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Analysis of changes in acute-phase plasma proteins in an acute inflammatory response and in rheumatoid arthritis using two-dimensional gel electrophoresis

Two-dimensional (2-D) gel analysis was used to examine differences in the levels of 19 plasma proteins: before and after an acute inflammatory reaction (parenteral typhoid vaccination) in normal subjects, between rheumatoid arthritis (RA) patients and normals and in RA patients treated with tenidap (120 mg) and piroxicam (20 mg). Typhoid vaccination increased levels of SAA, haptoglobin α_1, haptoglobin α_2, haptoglobin β and α_1-anti-chymotrypsin but decreased transthyretin and apolipoprotein E. In RA patients, serum amyloid A (SAA), haptoglobin α_2, haptoglobin β, α_1-antichymotrypsin and C3 proactivator levels were elevated while apolipoprotein A-I, apolipoprotein A-IV, transthyretin, Gc-globulin, α_2-HS glycoprotein, α_2-macroglobulin and α_1-B glycoprotein levels were decreased, compared to normals. Compared to piroxicam, tenidap lowered levels of α_1-antiprotease and SAA but raised the levels of transthyretin, Gc-globulin, α_2-HS-glycoprotein and α_2-macroglobulin in RA patients. C-reactive protein (CRP) could not be quantified on 2-D gels but, when measured by rate nephelometry, levels were reduced after treatment with tenidap compared to piroxicam. The general pattern of the acute phase protein response to an acute inflammatory response to typhoid vaccination is similar to that in the chronic inflammatory condition, RA. The impact of tenidap on both positive and negative acute-phase proteins in RA patients could clearly be distinguished from that of piroxicam.

1 Introduction

Serum amyloid A (SAA) and C-reactive protein (CRP) are positive acute-phase proteins, *i.e.*, in response to a variety of injuries or inflammatory disease states the plasma levels of these proteins are markedly increased [1–3]. Levels of CRP and SAA have been shown to correlate with a number of clinical parameters in inflammatory diseases [4–8] and measurement of SAA and CRP have been incorporated into clinical trials of novel therapeutic agents [4, 9, 10]. The levels of many other proteins also change during the acute-phase response: some of these changes are decreases (negative acute-phase proteins, *e.g.*, albumin, transthyretin, GC-globulin) [11, 12]. This complex pattern of changes could contain information about the disease process, particularly the involvement of cytokines [1, 3] and response to therapy [4]. However, information on the overall pattern of acute-phase protein changes is not routinely available because of the cost, complexity and sample requirements for performing independent assays for each protein studied. Therefore, in an effort to determine whether there is clinically useful information contained in the complex pattern of changes seen in plasma proteins during inflammation, we have utilized two-dimensional (2-D) gel electrophoresis. This technology has been advanced to the point where it is possible to separate and quantify many proteins in a single analysis [13]. The present report describes the application of 2-D gel technology to the analysis of nineteen plasma proteins in two inflammatory conditions: normal volunteers during an acute-phase response induced by parenteral typhoid vaccine, and rheumatoid arthritis (RA). In addition, it has been used to compare the effects of two drugs (piroxicam and tenidap) on the levels of these nineteen plasma protein in RA patients.

2 Materials and methods

2.1 Typhoid vaccination study

After giving informed consent, ten normal healthy volunteers (Table 1) were administered 0.5 mL of phenol- and heat-killed monovalent typhoid vaccine (Wyeth) by subcutaneous injection. Blood samples for preparation of EDTA plasma were collected prior to and 24 and 48 h after vaccination. At each post-vaccination time, the volunteers were asked to complete a questionnaire (see Table 1) to document symptoms.

2.2 RA patients

The RA patients were part of a larger drug study [14] and were required to have rheumatoid arthritis (American Rheumatism Association 1987 Revised Criteria), active disease despite current treatment and have serum CRP levels $\geq$ 1.5 mg/dL. Patients receiving disease modifying

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Abbreviations: **CRP**, C-reactive protein; **DMARD**, disease modifying antirheumatic drugs; **ESR**, erythrocyte sedimentation rate; **RA**, rheumatoid arthritis; **SAA**, serum amyloid A

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Table 1. Clinical signs reported by subjects administered typhoid vaccine

		Clinical response after typhoid vaccination						
Subject #	Age/ Gender	Fever	Pain	Swelling	Redness	Nausea	Chills	Headache
1	31/F			+	+			
2	35/F		+	+				
3	34/M		+		+		+	+
4[a)]	29/M	+	+	+	+			
5	30/M		+	+	+			±
6[b)]	39/M		+	+	+			
7	36/M		+					
8	31/M		+	+	+		+	
9[c)]	27/F		+	+	+			
10	25/F		+	+	+			

a) Subject 4 reported local itch

b) Subject 6 reported swelling and severe pain of left thigh and difficulty walking 36 h after vaccination. Subject attributed the response to exacerbation of osteoarthritis

c) Subject 9 reported severe abdominal cramps 3 to 6 h after vaccination followed by myalgias, fever, arthralgia, dyspnea and weakness about 8 days following vaccination. The subject was treated briefly with systemic prednisone and recovered fully

