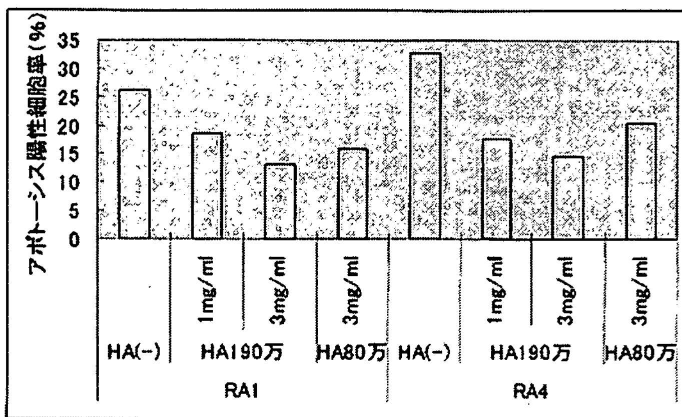

図11　高分子HAによる軟骨アポトーシスの抑制

RA関節液を加えたヒト軟骨細胞培養系に高分子HA（分子量190万：1 mg/ml，3 mg/ml，分子量80万：3 mg/ml）を添加し，それぞれ一晩培養した後，TUNEL染色でアポトーシスに陥った細胞を検出した．その結果，分子量190万のHAでは80万のものに比較してアポトーシスの抑制効果は大きかった（文献2より引用）．

のHAによる反応性が最も強く，変性軟骨組織と強固に反応していることが証明された（図10）．

2．RA患者関節液による軟骨細胞のアポトーシス誘導と高分子HAによるアポトーシス抑制[2)]

ヒト培養軟骨細胞に対してRA患者の関節液を与えるとアポトーシスが起こることがTUNEL法で証明された．この系に分子量80万と190万のHAをそれぞれ添加すると，TUNEL陽性細胞数は減少したが，190万のHAを加えた場合によりアポトーシスの抑制効果が強いことが証明された（図11）．これはHoechst33342染色によるアポトーシス検出方法を用いても同じ傾向を示した．

以上の結果より，変性軟骨に対してHAを投

与すると高分子の HA ほど変性した軟骨基質への新和性や軟骨細胞へのアポトーシス抑制効果が強いことが証明された．

3．RA 患者の関節腔への投与結果[18]

RA 患者に高分子 HA を投与し，投与前後での滑膜組織中の炎症性細胞浸潤の程度をマクロファージ，リンパ球などのマーカーに対する抗体を用いて免疫染色を行って検討した．その結果，有意の差はみられなかったものの，投与後の滑膜組織での炎症性細胞数(マクロファージ，Tリンパ球，Bリンパ球）の減少傾向が確認された．

HA 治療における今後の問題点

本研究の今後の問題としては，関節組織における高分子 HA の分解の阻止が可能であるか，あるいは RA 患者の関節液によるアポトーシスの誘導が HA により如何なる機序で抑制されるか，さらに RA 患者の血中で増加している HA の分子量は高分子か，低分子かということでありいずれも興味のある問題である．

まとめ

1）RA の滑膜組織では高分子 HA が減少し，低分子 HA の合成が亢進している．
2）軟骨では周囲の炎症性細胞から産生される蛋白分解酵素，関節液からの影響，軟骨細胞自身の蛋白分解酵素の産生や NO の発現などにより HA の分解が亢進している．
3）RA 患者の関節液では，正常に比較して HA の分子量，濃度，粘度の低下が証明された．
4）血中の HA 濃度は関節破壊の亢進している症例で上昇していた．
5）高分子 HA は低分子 HA に比較して変性軟骨との親和性がよく，RA 患者の関節液によって引き起こされる培養軟骨細胞のアポトーシスを抑制した．

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ORIGINAL ARTICLE

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Adrenocorticotropic hormone and dehydroepiandrosterone sulfate levels of rheumatoid arthritis patients treated with glucocorticoids

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Abstract To assess adrenal function with respect to the presence or absence of steroid therapy, we investigated differences in the blood levels of adrenocorticotropic hormone (ACTH) and dehydroepiandrosterone sulfate (DHEAS) in relation to steroid (prednisolone) administration in 123 patients with rheumatoid arthritis (RA). Levels of ACTH and DHEAS were significantly lower in the steroid-treated group than in the non-treated group (ACTH: 11.79 pg/ml vs 27.92 pg/ml) (DHEAS: 418.12 ng/ml vs 883.91 ng/ml) ($P < 0.0001$). We observed no steroid dose-related differences in ACTH levels. However, DHEAS levels showed a slight decrease at a prednisolone dose of 2.5 mg/day, with a significant decrease being observed at a dose of 5 mg/day when statistical adjustments were made for age and sex ($P < 0.0001$). At doses of 7.5 mg/day or greater, DHEAS levels were significantly lower than those for 5 mg/day ($P < 0.0006$). These results suggest that low-dose prednisolone reduces adrenal function in patients with RA. We recommend that doses of prednisolone should be limited to 5 mg/day or less in consideration of adrenal function when treating RA patients. The measurement of ACTH and DHEAS may be useful for evaluating adrenal function in patients with RA.

Key words Adrenocorticotropic hormone (ACTH) · Dehydroepiandrosterone sulfate (DHEAS) · Rheumatoid arthritis (RA) · Steroid administration · Stress

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Introduction

Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) are secreted by the adrenal cortex and are classified as sex hormones.[1] Dehydroepiandrosterone sulfate levels markedly increase during adolescence, and reach a peak at about 20 years of age, after which they decrease with age. As such, DHEAS may be used as a parameter of aging.[2,3] Previous studies have suggested that DHEAS prevents osteoporosis, cardiovascular disease and arteriosclerosis, and it is becoming increasingly recognized that DHEAS is important for maintaining a healthy state.[4,5] In patients with rheumatoid arthritis (RA), there have been reports of normal, increased, and decreased DHEAS values.[6–9] However, steroids are often administered to these patients, and may decrease the levels of adrenocorticotropic hormone (ACTH) and DHEAS via negative feedback mechanisms.[1] Furthermore, a previous study has reported that blood levels of DHEAS are constant for a long period,[8] but that mental or physical stress and inflammatory responses may influence DHEAS levels via ACTH.[1,10] In this study, we investigated differences in the blood levels of ACTH and DHEAS in subjects on and off of various doses of steroids. In addition, we examined changes in ACTH and DHEAS levels in 14 patients for whom these parameters were measured twice.

Patients and methods

Of 130 RA patients meeting the criteria established by the American College of Rheumatology,[11] for whom levels of ACTH and DHEAS were measured between February 2002 and September 2002, there were 123 patients (137

samples) for whom confirmation of the undertaking and dosages or non-undertaking of prednisolone therapy could be made (patients treated with betamethasone were excluded). In the 14 patients for whom these parameters were measured twice, the values at the second measurement were also evaluated. Subjects were 123 patients (26 males, 97 females) consisting of 19 inpatients and 104 outpatients, with a mean age of 57.24 years (±12.58) at the time of examination. Steroids had been administered to 78 of these patients, the daily prednisolone doses were 2.5mg in 16 patients, 5mg in 47 patients, 7.5mg in 9 patients, and 10mg in 6 patients (Table 1).

Adrenocorticotropic hormone levels were determined by a one-step IRMA (immunoradiometric assay) method using biotinylated antibodies, and, for B/F (bound/free) isolation, avidin-bound beads were used (Allegro HS-ACTH IRMA kit: Nichols Diagnostics, San Juan Capistrano, CA, USA). Serum DHEAS was determined after serum was allowed to react competitively with I-labeled DHEAS in a DHEAS antibody coated tube (DHEA-S kit: Coat-A Count DHEA-SO_4, Mitsubishi Kagaku Iatron, Tokyo, Japan). In addition, we investigated the correlations of ACTH and DHEAS values with age, erythrocyte sedimentation rate (ESR), matrix metalloproteinase (MMP_3), red blood cell count (RBC), and rheumatoid factor (RF). Blood samples were measured between 08:30 and 11:00h. Before the measurement of these values, informed consent was obtained from all patients.

Reproducibility of ACTH and DHEAS measurements

Using 3 samples of ACTH with different concentrations, Intra-day ACTH concentrations were evaluated 10 times on 1 day. The coefficients of variation (CV) were 3.8%, 2.4%, and 2.2%. Inter-day ACTH concentrations were evaluated daily over a period of 5 days. The CV were 1.3%, 5.1%, and 3.6%. Using 3 samples of DHEAS with different concentrations, intra-day DHEAS concentrations were evaluated 20 times on 1 day. The CV were 7.2%, 4.7%, and 3.4%. Inter-day DHEAS concentrations were evaluated daily over a period of 10 days. The CV were 10.6%, 4.9%, and 4.2%. These values confirmed the reproducibility of ACTH and DHEAS measurements.

Table 1. Characteristics of 123 rheumatoid arthritis (RA) patients treated with various doses of prednisolone

Steroid dose (mg)	No. of cases	Sex		Age (years)[a]
		Male	Female	
0	45	6	39	54.76 ± 12.37
2.5	26	1	15	61.13 ± 7.91
5	47	11	36	56.45 ± 14.65
7.5	9	5	4	60.33 ± 8.02
10	6	3	3	67.00 ± 3.03
Total	123	26	97	57.24 ± 12.58

[a]Mean ± SD

Statistical analysis

Differences between the ACTH and DHEAS levels of steroid-treated and non-treated RA patients were compared using a Wilcoxon rank sum test. In addition, the effects of steroids on ACTH and DHEAS levels were evaluated by analysis of variance with steroid dosage as a factor, and by analysis of covariance with steroid dosage as a factor and with age and sex as covariates. Prior to the comparison, it was confirmed that there were no correlations between ACTH or DHEAS levels and age, CRP, ESR, MMP_3, RBC, or RF through simple correlation coefficients and partial correlation coefficients. P values of less than 0.05 were considered to be significant.

Results

Differences in ACTH and DHEAS levels related to the steroid administration and dosage

In the steroid-treated group, ACTH and DHEAS levels were lower (Wilcoxon's test, $P < 0.0001$) (Table 2) than the non-treated group. There were no dose-related differences in ACTH levels (Table 3). However, DHEAS levels in the steroid-treated group were lower than in the non-treated group at a prednisolone dose of 2.5mg/day (Table 4), and significant decreases in DHEAS levels were observed at doses of 5mg/day ($P < 0.0002$) or greater after adjustments were made for age and sex (Table 4). In addition, at doses of 7.5mg/day or higher, DHEAS levels were significantly lower than at doses of 5mg/day ($P < 0.0006$).

Table 2. Adrenocorticotropic hormone (ACTH) and dehydroepiandrosterone sulfate (DHEAS) levels in RA patients on and off steroid treatments

Steroid administration	No. of cases	ACTH (pg/ml)	DHEAS (ng/ml)
No	45	27.92 ± 19.74	883.91 ± 655.23
Yes	78	11.79 ± 10.13	418.12 ± 442.83

Values are mean ± SD
$P < 0.0001$

Table 3. ACTH levels in RA patients according to prednisolone dose

Dose (mg)	No. of cases	Mean (pg/ml) ± SD	P[a]
0	45	27.92 ± 19.74	
2.5	16	13.31 ± 8.50	0.0004*
5	47	11.51 ± 8.47	<0.0001*
7.5	9	7.67 ± 4.56	0.0003*
10	6	16.17 ± 24.70	0.0514

[a] Adjusted for age and sex
* Significant difference compared with 0mg dose

Table 4. DHEAS levels in RA patients according to prednisolone dose

Dose (mg)	No. of cases	Mean (ng/ml) ± SD	P^a
0	45	883.91 ± 655.23	
2.5	16	443.94 ± 369.54	0.1648
5	47	482.43 ± 503.51	0.0002*
7.5	9	196.11 ± 173.38	0.0002*
10	6	178.50 ± 176.39	0.0271*

[a] Adjusted for age and sex
* Significant difference compared with 0mg dose

Changes in ACTH and DHEAS levels in 14 patients for whom these parameters were measured twice

In one of the 14 patients, a steroid-free treatment approach was switched to prednisolone therapy at a dose of 5mg/day. In another patient, the daily steroid dose was increased from 5 to 7.5mg. In both of these patients, DHEAS levels decreased (Fig. 1). Some of the remaining 12 patients showed changes in ACTH and DHEAS levels, despite the constant steroid dosage (Fig. 2).

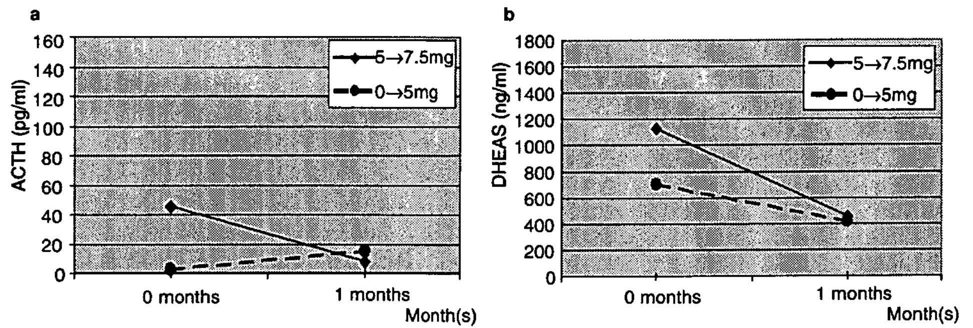

Fig. 1. Changes in adrenocorticotropic hormone (*ACTH*) and dehydroepiandrosterone sulfate (*DHEAS*) levels after increased doses of prednisolone in two patients

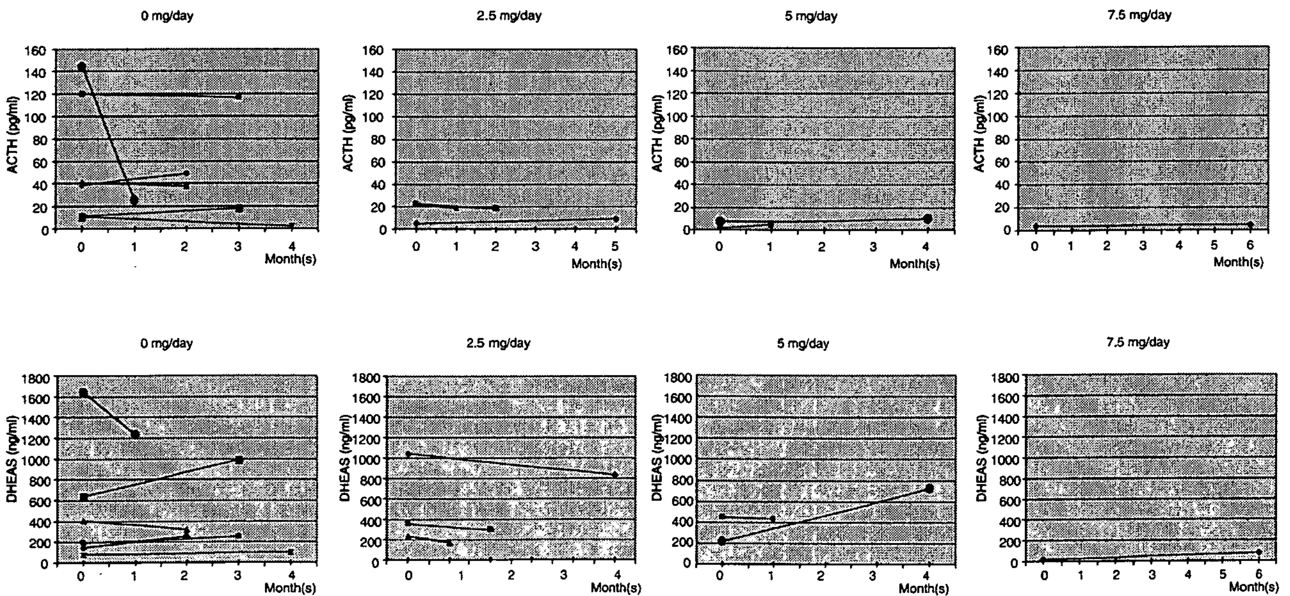

Fig. 2. Changes in adrenocorticotropic hormone (*ACTH*) and dehydroepiandrosterone sulfate (*DHEAS*) levels in patients on fixed doses of steroids