antirheumatic drugs (DMARDs, *i.e.*, injectable gold, auranofin, D-penicillamine, methotrexate or hydroxychloroquine) had to be on stable therapy for three months prior to and during the study. Patients receiving corticosteroid therapy equivalent to an average daily dose of $\leq$ 10 mg of prednisone were admitted provided that the dosage was stable for one month prior and during the study. Patients with other conditions considered likely to affect acute-phase protein levels (*e.g.*, other inflammatory diseases, burns, major surgical procedures) were excluded. Because of the criteria for their selection, patients were expected to display the characteristic RA acute-phase protein pattern regardless of their current therapy. The study was double-blind, random sequence and crossover in design. At the baseline visit, patients were randomly assigned to double-blind therapy with either tenidap or piroxicam. After treatment in the first six-week period, patients were switched, without a washout period, to treatment with the other agent for the second six-week period (Table 2). Blood samples were collected at baseline and at the end of each six-week treatment period for measurements of total protein, albumin, globulin, erythrocyte sedimentation rate (Westergren, ESR), CRP and transthyretin (rate nephelometry, SmithKline Bio-Science Laboratories), SAA (ELISA assay, Hemagen, Waltham, MA), IL-6 (ELISA assay, R & D Systems, Minneapolis, MN) and 2-D gel analysis (described below). For comparison, blood was collected from a group of healthy individuals, selected so that their age and sex distribution matched that of the patient group (Table 3).

2.3 2-D gel electrophoresis

The methodology used for 2-D gel analysis of proteins has been described previously [15–17] and is summarized briefly here. Two µL aliquots of plasma were mixed with three parts of 2% sodium dodecyl sulfate, 0.05 M CHES buffer, pH 9.5, 1% dithiothreitol, 10% glycerol to denature the proteins. Sample proteins were resolved using the 20 × 25 cm ISO-DALT® 2-D gel system operating with 20 gels per batch. First-dimensional isoelectric focusing gels were prepared using Pharmacia 3–10 carrier ampholytes and run for 33 000–34 000 Vh. An Angelique computer-controlled gradient casting system was used to prepare second-dimensional SDS-gradient slab gels in which the top 5% of the gel was 11%T acrylamide, and the lower 95% of the gel varied linearly from 11% to 18%T. Each gel was identified by a filter paper polymerized into the gel. First-dimension gels were loaded directly onto the slab gels without equilibration and were held in place by polyester fabric wedges (Wedgies™) to avoid the use of hot agarose. Following SDS electrophoresis, the slab gels were stained for protein using a colloidal Coomassie Blue G-250 procedure in covered plastic boxes, 10 gels per box. This procedure involves fixation in 1.5 L of 50% ethanol / 2% phosphoric acid for 2 h, three 30 min washes in 2 L of cold tap water and transfer to 1.5 L of 34% methanol / 17% ammonium sulfate / 2% phosphoric acid for 1 h followed by addition of 1 g of powdered stain. Staining requires approximately 4 days to reach equilibrium density (destaining is not necessary). Each stained slab gel was digitized in red light at 134 µ resolution using an Eikonix 1412 scanner and the digitized gel images processed using the Kepler® software system with an optimized image processing procedure to yield 'spotlists' giving position, shape and density information on each detected spot. Gels were matched to a master 2-D pattern of human plasma proteins, and the abundances of the set of spots representing the variously glycosylated forms of each reported protein were combined to yield an estimate of that protein's abundance. CRP, SAA, and their respective antibodies used for spot identification on Western blots, were purchased from Calbiochem (La Jolla, CA).

2.4 Data analysis

In order to make quantitative comparisons of plasma proteins among different 2-D gels, the integrated staining intensity of each protein spot was normalized by dividing its value by the sum of the intensities on the same gel of all 19 proteins under study. Statistical analyses were carried out using the following software: SAS (SAS Institute, Cary, NC), RS-1 (Bolt Beranek &

Table 2. Background data on RA patients

Subject #	Age/ Gender	Disease duration (years)	RF Titer	MTX[a)]	PRED[b)]	ESR[c)]	Treatment order[c)]
1	54/F	18	2560	7.5		50	PT
2	60/F	10	2560		5	40	PT
3	57/M	1.5	320			70	PT
4	46/F	14	1280	7.5	5	52	PT
5	62/F	18	<20		5	12	PT
6	57/M	15	2560		5	15	PT
7	35/M	3	<20	7.5		114	PT
8	67/M	2	10240	15	10	88	TP
9	48/M	12	10240	15	5	93	TP
10	45/F	3	5120		2	114	TP
11	48/F	17	81920		10	116	TP
12	66/M	10	5120		10	66	TP
13	43/F	12	<20		5	110	TP
14	68/F	21	5120	10		15	TP
15	38/F	3	640			70	TP
16	56/F	3	1280	12.5		74	TP

a) Dose of methotrexate (mg/week)
b) Dose of prednisone (mg/day)
c) mm/h

Table 3. Comparison of the age and gender distribution of the three groups of subjects

Group	Median age (Range)	Number of males	Number of females
Typhoid vaccination	30.5 (25–39)	6	4
RA patients	55 (25–68)	6	10
Matched controls	55 (39–66)	7	7