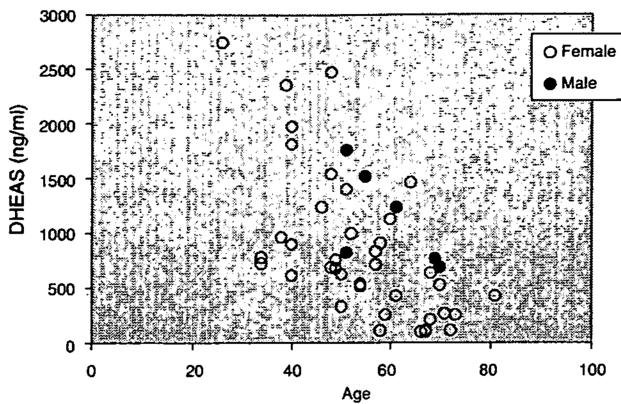

Fig. 3. Dehydroepiandrosterone sulfate (*DHEAS*) levels in rheumatoid arthritis patients without steroid treatment

Correlations of ACTH and DHEAS with age, ESR, CRP, MMP_3, and RBC values

We observed a correlation between ACTH and DHEAS ($r = 0.37125$). Dehydroepiandrosterone sulfate was negatively correlated with age ($r = -0.43464$). In addition, CRP values were correlated with ESR ($r = 0.50421$), RF ($r = 0.46516$), and MMP_3 ($r = 0.39224$) ($r > 0.3000$ or more was extracted).

Discussion

Dehydroepiandrosterone sulfate levels decrease with age,[2,3] and a high blood DHEAS level is related to a long life span.[12] It is also thought that DHEAS may exhibit antiaging actions. In our patients, DHEAS was negatively correlated with age, supporting that supposition. Furthermore, cortisol and DHEAS are secreted by the adrenal cortex as a result of stimuli from ACTH secreted by the pituitary gland.[1] However, cortisol has a negative feedback mechanism, and blood levels of cortisol show a marked circadian variation.[1,13] In addition, steroids are administered to a high percentage of patients with RA. In comparison to cortisol, DHEAS does not have any negative feedback mechanism, and there may be less marked circadian variations,[1] meaning that the measurement of ACTH and DHEAS levels may be more useful for evaluating adrenal function.

Studies have reported that the blood levels of DHEAS in patients with RA are lower,[6-8] or similar[9] to those of normal controls; however, in the Hall et al.[7] study involving a large number of samples, it was reported that blood levels of DHEAS are lower in patients with RA, and that these levels are further reduced in patients receiving steroids or in those with a history of steroid use. We found that the levels of DHEAS and ACTH were decreased in the group receiving steroid therapy. There were no steroid dose-related differences in ACTH levels. However, DHEAS levels were reduced at a prednisolone dose of 2.5 mg/day, with levels significantly lower at a dose of 5 mg/day, and with further decreases at doses of 7.5 mg/day or higher. We consider a negative feedback mechanism to be involved in steroid therapy-related decreases in the levels of DHEAS and ACTH. While we observed significant differences in levels of DHEAS, there were no significant differences in ACTH levels related to steroid dose, possibly because there was circadian variation in ACTH[1] and because samples were not collected at a specific time point (samples were collected between 08:30 and 11:00 h). Another study has reported that steroid therapy reduces the responsiveness of ACTH to hypoglycemic stress stimuli, although the function of ACTH is maintained.[14] Many of our subjects visited our hospital as outpatients from distant areas by train or bus, which may have influenced ACTH levels as a result of physical activity.[15] In addition, mental stress and increases in interleukin-6 and tumor necrosis factor-α may influence the ACTH level.[1,10] These factors may have contributed to no steroid dose related differences being noted in the ACTH levels, which differed from our results for DHEAS levels.

A previous study has reported that blood DHEAS levels are constant during clinical courses.[8] However, in our study, some of our patients showed changes in ACTH and DHEAS levels, although the steroid dosage was not changed. This may be related to physical or mental stressors, including increased RA activity.[10,16-19] Concerning the relationship between stress and hormones, Nishikaze and colleagues hypothesized that urinary 17-hydroxycorticosteroid (17OHCS mg/g Cr.) and 17-ketosteroid sulfates (17KS-S mg/g Cr.), respectively, reflect the level of stress and recovery ability, and reported that the level of stress and the ability to cope with stress can be assessed by measuring these two urinary hormones.[20-22] Briefly, 17OHCS levels increase in the alarm reaction and resistance phases of Syle's general adaptation syndrome.[23] In the exhaustion phase, the 17OHCS and 17KS-S levels decrease. In the recovery phase, the 17KS-S level increases. We previously reported that the level of stress could be evaluated by measuring urinary levels of 17OHCS and 17KS-S in patients with RA.[24] Urinary 17OHCS is a metabolite of blood cortisol, and 17KS-S is a metabolite of blood DHEA(S).[20] In the presence of ACTH stimulation, cortisol is secreted by the adrenal cortex. We consider that the level of stress in patients with RA and their recovery ability can be evaluated by measuring ACTH and DHEAS levels, and urinary levels of 17OHCS and 17KS-S. Based on Syle's general adaptation syndrome, it is considered that the levels of ACTH and cortisol may increase in the alarm reaction and resistance phases. However, we speculate that when ACTH stimulation shifts to cortisol in these phases, there may be no change or a reduction in DHEAS levels.[9,25] In the exhaustion phase, the adrenal gland function appears to become inhibited, with cortisol and DHEA levels decreasing despite an increase in ACTH levels. In addition, when functional disorders of the hypothalamic-pituitary-adrenocortical (HPA) system occur, the levels of ACTH and DHEAS decrease.[26] Increases in ACTH and decreases in DHEAS were observed in cases of adrenal hypofunction.[1] In this study, as was reported by Hall et al.,[19] DHEAS was not

correlated with ESR or CRP. However, some studies have found correlations between DHEAS and these parameters.[27,28] We speculate that DHEAS levels are increased in patients with marked inflammatory responses, involving ESR and CRP, in the resistance phase described by Syle.[9,10] Thus, the measurement of ACTH and DHEAS may be useful for evaluating HPA system function and stress.

An association between blood levels of DHEAS and osteoporosis has also been suggested,[4,29,30] and it has been suggested that DHEA intake helps prevent osteoporosis.[31–33] On the other hand, it has been reported that prednisolone at doses of more than 5 mg/day may increase the risk of osteoporosis.[34,35] This was supported by the results of our study. In short, DHEAS levels in patients receiving prednisolone at 7.5 mg/day or higher are lower than in those who receive 5 mg/day, thus increasing the risk of osteoporosis.[36] We therefore consider DHEAS may be a possible blood marker of osteoporosis in patients with RA. Dehydroepiandrosterone sulfate levels in the 45 patients not on steroid therapy are presented in Fig. 3. There was a marked variation in DHEAS levels. In patients with decreases in DHEAS, adrenal function may have been reduced. In this group with decreases in the DHEAS levels, adrenal hypofunction may have been associated with the various factors described above. Prior to steroid therapy, adrenal function in patients with RA should be evaluated by measuring blood levels of ACTH and DHEAS.