Newman, Cambridge, MA) and Statistica (StatSoft Inc., Tulsa, OK). The change in plasma concentration of each protein due to typhoid vaccination was estimated by subtracting the normalized intensity of the protein at baseline from its normalized intensity 24 and 48 h after administration of the vaccine. Nonparametric Wilcoxon Signed Rank tests for paired observations, with no correction of *P*-values for multiple comparisons, were used to test the null hypothesis of no change in concentration of each protein. In comparing 2-D gel data in RA patients with matched control subjects, the Wilcoxon Rank Sum test for unpaired data was used to test for null difference for each protein. The immunoassays for transthyretin, CRP and SAA give the absolute concentrations of these proteins (μg/mL). At baseline in the vaccination study, concentrations are normally distributed and means are reported. Following typhoid vaccination, some patients experienced large increases in both CRP and SAA and data distributions were skewed; therefore, medians and ranges of changes from baseline are reported. For comparison of drug effects, statistical significances were computed for within-subject paired differences (values after tenidap treatment minus values after piroxicam treatment) by the Wilcoxon Signed Rank test.

3 Results

3.1 Identification and measurement of proteins on 2-D gels

Approximately 600 protein spots are seen in 2-D gels of human plasma (Fig. 1). It was possible to identify and quantify 19 plasma proteins with confidence, based on their reproducible location on the gels in relationship to previously created maps and absence of overlap with other spots [18, 19]. The relative abundance listed for these proteins are the summed values for all the isoforms identified by Western blotting with specific antibodies [20]. CRP and SAA were located using commercial standards and immunoblotting of RA sera using specific antibodies (essentially as described in [21]). SAA is located just below the haptoglobin α_{1F} chain as two spots corresponding very closely to the positions predicted from the respective sequences of SAA-1 and its des-Arg version (Fig. 1) and to the positions recently reported by Bini and co-workers [22]. The relative abundance of the combined SAA and des-Arg SAA spots was used in subsequent analyses and the limit of detection was 50 μg/mL. CRP comigrated with a single-charge modified form of Apo A-I lipoprotein and could not be quantified on 2-D gels. Measurements of changes in the abundance of haptoglobin α_1 and α_2 chains were not available for all subjects because these proteins form a polymorphic system of three gene products, only one or two of which are expressed in a given individual. This data could be used, however, in comparisons between time points for an individual (*e.g.*, in the typhoid vaccination study).

Correlations between relative concentration measured by 2-D gel methodology and levels measured independently by immunoassay were significant for both SAA ($P < 0.0001$, $r^2 = 0.5726$) and transthyretin ($P < 0.0001$, $r^2 = 0.3762$), validating the 2-D gel assay. Further comparison of the two methods was carried out using Morgan's test, which examines trends in the difference between assay methods versus the mean of normalized values, for each sample. No significant divergence between the assays was detected. The coefficients of assay variation of individual proteins in replicate assays of several of the serum samples were significantly higher for a few proteins than for the others. Proteins in the higher-variability group are ceruloplasmin, Cls, α_1-antichymotrypsin and C3 proactivator; they are suspected of greater lability under the assay conditions than the other proteins.

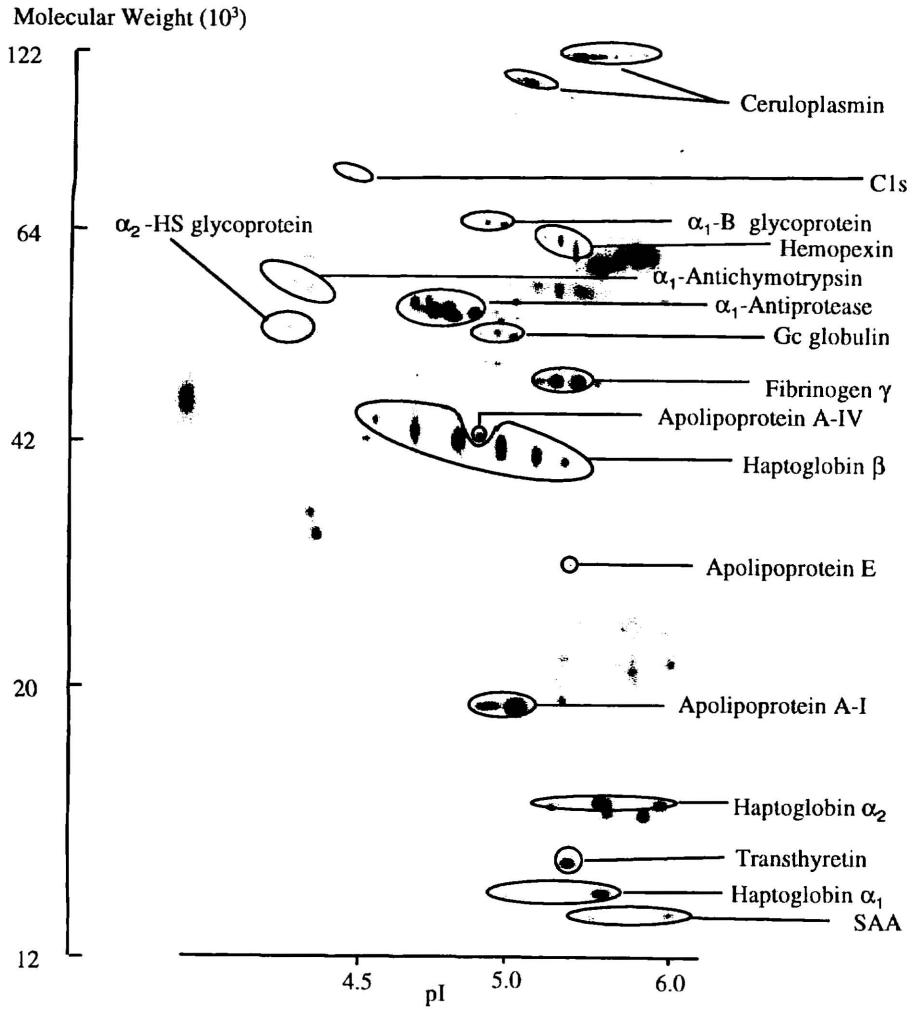

Figure 1. Representative 2-D gel map of RA patient plasma proteins showing the 19 proteins used in this analysis.

As noted above, the most satisfactory method of normalizing the 2-D gel electrophoresis staining intensity data to correct for possible differences among gels in loading, staining and denaturation was also the simplest, *i.e.*, to express all abundance data as a percent of the sum of intensities of all 19 proteins under study. This method gave the lowest coefficient of variation among replicate assays of serum samples, and the best correlation of 2-D gel *vs.* immunoassay for SAA and transthyretin. It allowed accurate estimation of differences in the relative abundance of each protein.

3.2 The acute phase response to typhoid vaccination

All the normal volunteers given the typhoid vaccination reported clinical responses of variable severity: local pain (9/10), swelling (8/10), itch (1/10) and redness (8/10) at the site of injection plus fever (1/10), chills (2/10) and headache (2/10). Volunteer # 6 exhibited exacerbation of left hip osteoarthritis and volunteer # 9 had severe abdominal cramps 3–6 h after injection, followed by myalgias, fever, arthralgia, dyspnea and weakness about 8 days following vaccination (Table 1). The subject was treated briefly with systemic prednisone and recovered fully. Similar responses to typhoid vaccination have been reported previously [23]. CRP and SAA measured by immunoassay both showed clear increases in all ten subjects (Table 4). The levels of these two acute-phase proteins were correlated when data from all time points were pooled (Pearson correlation coefficient of log values = 0.725, $P < 0.0001$). At 24 h after vaccination, 2-D gel analysis detected statistically significant ($P < 0.05$) increases in the level of haptoglobin β chain; none of the other protein changes at this time were statistically significant (Table 5). Forty-eight hours after vaccination, statistically significant increases in the abundance of haptoglobin α_1, haptoglobin α_2, haptoglobin β, and α_1-antichymotrypsin were observed (Table 5). Baseline levels of SAA in normal subjects were below the limit of detection by 2-D gel analysis. However, following vaccination, SAA levels were detectable by 2-D gel analysis in those subjects whose level was above approximately 50 μg/mL as measured by immunoassay (4 of the 10 subjects 24 h after typhoid vaccination and in 7 of 10 at 48 h, Table 5). In addition, 48 h after vaccination the level of apoE lipoprotein and transthyretin were significantly below baseline (Table 5).

3.3 Comparison of the plasma levels of 19 proteins in RA patients and normals

Baseline levels of the 19 proteins analyzed by 2-D gels in the 16 RA patients with active disease and elevated CRP were compared with levels in a group of 14 age- and gen-

Table 4. The SAA and CRP response (measured by ELISA and rate nephelometry, respectively) to typhoid vaccination in 10 normal subjects

		Hours after vaccination					
	Baseline[a]	24			48		
		Change[b]	Range	*P*-value	Change[b]	Range	*P*-value
SAA	6.8	21.5	3.0–224	.002	43.0	3.0–1108	.002
CRP	2.7	6.3	1.1–27.5	.002	11.3	2.8–94.4	.002

a) Expressed as mean μg/mL in plasma of 10 normal subjects

b) Change is the concentration at 24 or 48 h minus the concentration at baseline, expressed as the median. The range of observed changes are shown. *P*-values are calculated by Wilcoxon Signed Rank test

Table 5. The acute-phase response of 19 proteins measured by 2-D gel electrophoresis in 10 subjects administered typhoid vaccine

	Baseline	24 h		48 h	
Protein	Relative abundance[a]	Change[b]	*P*-value	Change[b]	*P*-value[c]
Apolipoprotein A-I	20.41	−0.52	0.43	−1.74	0.19
Apolipoprotein A-IV	1.37	−0.24	0.16	−0.19	0.25
Apolipoprotein E	0.81	−0.07	0.23	−0.13	**0.01**[c]
SAA	d)	0.42	0.13	0.57	**0.02**
α_1-Antiprotease	23.14	−0.95	0.23	−1.37	0.11
α_1-Antichymotrypsin	0.26	0.12	0.15	0.18	**0.01**
α_2-Macroglobulin	4.13	−0.32	0.05	−0.24	0.28
Ceruloplasmin	1.92	−0.24	0.32	−0.19	0.38
C3 proactivator	1.39	0.09	0.63	0.46	0.064
Transthyretin	3.47	−0.18	0.23	−0.56	**0.03**
Gc globulin	4.10	−0.15	0.23	−0.44	0.084
α_2-HS glycoprotein	2.80	−0.01	1.00	−0.38	0.23
α_1-B glycoprotein	2.08	0.00	0.70	−0.07	0.56
Hemopexin	5.70	−0.27	0.28	−0.29	0.38
Cls	0.26	0.02	0.49	0.02	0.77
Fibrinogen γ	11.51	0.28	0.70	0.63	0.50
Haptoglobin α_1	1.31	0.34	0.11	0.80	**0.02**
Haptoglobin α_2	4.37	0.55	0.36	1.80	**0.01**
Haptoglobin β	10.8	1.45	**0.004**	3.11	**<0.01**