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Anti–Ribosomal P Protein Antibody in Human Systemic Lupus Erythematosus Up-Regulates the Expression of Proinflammatory Cytokines by Human Peripheral Blood Monocytes

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Objective. **Autoantibodies to ribosomal P proteins (anti-P antibodies) are detected in 12–16% of patients with systemic lupus erythematosus (SLE) and have been found to be associated with some manifestations of the disease, including lupus psychosis and hepatitis. Recent studies have disclosed that anti-P antibodies react with activated T cells but not with B cells, suggesting possible direct effects of anti-P antibodies on immune regulation. The present study was designed to explore the presence of the epitope recognized by anti-P antibodies on human peripheral blood monocytes.**

Methods. **Highly purified peripheral blood monocytes obtained from healthy donors were cultured with or without interferon-γ (IFNγ) in the presence of either anti-P antibodies purified by affinity chromatography from the sera of patients with SLE or control IgG.**

Results. **Flow cytometry analysis disclosed that fresh (day 0) monocytes did not express the ribosomal P epitope, whereas expression of the ribosomal P epitope was induced on annexin V–negative monocytes after activation through plastic adherence for 48 hours. More important, anti-P antibodies (compared with normal IgG or IgG from SLE patients devoid of anti-P antibodies) enhanced the production of tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) by activated monocytes. Accordingly, anti-P antibodies also up-regulated the expression of TNFα and IL-6 messenger RNA in activated monocytes. Of note, $F(ab')_2$ fragments of anti-P antibodies, which do not result in Fcγ receptor (FcγR) crosslinking, also effectively up-regulated the expression of TNFα and IL-6.**

Conclusion. **These results indicate that human peripheral blood monocytes express the ribosomal P epitope upon activation, irrespective of induction of apoptosis. Moreover, the data suggest that anti-P antibodies might modify a variety of inflammatory responses through up-regulation of the expression of proinflammatory cytokines in monocytes, in a manner that does not involve FcγR crosslinking.**

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by the expression of a variety of autoantibodies. Autoantibodies to ribosomal P proteins (anti-P antibodies) are detected in 12–16% of patients with SLE (1). Anti-P antibodies are directed to 3 phosphoproteins (P0, P1, and P2), which are located on the larger 60S subunit of eukaryotic ribosomes and have molecular weights of 38 kd, 19 kd, and 17 kd, respectively (1). Ribosomal P proteins share a common linear determinant that is present in the carboxyl-terminal 22–amino acid sequence (1). Previous and recent studies have disclosed the association of anti-P antibodies with neuropsychiatric disease in SLE (2,3). Moreover, it was recently shown that anti–ribosomal P is strongly associated with hepatitis and

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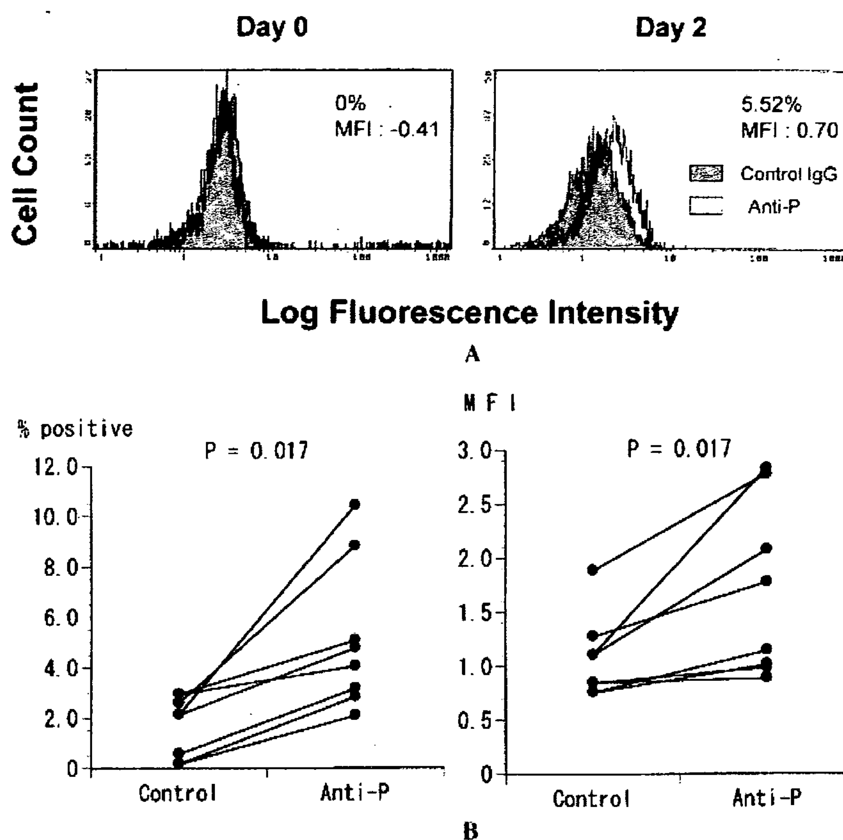


Figure 1. Flow cytometric analysis of peripheral blood monocytes stained with anti–ribosomal P antibodies (anti-P). Highly purified monocytes (1×10^6/ml) were cultured for 48 hours, after which the cells were stained with purified anti-P or normal IgG (5 μg/ml), followed by counterstaining with fluorescein isothiocyanate (FITC)–conjugated goat $F(ab')_2$ anti-human IgG, and propidium iodide (PI). The cells were then analyzed by flow cytometry. **A,** Representative FITC stainings on viable cells gated by negative staining with PI. The percent positive as well as the mean fluorescence intensity (MFI) for specific anti-P staining are indicated. **B,** Binding of anti-P on activated monocytes in different experiments. Each line is representative of anti-P and control IgG in the same experiment. Statistical significance was evaluated by Wilcoxon's signed rank test.

nephritis in SLE (4). However, the pathogenic roles of anti-P antibodies in SLE have not been fully delineated.

The presence of an epitope that is antigenically related to the carboxyl terminus of ribosomal P proteins has been demonstrated on the surface of human hepatoma cells, neuroblastoma cells, fibroblasts, and endothelial cells (5). In addition, a previous study showed that anti-P antibodies bound to the surface of transformed human T cell lines as well as to circulating nontransformed T cells from healthy adults, children, and cord blood (6). We have also demonstrated that the ribosomal P epitope is expressed on the surface of activated CD4+ and CD8+ T cells, but on neither fresh CD4+ and CD8+ T cells nor resting or activated B cells (7). Thus, it is suggested that expression of the ribosomal P epitope might be specific for activated or transformed T cells (7). However, it remains unclear whether monocytes might express the ribosomal P epitope. The current study was undertaken to explore the presence of the ribosomal P epitope on the surface of monocytes and to delineate the effects of anti-P antibodies on the function of monocytes.

MATERIALS AND METHODS

Ribosomal P peptides. A synthetic peptide corresponding to the carboxyl-terminal 22–amino acid sequence of the ribosomal P proteins (residues 90–111 of the *Artemia salina* protein P2) was synthesized by a solid-phase method (7). The purity of the peptide was confirmed as >99% by analytic high-pressure liquid chromatography, amino acid analysis, and microsequencing (7). The synthetic ribosomal P peptide was conjugated to human serum albumin (HSA; Miles, Elkhart, IN) with the use of glutaraldehyde (7).