a) Relative abundance of each protein is expressed as a percent of the sum of the intensities of all 19 proteins under study

b) Change is the relative abundance at 24 or 48 h minus the relative abundance at baseline, expressed as the median. *P*-values are calculated by Wilcoxon Signed Rank test

c) *P*-values <0.05 are shown boldface

d) Below 50 μg/mL, the limit of detection

der-matched normal volunteers. *P*-values for the differences between the groups were calculated for each protein in 19 individual Wilcoxon (nonparametric) Rank Sum tests. This comparison was used to give a general indication of the nature and magnitude of the acute-phase response in RA patients. The results are presented in Table 6 and represented graphically in Fig. 2A. For 12 of the 19 proteins examined, arthritic subjects are significantly ($P \leq 0.05$) different from normal subjects. Levels of five proteins were significantly ($P \leq 0.05$) higher in RA patients than in normal subjects: C3 proactivator, haptoglobin β, haptoglobin α_2, SAA and α_1-antichymotrypsin. Note that SAA levels were undetectable by 2-D gel in the group of normals, and that data for the haptoglobin α_1 chain is complicated by the unequal distribution of this genetically polymorphic form between the two groups and cannot be adequately analyzed. Levels of seven proteins were significantly ($P \leq 0.05$) lower in arthritic than in normal subjects: α_2-macroglobulin, α_1-B glycoprotein, Gc-globulin, apoA-I lipoprotein apoA-IV lipoprotein, α_2-HS glycoprotein, and transthyretin.

3.4 Comparison of the changes in plasma protein levels induced by an acute-phase response (typhoid vaccination) to the changes seen in RA

The pattern of changes elicited in the acute inflammatory response to typhoid vaccination was compared to differences between RA patients and matched controls by expressing both sets of data as ratios with respect to the relevant controls. The relative abundance value of each protein after typhoid vaccination was divided by the corresponding baseline value, and the mean ratio for all volunteers was determined. The mean relative abundance values of each protein in RA patients was divided by the corresponding mean value in the age- and gender-matched controls. There was a clear linear correlation (Pearson's $R = 0.7848$, $P = 0.00069$) between the two sets of logarithmically transformed ratios (Fig. 3), indicating that the pattern of changes in the two conditions was remarkably similar. Changes due to RA were consistently greater than those caused by typhoid vaccination although the directions of change were consistent. Two

Table 6. Comparison of the relative abundance of 19 plasma proteins measured by 2-D gel electrophoresis in normal individuals and RA patients

Protein	Relative abundance (mean)		
	Normal (N=14)	RA (N=15)	P-value[a]
Apolipoprotein A-I	18.31	13.82	**0.001**[b]
Apolipoprotein A-IV	1.19	0.85	**0.025**
Apolipoprotein E	0.56	0.45	0.21
SAA	b)	0.92	**0.0001**
α_1-Antiprotease	22.21	21.93	0.55
α_1-Antichymotrypsin	0.40	0.89	**0.003**
α_2-Macroglobulin	4.26	3.39	**0.011**
Ceruloplasmin	1.15	1.91	0.058
C3 proactivator	0.74	1.93	**0.0001**
Transthyretin	3.58	1.80	**0.0001**
Gc globulin	4.30	2.88	**0.0001**
α_2-HS glycoprotein	3.09	1.77	**0.0002**
α_1-B glycoprotein	2.51	1.66	**0.007**
Hemopexin	5.59	5.16	0.68
Cls	0.24	0.25	0.82
Fibrinogen γ	12.13	12.18	0.91
Haptoglobin α_1	3.09	4.16	c)
Haptoglobin α_2	6.39	10.31	**0.017**
Haptoglobin β	13.12	20.94	**0.0006**

a) P-values <0.05 are shown boldface, calculated by the Wilcoxon Rank Sum test

b) Below 50 μg/mL, the limit of detection

c) Data for the haptoglobin α_1 chain is complicated by the unequal distribution of this genetically polymorphic form between the two groups and cannot be adequately analyzed

proteins lay outside the 98% bivariate confidence bounds: for C3 proactivator and haptoglobin α_2 the increase due to RA was proportionally larger than the increase due to typhoid vaccination (Fig. 3).

3.5 Age and gender differences in plasma proteins in normals

Effects of age and gender on relative abundance of individual proteins determined from the 2-D gels were examined in data pooled from the two groups of normal subjects: normal controls matched to RA patients, and baseline measurements of normal volunteers for the typhoid vaccination study. Linear regression analysis of the relative abundance of each protein against age revealed a significant ($P < 0.01$) decrease with increasing age for two of the 19 proteins studied: ceruloplasmin and C3-proactivator. Note that C3-proactivator was one of the two outliers when the changes induced by typhoid vaccination were compared with the differences between RA patients and age- and gender-matched controls (Fig.3). This analysis of age effects is hampered by the fact that neither group covers a sufficient age range to support within-group analysis and there is minimal overlap in ages between the two control groups (see Table 3). Thus, the age-related trends observed could be due to either age or an unknown categorical difference between the two groups. In an analysis of molecular variance (ANOVA) with gender nested within groups, women were found to have significantly ($P < 0.05$) higher levels of α_1-antichymotrypsin.