Human anti-P sera and affinity purification of anti-P antibodies. Sera were obtained from 8 patients with SLE satisfying the American College of Rheumatology 1982 revised criteria for the classification of SLE (8). All patients provided informed consent. IgG fractions were purified from the sera of SLE patients and from the sera of healthy individuals, using a protein G–Sepharose 4FF column (Amersham Pharmacia Biotech, Uppsala, Sweden). Anti-P antibodies were purified from the IgG fractions of SLE sera using an *N*-hydroxysuccinimide–activated Sepharose HP column (Amersham) coupled with ribosomal P peptide–HSA conjugates, as previously described (7). Anti-P antibodies thus purified reacted strongly with ribosomal P peptide–HSA conjugates, but not with HSA alone in an enzyme-linked immunosorbent assay (ELISA). It was also confirmed that purified anti-P antibodies reacted with native ribosomal proteins (P0, P1, P2) on Western blot analysis.

Purified anti-P antibodies did not contain rheumatoid factor, anti-Sm, or anti-DNA. Only 1 preparation of IgG devoid of anti-P antibodies contained modest amounts of anti-Sm. In some experiments, $F(ab')_2$ fragments of IgG were obtained by digesting the IgG fractions of SLE sera with pepsin, followed by gel filtration with a Sephadex G-100 column. The generation of $F(ab')_2$ fragments was confirmed by Western blotting. $F(ab')_2$ fragments of anti-P antibodies were further purified from these $F(ab')_2$ fragments of total IgG using an *N*-hydroxysuccinimide–activated Sepharose HP column coupled with ribosomal P peptide–HSA conjugates. The concentration of endotoxin in purified IgG anti-P antibodies and $F(ab')_2$ fragments of anti-P antibodies was <0.8 pg/ml.

Cell preparation and cultures. Monocytes were purified from peripheral blood mononuclear cells (PBMCs) obtained from healthy adult volunteers, using Monocyte Isolation Kit II (Miltenyi Biotec, Tokyo, Japan). The monocytes contained >95% CD14+ monocytes, <0.1% CD3+ T cells, and <0.1% CD19+ B cells, as determined by analysis with flow cytometry. Purified monocytes (1×10^6/ml) were cultured in flat-bottomed wells of Costar microtiter plates (Corning, NY) in the presence of control IgG or purified anti-P antibodies (5 μg/ml).

Immunofluorescence staining and analysis. Fresh or cultured monocytes ($4–10 \times 10^5$/sample) were stained with affinity-purified anti-P antibodies (5 μg/ml) or control human IgG (5 μg/ml), followed by counterstaining with fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ anti-human IgG (Cappel, West Chester, PA), as previously described (7). After staining, the cells were treated in saline with 50 μg/ml propidium iodide (PI; Sigma, St. Louis, MO) for more than 5 minutes at room temperature, followed by analysis using an EPICS XL flow cytometer (Coulter, Hialeah, FL). The gating threshold for PI staining to identify viable cells was determined using cells without PI staining (7). In some experiments, the cells were stained with phycoerythrin (PE)–conjugated annexin V (BioVision, Mountain View, CA). The percentages of cells that were stained positively for anti-P antibodies were determined in relation to the percentage of staining with control human IgG. The density of staining was expressed as the change in mean fluorescence intensity (MFI) for staining with anti-P antibodies, which was calculated by subtracting the MFI of staining with control IgG.

Measurement of interleukin-6 (IL-6) and tumor necrosis factor α (TNFα). The concentration of TNFα in the culture supernatants was measured using the Human TNFα ELISA Development Kit (PeproTech, London, UK). The concentration of IL-6 in the culture supernatants was determined by bioassay using MH60.BSF2 cells, as previously described (9). Preliminary experiments confirmed that neither control IgG nor anti-P antibodies affected the growth of MH60.BSF2 cells supported by 0.1 units/ml IL-6.

RNA isolation and real-time quantitative polymerase chain reaction (PCR). Total RNA was isolated from cultured cells using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's specifications. Complementary DNA 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 PCRs. Real-time quantitative PCR was performed using the LightCycler rapid thermal cycler system (Roche Diagnostics, Lewes, UK) with primer sets for IL-6, TNFα, or β-actin (LightCycler-Primer Set; Roche Diagnostics, Heidelberg, Germany) and LightCycler-Fast Start DNA Master SYBR Green I (Roche Diagnostics, Heidelberg, Germany). Quantitative analysis was performed using LightCycler version 3.5 software.

RESULTS

Expression of ribosomal P epitope on the surface of peripheral blood monocytes. Our initial experiments examined expression of the ribosomal P epitope on the surface of peripheral blood monocytes, using affinity-purified anti-P antibodies. Figure 1A shows results of a representative immunostain of monocytes with anti-P antibodies. Expression of the ribosomal P epitope on the surface of resting monocytes that were negative for PI staining was negligible. However, after activation with plastic adherence for 48 hours, expression of the ribosomal P epitope on the surface of monocytes was up-regulated. The up-regulation of the ribosomal P epitope on the surface of monocytes was statistically significant (Figure 1B). The binding of purified anti-P antibodies to the surface of activated monocytes was completely blocked by synthetic ribosomal P peptide, confirming the presence of the ribosomal P 22-mer

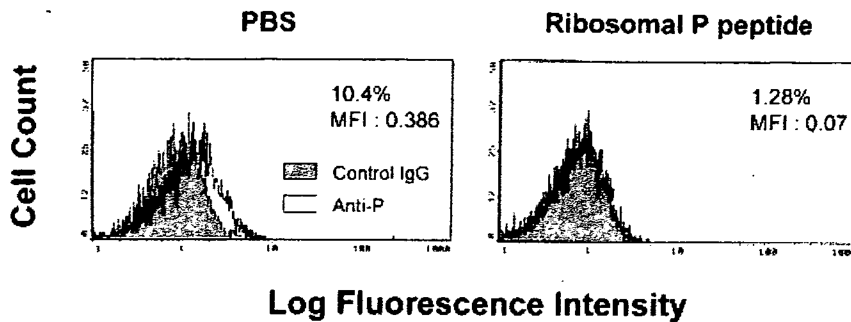


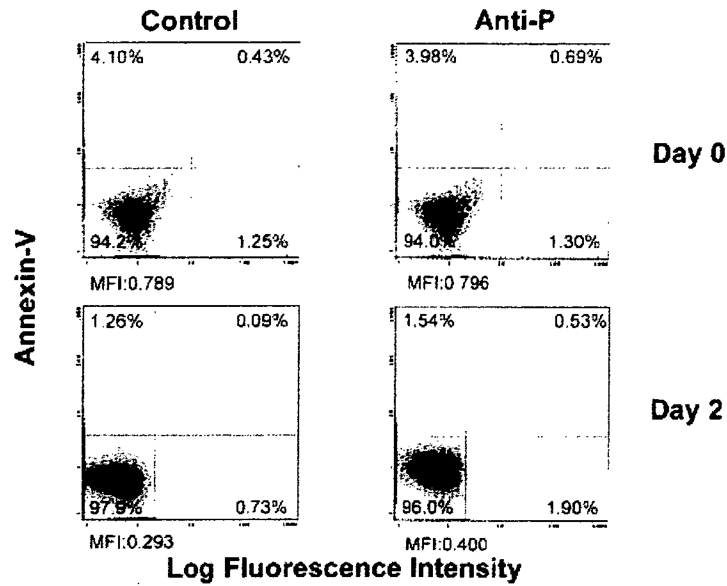
Figure 2. Reversal of binding of anti-P on peripheral blood monocytes. Highly purified monocytes (1×10^6/ml) were cultured for 48 hours, after which the cells were stained with purified anti-P or normal IgG (5 μg/ml) in the presence or absence of the synthetic 22–amino acid peptide (800 μg/ml). The cells were then counterstained with FITC-conjugated goat F(ab')$_2$ anti-human IgG and PI. Shown are FITC stainings on viable cells gated by negative staining with PI. The percent positive as well as the MFI for specific anti-P stainings are indicated. Data are representative of 2 independent experiments. PBS = phosphate buffered saline (see Figure 1 for other definitions).

mimetope on activated human peripheral blood monocytes (Figure 2).

Figure 3A shows representative results of 2-color flow cytometry of activated monocytes stained with PE-conjugated annexin V and with anti-P antibodies or control IgG followed by counterstaining with FITC-conjugated goat F(ab')$_2$ anti-human IgG. Resting monocytes contained ~4.5% annexin V–positive cells, whereas monocytes activated through plastic adherence for 48 hours contained only 2% annexin V–positive cells. Although annexin V–positive activated monocytes contained a significant number of anti-P antibody–positive cells, the fluorescence intensity for anti-P antibody staining of annexin V–negative activated monocytes was higher than that for control IgG staining. Accordingly, annexin V–negative resting monocytes (day 0) did not express the ribosomal P epitope, whereas annexin V–negative activated monocytes (day 2) did express the ribosomal P epitope (Figure 3B). Therefore, the results confirm that the positive binding of anti-P antibodies might not result from translocation of the intracellular antigens to the cell surface due to apoptosis. The data thus demonstrate that the ribosomal P epitope is not present on resting monocytes but is induced after activation.

Enhancement of production of TNFα and IL-6 by human peripheral blood monocytes by anti-P antibodies. The next experiments examined whether anti-P antibodies might influence the function of human monocytes. Because production of proinflammatory cytokines is an important feature of monocytes in immune regulation, the effects of anti-P antibodies on the production of TNFα and IL-6 were explored. Highly purified peripheral blood monocytes were cultured in flat-bottomed wells of microtiter plates in the presence of affinity-purified anti-P antibodies or control IgG. After 120 hours of culture, the supernatants were harvested and assayed for TNFα and IL-6. As shown in Figure 4A, anti-P antibodies from 1 SLE patient significantly enhanced the production of TNFα and IL-6 by monocytes from 6 different healthy donors compared with normal IgG or IgG devoid of anti-P antibodies from the same SLE patient. Moreover, anti-P antibodies from 8 different SLE patients also significantly enhanced the production of TNFα and IL-6 by monocytes from 1 healthy donor compared with 8 different IgG devoid of anti-P antibodies from the corresponding SLE patients (Figure 4B). These results indicate that anti-P antibodies up-regulate the production of TNFα and IL-6 by peripheral blood monocytes.

Up-regulation of expression of TNFα and IL-6 messenger RNA (mRNA) in human peripheral blood monocytes by anti-P antibodies. The final experiments explored the mechanism by which anti-P antibodies enhance production of TNFα and IL-6 by human monocytes. Highly purified peripheral blood monocytes were cultured in flat-bottomed wells of 24-well microtiter plates in the presence of affinity-purified anti-P antibodies or control IgG. After 48 hours of culture, RNA was extracted from the cells and subjected to real-time PCR analysis. As shown in Figure 5A, anti-P antibodies from 1 SLE patient significantly up-regulated expression of TNFα and IL-6 mRNA by monocytes from 6 different healthy donors compared with normal IgG or IgG devoid of anti-P antibodies from the same SLE patient. Moreover, anti-P antibodies from 7 different SLE pa-



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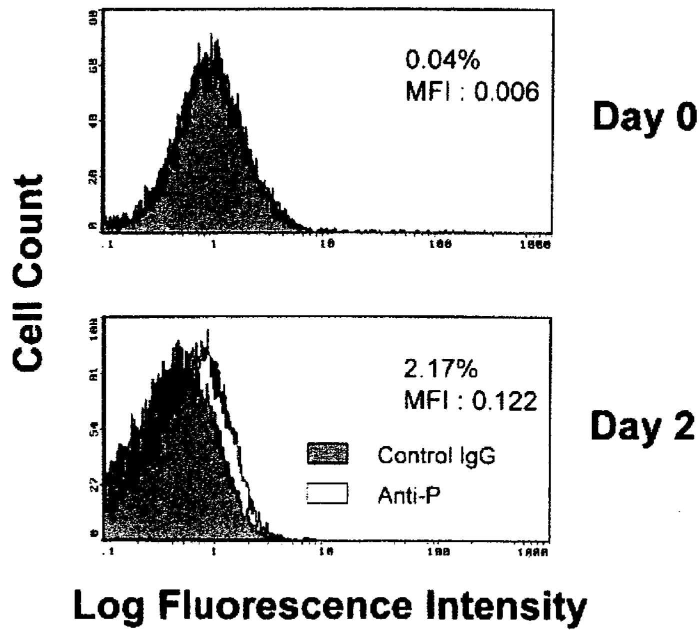

B

Figure 3. Results of 2-color flow cytometric analysis of peripheral blood monocytes stained with anti-P antibodies and annexin V. Highly purified monocytes (1×10^6 /ml) were cultured for 48 hours, after which the cells were stained with phycoerythrin (PE)–conjugated annexin V and purified anti-P or normal IgG (5 μg/ml), followed by counterstaining with FITC-conjugated goat F(ab')$_2$ anti-human IgG. The cells were then analyzed by flow cytometry. **A,** Percentage of cells above the upper limit of control staining without annexin V. **B,** Histograms for FITC staining in gated annexin V–negative cells as shown in **A.** The percent positive as well as the MFI for specific anti-P stainings are indicated. See Figure 1 for definitions.