3.6 Relative abundance of acute-phase proteins in normals

Correlation analysis was carried out for all pairs of proteins by Spearman's rank method using data from the 24 normal individuals available (baseline values for the ten normal volunteers in the typhoid vaccination study and the 14 matched controls in the RA study). Relationships were identified which resembled those seen in inflammatory conditions, *i.e.*, the levels of positive acute-phase proteins were positively correlated with each other and were inversely related to the levels of negative acute-phase proteins. For example, there were significant ($P < 0.01$) positive correlations among the three haptoglobin species but negative correlations between the haptoglobins and Gc globulin, α_2-HS glycoprotein, α_1-B glycoprotein and apolipoproteins A-I and A-IV.

3.7 Comparison of the effects of tenidap and piroxicam in RA patients

The differences between the value at the end of each drug treatment period and the baseline value, without regard to the order in which the drugs were administered, are tabulated (Table 7). Statistical analysis utilized the paired differences at the end of each treatment period to identify differences between therapy with tenidap and piroxicam. The 2-D gel data revealed significantly ($P \leq 0.05$) lower levels with tenidap treatment of two positive acute phase proteins, α_1-antiprotease and SAA, in comparison to piroxicam (Table 7). In addition, the levels of four negative acute phase proteins, Gc globulin, transthyretin, apolipoprotein E and α_2-macroglobulin, were significantly elevated after tenidap compared to piroxicam (Table 7). In Fig. 2B these changes are illustrated graphically and compared with the differences between these RA patients and the age- and sex-matched normals (Fig. 2A).

Measurement of SAA by ELISA confirmed the results from 2-D gels: treatment with tenidap reduced the elevated levels of this positive acute-phase protein while treatment with piroxicam was associated with higher SAA levels (Table 8). Transthyretin levels measured by rate nephelometry also confirmed the 2-D gel results in that levels of this negative acute-phase protein were higher after tenidap (*i.e.*, closer to normal values) than after piroxicam. However, this difference was a result of a greater decrease with piroxicam than tenidap (Table 8). The absolute amounts of these two proteins measured by immunoassay showed good correlation with their relative abundance measured by 2-D gel electrophoresis ($P < 0.001$). In addition, for the sixteen subjects reported here, levels of CRP (a positive acute-phase protein, measured by rate nephelometry) were reduced after treatment with tenidap compared to piroxicam (Table 8). The ESR, a positive acute-phase reactant, was also reduced by tenidap compared to piroxicam (Table 8). IL-6 levels were lower after tenidap than after piroxicam but this difference did not reach statistical significance ($P = 0.07$, Table 8). Levels of total serum protein, albumin or globulins showed no treatment-related differences.