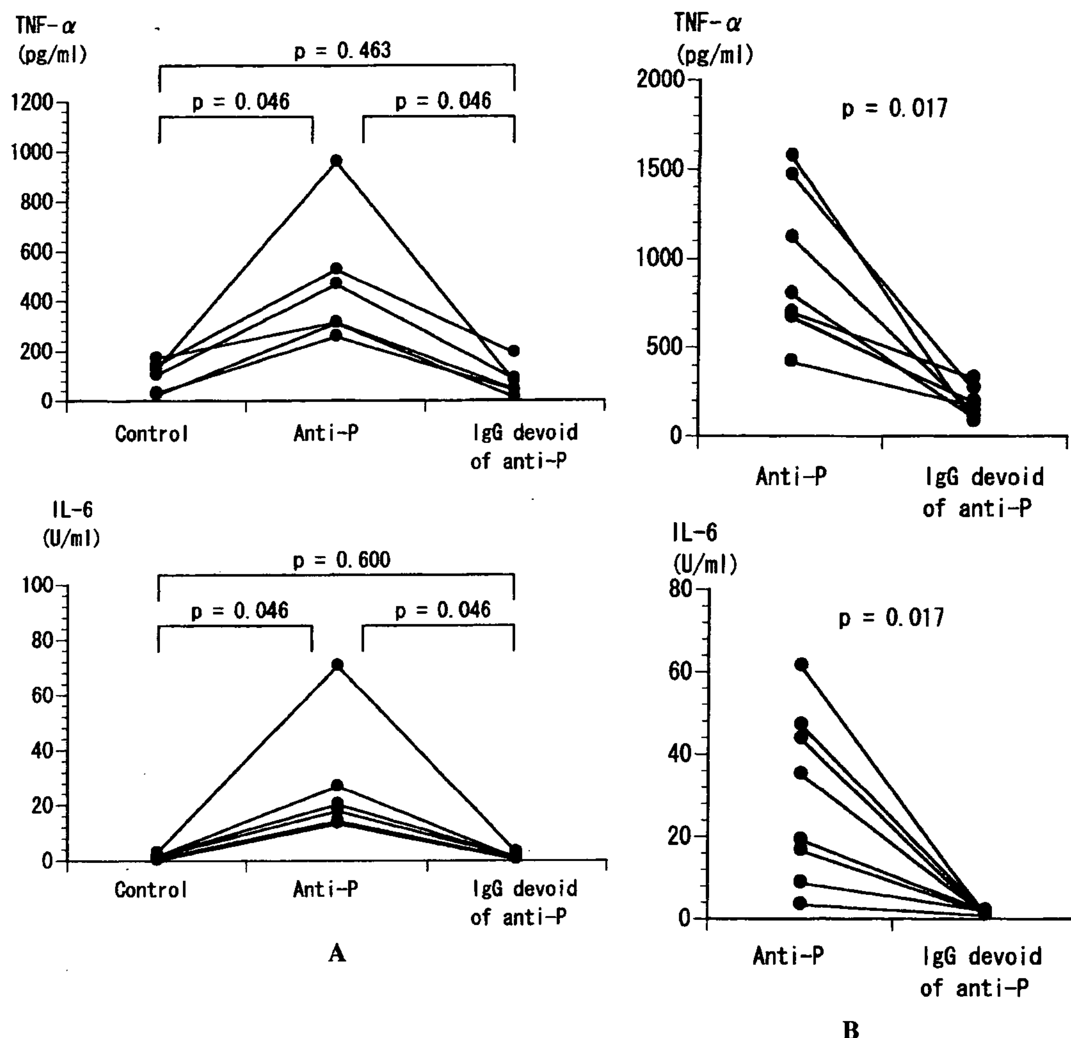

Figure 4. Effects of anti–ribosomal P (anti-P) antibodies on the production of tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) in peripheral blood monocytes. Highly purified monocytes (1×10^6/ml) were cultured in the presence of affinity-purified anti-P or control IgG (5 μg/ml). After 120 hours of incubation, the supernatants were assayed for TNFα and IL-6, as described in Materials and Methods. **A,** Effect of anti-P antibodies from 1 patient with systemic lupus erythematosus (SLE) on monocytes from 6 different healthy donors. Each line on the graph is representative of the same monocyte preparation from the same donor. The mean ± SD values for TNFα are as follows: for control, 100.18 ± 60.27 pg/ml; for anti-P antibodies, 473.37 ± 259.86 pg/ml; for IgG devoid of anti-P antibodies, 77.73 ± 62.45 pg/ml. The mean ± SD values for IL-6 are as follows: for control, 1.46 ± 0.90 units/ml; for anti-P antibodies, 27.22 ± 21.80 units/ml; for IgG devoid of anti-P antibodies, 1.35 ± 0.97 units/ml. **B,** Effect of anti-P antibodies from 8 different SLE patients on monocytes from a single donor. Each line is representative of anti-P antibodies and IgG devoid of anti-P from the same SLE patient. The mean ± SD values for TNFα are as follows: for anti-P antibodies, 926.20 ± 414.41 pg/ml; for IgG devoid of anti-P antibodies, 186.24 ± 81.33 pg/ml. The mean ± SD values for IL-6 are as follows: for anti-P antibodies, 29.43 ± 20.51 units/ml; for IgG devoid of anti-P antibodies, 1.42 ± 0.46 units/ml. Statistical significance was evaluated by Wilcoxon's signed rank test.

tients also significantly up-regulated expression of TNFα and IL-6 mRNA by monocytes from 1 healthy donor compared with 7 different IgG devoid of anti-P antibodies from the corresponding SLE patients (Figure 5B). These results indicate that anti-P antibodies enhance production of TNFα and IL-6 by peripheral blood

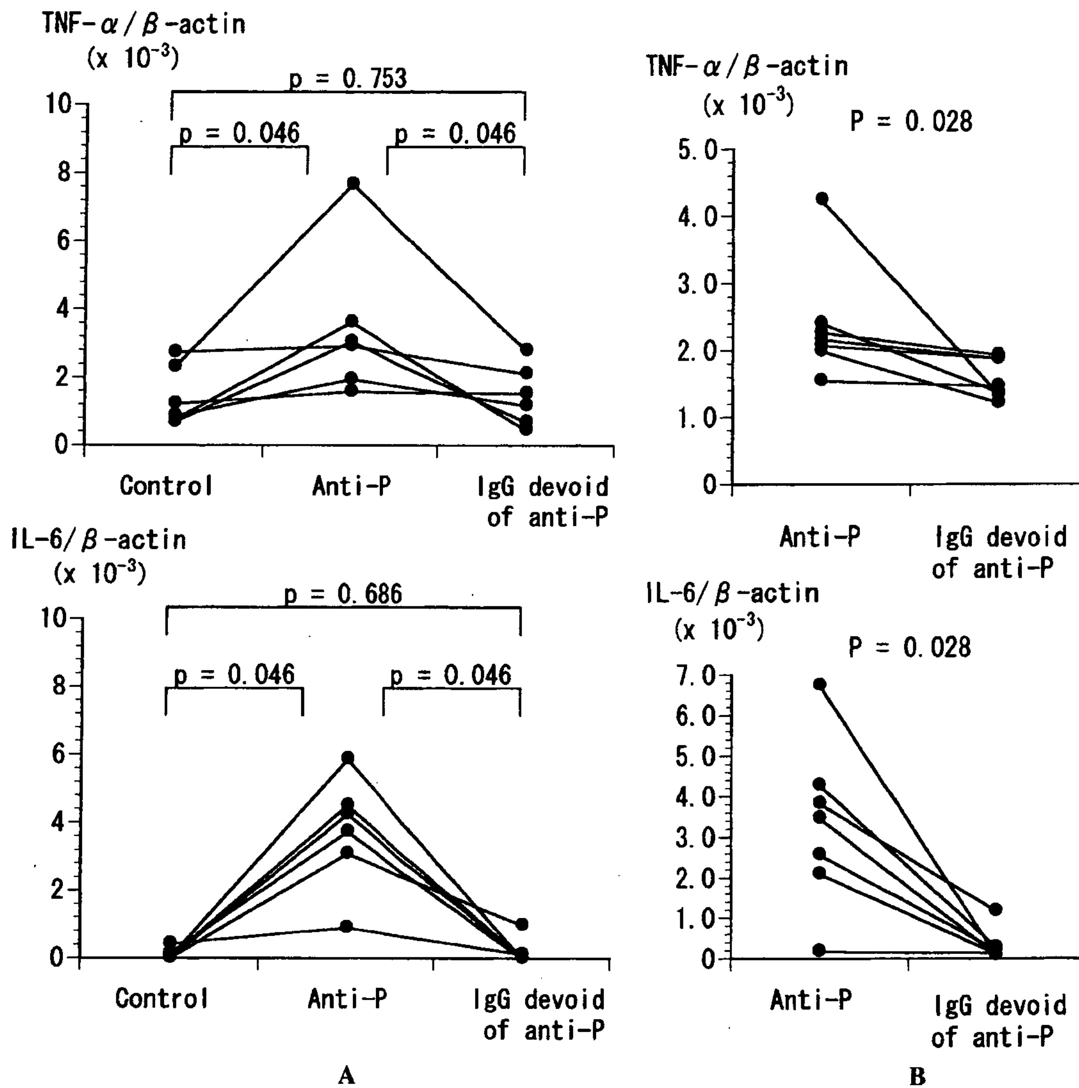

Figure 5. Effects of anti-P antibodies on TNFα and IL-6 mRNA expression in peripheral blood monocytes. Highly purified monocytes (1×10^6/ml) were cultured in the presence of affinity-purified anti-P antibodies or control IgG (5 μg/ml). After 48 hours of incubation, total RNA was isolated, and real-time quantitative polymerase chain reaction was performed as described in Materials and Methods. All results were calibrated to the copy number of β-actin (10^3 copies) from each cDNA sample. **A,** Effect of anti-P antibodies from 1 SLE patient on monocytes from 6 different healthy donors. Each line on the graph is representative of the same monocyte preparation from the same donor. The mean ± SD values for TNFα are as follows: for control, $1.602 \pm 1.056 \times 10^{-3}$; for anti-P antibodies, $3.461 \pm 2.193 \times 10^{-3}$; for IgG devoid of anti-P antibodies, $1.449 \pm 0.886 \times 10^{-3}$. The mean ± SD values for IL-6 are as follows: for control, $0.060 \pm 0.146 \times 10^{-3}$; for anti-P antibodies, $3.720 \pm 1.668 \times 10^{-3}$; for IgG devoid of anti-P antibodies, $0.047 \pm 0.051 \times 10^{-3}$. **B,** Effect of anti-P antibodies from 7 different SLE patients on monocytes from a single donor. Each line is representative of anti-P antibodies and IgG devoid of anti-P antibodies from the same SLE patient. The mean ± SD values for TNFα are as follows: for anti-P antibodies, $2.382 \pm 0.863 \times 10^{-3}$; for IgG devoid of anti-P antibodies, $1.578 \pm 0.301 \times 10^{-3}$. The mean ± SD values for IL-6 are as follows: for anti-P antibodies, $3.309 \pm 2.034 \times 10^{-3}$; for IgG devoid of anti-P antibodies, $0.296 \pm 0.391 \times 10^{-3}$. Statistical significance was evaluated by Wilcoxon's signed rank test. See Figure 4 for definitions.

Table 1. Effect of $F(ab')_2$ fragments of anti-P antibodies on production of TNFα and IL-6 in peripheral blood monocytes*

Experiment, addition	Cytokine production	
	TNFα, pg/ml	IL-6, units/ml
1		
Control IgG	<16.0	1.0
IgG anti-P (KK)	1,207.3	140.9
$F(ab')_2$ anti-P (KK)	586.3	48.1
2		
Control IgG	554.7	5.6
IgG anti-P (KK)	2,387.0	1,555.0
IgG devoid of anti-P (KK)	1,059.0	17.9
$F(ab')_2$ anti-P (KK)	4,643.0	773.0
3		
Control IgG	189.0	9.9
IgG anti-P (MR)	305.0	21.5
$F(ab')_2$ anti-P (MR)	1,173.0	596.1

* Highly purified monocytes (1×10^6/ml) were cultured in the presence of affinity-purified IgG anti–ribosomal P (anti-P), $F(ab')_2$ anti-P (affinity-purified anti-P from $F[ab']_2$ fragments of total IgG), or control IgG (3 μg/ml). After 120 hours of incubation, the supernatants were assayed for tumor necrosis factor α (TNFα) and interleukin-6 (IL-6), as described in Materials and Methods. The initials of the original patients from whom anti-P antibodies were derived are indicated in parentheses.

monocytes through up-regulation of their expression of mRNA.

Up-regulation of expression of TNFα and IL-6 in human peripheral blood monocytes by anti-P antibodies without involvement of Fcγ receptor (FcγR) crosslinking. To investigate the mechanism by which anti-P antibodies up-regulate the expression of TNFα and IL-6 in human monocytes, $F(ab')_2$ fragments of anti-P antibodies were prepared. As shown in Table 1, the $F(ab')_2$ fragments of anti-P antibodies up-regulated the production of TNFα and IL-6 by human monocytes as effectively as did IgG anti-P antibodies. The data indicate that the up-regulation of the expression of TNFα and IL-6 by anti-P antibodies does not require the crosslinking of FcγRs.

DISCUSSION

Because ribosomal antigens are abundant in the cytoplasm, even minor damage to plasma membranes might result in binding of anti-P antibodies with cytoplasmic ribosomal antigens. In fact, previous studies demonstrated that treatment of PBMCs with paraformaldehyde and saponin markedly enhances binding of anti-P antibodies to the cells, whereas anti-P antibodies do not bind to viable PBMCs (7). It is therefore conceivable that resting monocytes do not express the ribosomal P epitope. Accordingly, in the present study the analysis of viable cells gated by negative staining for PI on flow cytometry failed to detect the presence of the ribosomal P epitope on the surface of resting monocytes. In contrast, expression of the ribosomal P epitope was induced on monocytes after activation through plastic adherence in the presence (data not shown) or absence of IFNγ. Of note, the ribosomal P epitope was also induced on T cells after activation with immobilized anti-CD3, although it was absent on the surface of resting T cells or on the surface of resting or activated B cells (7). In this regard, the surface expression of the ribosomal P epitope on monocytes is comparable with that on T cells, confirming that the regulatory mechanism of the expression of this epitope is different depending on the lineage of immunocompetent cells as well as on their state of activation (7).

The binding of affinity-purified anti-P antibodies with the surface of activated monocytes was completely blocked by the addition of the synthetic 22-mer ribosomal P peptide. The data therefore demonstrate that the binding of anti-P antibodies is specific for the 22-mer mimetope, although further studies are required to delineate whether activated monocytes express the whole sequences of ribosomal P proteins. Of note, annexin V–positive monocytes showed enhanced binding with anti-P antibodies, indicating that the ribosomal P epitope is expressed on cells undergoing apoptosis, presumably due to the translocation of the intracellular antigens. However, the ribosomal P epitope was also detected on annexin V–negative activated monocytes but not on annexin V–negative fresh monocytes. Therefore, the results indicate that expression of the ribosomal P epitope is an active process of activation of human peripheral blood monocytes.

The results of the present study demonstrate that anti-P antibodies up-regulate the expression of TNFα and IL-6 in human peripheral blood monocytes, at the protein level as well as the mRNA level. Of note, previous studies revealed that proteinase 3 antineutrophil cytoplasmic antibodies (PR3 ANCA) induce IL-8 release by human peripheral blood monocytes through binding to PR3 expressed on the surface of monocytes (10). It remains unclear whether PR3 ANCA up-regulate expression of IL-8 mRNA in monocytes or simply induce release of preformed IL-8 from monocytes. Because Fab or $F(ab')_2$ fragments of anti-PR3 IgG did not induce IL-8 release, the induction of IL-8 release was considered to involve FcγR crosslinking (10). In contrast, in the present study $F(ab')_2$ fragments of anti-P antibodies up-regulated expression of TNFα and IL-6 in

activated monocytes as effectively as IgG anti-P antibodies. The data therefore demonstrate that up-regulation of the expression of TNFα and IL-6 in monocytes by anti-P antibodies does not involve FcγR crosslinking. In this regard, the mechanism of action of anti-P antibodies is different from that of PR3 ANCA. Further studies are required to clarify the precise mechanism by which anti-P antibodies up-regulate expression of TNFα and IL-6 in monocytes. Nonetheless, the data in the current studies underscore the pathogenic role of anti-P antibodies through induction of proinflammatory cytokines in monocytes.

Although serum anti-P antibodies are closely associated with lupus psychosis (2,3), the mechanism by which anti-P antibodies lead to the development of lupus psychosis remains unclear. The ribosomal P epitope has been shown to be expressed on the surface of human neuroblastoma cells (5). However, anti-P antibodies have not been detected in cerebrospinal fluid from patients with lupus psychosis (3). Thus, there has been no evidence for the direct interaction of anti-P antibodies with neuronal cells in vivo. In contrast, activation of the immune system within the central nervous system (CNS) has been found to play a pivotal role in the pathogenesis of lupus psychosis, as evidenced by enhanced intrathecal immunoglobulin synthesis (11). Of note, monocytes have been shown to modulate the function of endothelial cells (12–14). Thus, TNFα released by coexisting monocytes promotes transendothelial migration of activated lymphocytes (12,13). In addition, IL-6 has been shown to increase adherence of endothelial cells for lymphocytes, facilitating the recruitment of lymphocytes into nonlymphoid tissues (14). It is therefore possible that anti-P antibodies might be involved in the recruitment of immunocompetent cells into the site of inflammation, including the CNS, by stimulating their transendothelial migration through up-regulation of monocyte production of TNFα and IL-6.

Because the ribosomal P epitope is expressed on endothelial cells (10), further studies to examine the direct effects of anti-P antibodies on the functions of endothelial cells would be helpful for complete understanding of the pathogenic role of anti-P antibodies. Finally, although the current studies demonstrate the direct interactions of anti-P antibodies with monocytes to up-regulate their expression of IL-6 and TNFα mRNA, further studies using monocytes from SLE patients would be required to explore the in vivo relevance of our observations.

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