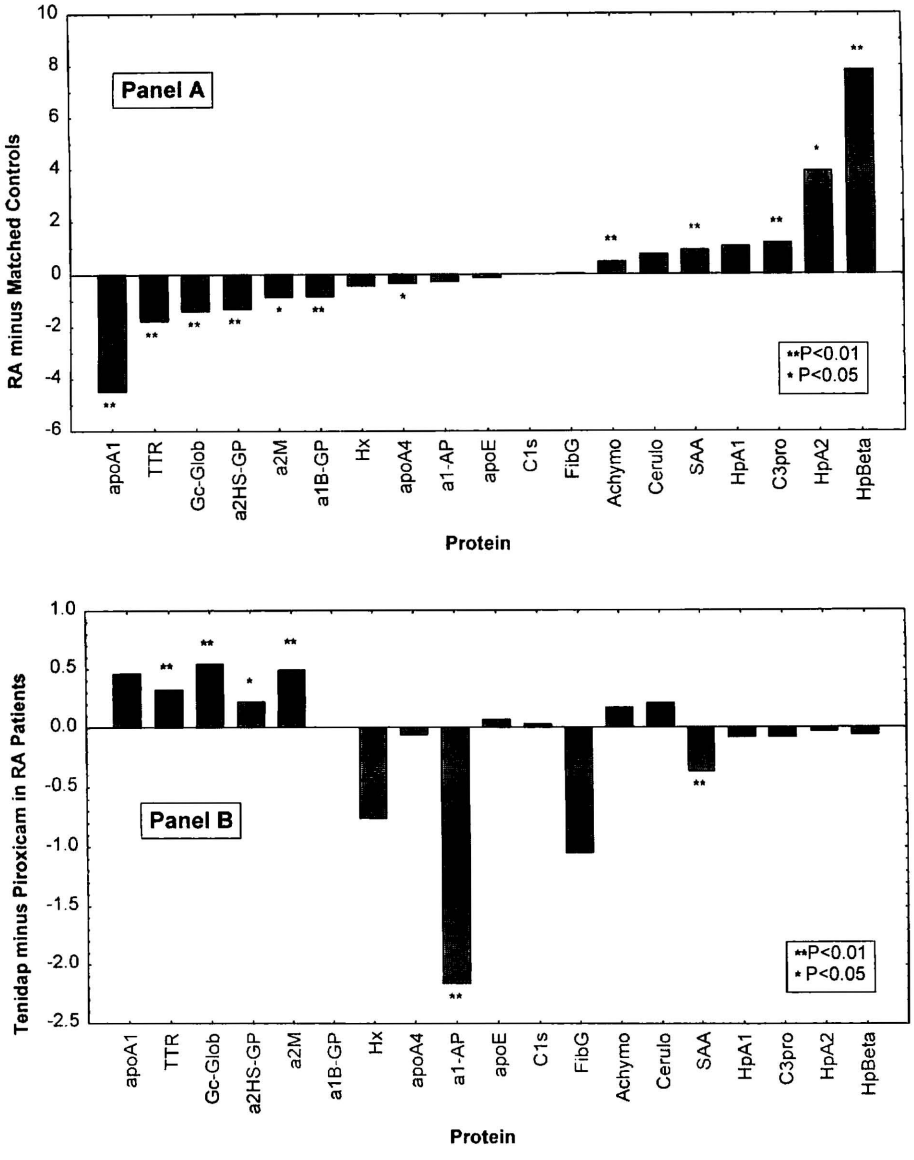

Figure 2. Differences in the abundance of plasma proteins (measured by 2-D gel electrophoresis) in RA patients *versus* matched controls (A) and the differences between the changes in abundance induced by treatment of RA patients with tenidap compared to the changes induced by treatment with piroxicam (B).

4 Discussion

Typhoid vaccination has previously been shown to induce elevations of the positive acute-phase proteins CRP and SAA [24, 25]. The present data demonstrate that these responses are accompanied by changes in six additional proteins. The directions of these changes reflect those predicted by the other studies which have characterized them as either positive or negative reactants [11, 12]. The fall in apolipoprotein E is consistent with changes seen in the acute-phase response in hamsters [26] and rats [12] but is not widely reported in humans. However, the acute-phase behavior of a number of apolipoproteins is documented; apolipoprotein (a) [27, 28] and SAA [11] behave as positive acute-phase reactants while apolipoproteins A-I, A-II and B behave as negative acute-phase reactants [29–31]. Since the time course of the change in plasma level varies markedly from protein to protein [11] and later time points were not studied, it is not clear whether the changes observed at 48 h after typhoid vaccination are maximal or whether additional proteins may change at later times.

In RA patients, levels of more proteins were significantly different from baseline levels in "normals" than were significantly altered in the response to typhoid vaccination in normals. This could be interpreted as evidence that the two responses are qualitatively different. However, when the typhoid vaccination-induced changes were compared with differences between RA patients and "normals", a remarkable concordance was seen. This concordance is remarkable given the large age difference between the groups providing the two sets of data. In fact, the discrepancy seen with one of the two outliers in this comparison, C3 convertase, can be attributed to the age difference between the two groups since it showed an age-related decrease in "normals". In addition, there was a strong correlation between levels of CRP and SAA measured by immunoassay when data were pooled from "normals", vaccinated volunteers and RA patients, indicating that these two proteins responded in parallel in the conditions studied. Therefore, at least within the 19 proteins studied, there is little to distinguish between the acute-phase protein response induced by an acute inflammatory/immunological response to a foreign

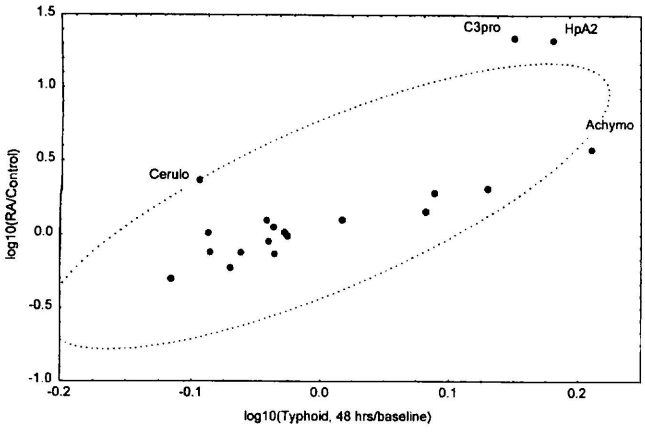

Figure 3. Comparison of the ratios of abundance of proteins, RA patients/matched controls *versus* post-typhoid vaccination/baseline. Each symbol represents a separate protein, and the four proteins nearest the 95% confidence ellipse are labeled. The rank correlation coefficient is $r = 0.7848$, $P = 0.00069$.

Table 7. Change in the abundance of plasma proteins (measured by 2-D gel electrophoresis) in RA patients treated with tenidap and piroxicam

Protein	Baseline[a]	Tenidap minus baseline[b]	Piroxicam minus baseline[b]	*P*-value[c]
Apolipoprotein A-I	13.82	−0.42	−0.88	0.900
Apolipoprotein A-IV	0.85	−0.08	−0.02	1.000
Apolipoprotein E	0.45	0.02	−0.05	0.073
SAA	0.92	−0.27	0.10	**0.004**
α_1-Antiprotease	21.93	−2.45	−0.29	**0.003**
α_1-Antichymotrypsin	0.89	0.20	0.03	1.000
α_2-Macroglobulin	3.39	0.28	−0.21	**<0.001**
Ceruloplasmin	1.91	−0.47	−0.68	0.105
C3 proactivator	1.93	−0.14	−0.06	0.489
Transthyretin	1.80	0.06	−0.26	**0.001**
Gc globulin	2.88	0.58	0.04	**0.001**
α_2-HS glycoprotein	1.77	−0.21	−0.43	**0.041**
α_1-B glycoprotein	1.66	0.05	0.05	0.860
Hemopexin	5.16	−0.43	0.33	0.105
Cls	0.25	0.02	−0.01	0.443
Fibrinogen γ	12.18	−0.58	−0.47	0.980
Haptoglobin α_1	4.16	0.00	0.08	0.970
Haptoglobin α_2	10.31	−0.08	−0.05	0.910
Haptoglobin β	20.94	0.88	0.94	0.782

a) Abundance expressed as percentage of total intensity of the 19 proteins under study

b) Differences from baseline are averages of the values calculated for each patient

c) *P*-values are calculated by Wilcoxon Signed Rank test for the paired comparison between tenidap and piroxicam

antigen (typhoid vaccination) and a chronic, inflammatory autoimmune disease (RA), suggesting that the regulatory mechanisms are similar in these two conditions. Furthermore, the relationships between levels of proteins in "normals" matched the changes induced by inflammation, suggesting that the primary regulatory factors acting on acute-phase proteins in "normals" are the same as during inflammation. However, there is some evidence which suggests that the acute-phase response is not a stereotypic response and can show qualitative differences in different situations. For example, it has been reported that in some systemic lupus erythematosus patients, CRP is elevated but ESR is not [32] and that in renal transplant patients, SAA is elevated but CRP is not [33]. Therefore, more detailed analysis of the pattern of changes in the acute-phase protein response may yield more clinically valuable information than has been obtained to date.

Table 8. Comparison of the effects of tenidap and piroxicam on other biochemical parameters in RA patients

Parameter	Baseline	Tenidap minus baseline	Piroxicam minus baseline	*P*-value[a]
IL-6 (pg/mL)	33.98	−8.68	1.96	0.074
ESR (mm/h)	65.93	−10.86	4.57	**0.007**
CRP (μg/mL)	46.3	−15.8	18.1	**0.00031**
SAA (μg/mL)	288.7	−91.8	111.8	**0.011**
Transthyretin (μg/mL)	215.0	−5.6	−36.0	**0.0015**
Total protein (g/dL)	7.16	−0.006	−0.006	0.87
Albumin (g/dL)	3.74	−0.013	−0.066	0.48
Globulin (g/dL)	3.41	0.006	0.056	0.73

a) *P*-values are calculated by Wilcoxon Signed Rank test for the paired comparison between tenidap and piroxicam. *P*-values <0.05 are shown boldface

It is surprising that no increase in the level of fibrinogen γ was observed in RA patients, since fibrinogen is believed to be a major factor in producing elevated ESR [34]. ESR was clearly elevated in our RA patients and fibrinogen levels, as measured by functional assays, are elevated in RA patients [35]. However, we can find no studies to compare with our data in which levels of the fibrinogen γ chain have been measured in RA patients; previous studies have used assays based on thrombin-induced clotting to estimate total fibrinogen. Based on immunoreactivity, location on the gels and abundance, the fibrinogen γ spots quantified in the present study likely correspond to the γA chain, the major form in plasma [36]. An additional cluster of spots of much lower abundance was identified with antibody to fibrinogen γ, the location of which was consistent with the splice variant, γ B [36], and its post-translationally modified forms [37]. Although these spots were not amenable to the rigorous mathematical analysis used above, there was no grossly discernible evidence of a disproportionate increase in the levels of these isoforms compared to fibrinogen γA. Therefore, there is no obvious explanation for the failure of 2-D gel technology to find an increase in fibrinogen levels.

Compared to piroxicam, tenidap treatment results in significantly lower levels of one positive acute-phase protein (SAA) and significantly raised levels of several negative acute-phase proteins (transthyretin, Gc-globulin, α_2-HS glycoprotein, α_2-macroglobulin). Although α_1-antiprotease is generally regarded as a positive acute-phase protein [1], no elevation in its level was detected in this cohort of RA patients compared to control subjects. Nonetheless, tenidap did significantly lower the levels of α_1-antiprotease compared to piroxicam. The effects of tenidap on one positive acute-phase protein (SAA) and one negative acute-phase protein (transthyretin) were confirmed by independent measurements using specific immunoassays, validating the 2-D gel methodology. The significant reductions with tenidap compared to piroxicam in CRP levels (measured by rate nephelometry since it could not be measured by 2-D gel electro-

phoresis) and ESR confirm earlier observations and emphasize the profound impact that tenidap has in normalizing the acute-phase response. These treatment differences in acute-phase protein levels cannot be attributed to inhibition of prostaglandin synthesis since both tenidap and piroxicam are potent inhibitors of cyclooxygenase [38]. The ability of tenidap to induce changes towards "normal" in the levels of both negative and positive acute-phase proteins indicates a general effect on the overall acute-phase response, perhaps secondary to effects on underlying disease mechanisms.

Detailed analysis of the acute-phase response, made possible by 2-D gel technology, may provide information of value in diagnosis, prognosis or monitoring of patients, beyond what is possible with individual measurements of a limited number of proteins. This method can also provide a more refined means to quantify and characterize responses to therapy. The need for such studies is enhanced by the evidence suggesting that some acute-phase proteins may have a pathogenic role [39].

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