

Quantitative and Qualitative Alterations of Acute-phase Proteins in Healthy Elderly Persons

STANLEY P. BALLOU, GERARD B. LOZANSKI, SALLY HODDER, DEBRA L. RZEWNICKI, LORRAINE C. MION, JEAN D. SIPE, AMASA B. FORD, IRVING KUSHNER

Summary

To assess acute-phase proteins in relation to ageing, we measured serum concentrations of C-reactive protein (CRP), serum amyloid-A protein (SAA), alpha-1 acid glycoprotein (AGP) and glycosylation microheterogeneity of AGP in 131 healthy elderly individuals (aged ≥ 65 years) living independently in the community, and 47 healthy younger individuals. Concentrations of CRP in the older persons (median = $3.0 \mu\text{g/ml}$) were significantly greater than in the younger group (median = $0.9 \mu\text{g/ml}$, $p = 0.0003$). Concentrations of SAA and AGP were similar in the two groups, but AGP glycosylation forms with reduced binding affinity for concanavalin-A (changes that have been observed in chronic inflammatory states) were increased in the elderly sample ($p < 0.0001$). These findings suggest that both quantitative and qualitative alterations of acute-phase proteins occur with physiological ageing in humans.

Introduction

The increased risk of infection that occurs with ageing is presumably related to impairment of local and systemic defence mechanisms. Intense investigation of immune function in elderly people has disclosed global defects in both cell-mediated and humoral immunity [1, 2], potentially contributing to an increased susceptibility to influenza, herpes zoster, tuberculosis, cancer, bacterial pneumonia, and lymphoproliferative disorders [3]. In contrast with the immune system, the inflammatory response, characterized by recruitment of inflammatory cells and elaboration of polypeptide mediators of inflammatory processes, has not been as rigorously studied. The possibility of abnormal inflammatory responses with ageing is none the less suggested by well established clinical observations, such as the reduced febrile response to infection in elderly subjects [4, 5].

Among the accompaniments of inflammatory processes is the acute-phase response, a systemic response to tissue injury or infection characterized by alteration in the synthesis and secretion of a large number of plasma proteins by hepatocytes [6]. The erythrocyte sedimentation rate (ESR), a non-specific indicator of inflammation that indirectly reflects serum concentrations of several acute-phase (and non-acute-phase) proteins, has long been known to increase with age in humans [7]. Few reports, however, have addressed variation in

actual concentrations of acute-phase proteins with ageing in man [8-13]. In the present study, we measured serum concentrations of the two major human acute-phase proteins, C-reactive protein (CRP) and serum amyloid A (SAA), in a group of 131 apparently healthy older individuals living independently in the community. We also measured both the concentrations and glycosylation patterns of α_1 -acid glycoprotein (AGP), an acute-phase glycoprotein whose quantity and pattern of glycosylation may vary independently during an acute-phase response [14]. The findings suggest that subtle quantitative and qualitative alterations of acute-phase proteins may occur with normal physiological ageing in humans.

Subjects and Methods

Sera used in this study were obtained from individuals enrolled in a large prospective study of respiratory infections in community-residing elderly persons [15]. Potential participants in the Respiratory Disease Study were identified from a volunteer registry of people over age 64 wishing to participate in research studies, a foster grandparents group, and from the inhabitants of several non-nursing home residences for older persons. Subjects were excluded from the Respiratory Disease Study if one or more of the following criteria were present: (1) dementia (defined by four or more errors on the Pfeiffer mental status test); (2) moribund (defined as presence of an illness likely to cause death in less than 1 year); (3) subject unable to co-operate; and (4) subjects

Table I. Selection of healthy elderly subjects

	Remaining n =
<i>All subjects available</i> (from initial enrolment)	574
<i>Exclusions based on admitting interview:</i>	
Poor or fair self-rating of health (237)	337
Have emphysema, tuberculosis, cancer, leukaemia or skin disorders (75)	262
Other problems affecting health (24)	238
Cortisone or antibiotics in past month (9)	229
Current smoker (29)	200
Needing help in any activity of daily living (7)	193
<i>Exclusions based on 4-month follow-up interview:</i>	
Ill since last visit (56)	137
Current illness (2)	135
<i>Exclusions at time of study:</i>	
Died <2 years after interview (1)	134
Samples lost (3)	131

planning to spend one or more months per year away from Cleveland. At intake, each subject was visited at home by a nurse practitioner and informed consent was obtained. During the intake interview, data about antecedent medical conditions and general health status were collected and a blood sample procured. Subjects were subsequently visited at 4-monthly intervals for a planned total of 3 years. At the interval visits, intake information was updated, histories of intercurrent illness were sought, and a blood sample collected. Subjects who completed the initial enrolment and first follow-up interview ($n = 574$) were eligible for inclusion in the present study. A number of exclusion criteria (Table I) were then applied to obtain a population of subjects who were free from known acute or chronic inflammatory disorders. This procedure resulted in sera from 131 subjects (aged 65–94), all of which were tested for concentrations of CRP, SAA, AGP, and patterns of AGP glycosylation. As a control group, sera were obtained from 47 randomly chosen, apparently healthy, young individuals (aged 17–46) and tested concurrently for each acute-phase protein. For all subjects, test specimens represented aliquots of fresh serum (≤ 0.5 ml) that were obtained and separated using standard sterile technique, and were stored at -20°C until assayed. The proteins assayed are stable for extended periods under these conditions.

Table II. Demographics of study participants

	Elderly (n = 131)	Young (n = 47)
Age (years): mean (SD)	75.4 (6.8)	31.6 (7.7)
Range	65–94	17–47
Men: number (%)	67 (51)	35 (74)
Women: number (%)	64 (49)	12 (26)
White: number (%)	94 (72)	43 (91)
Non-white: number (%)	37 (28)	4 (9)

Demographic characteristics of the study and control samples are presented in Table II.

Concentrations of CRP were measured by enzyme immunoassay (ELISA) using kits obtained from Hemagen Diagnostics, Inc. (Waltham, MA), and according to the manufacturer's instructions. The limit of detection of CRP in this assay was $0.1 \mu\text{g/ml}$. Acute-phase apoSAA isoforms (herein denoted SAA) were measured using ELISA kits (Hemagen Diagnostics) according to the manufacturer's instructions. Constitutive apoSAA [16], the concentration of which does not vary significantly, does not interfere with this assay. Duplicate aliquots of serum samples were assayed in microtitre wells, and absorbance at 450 nm was measured using a Molecular Devices ELISA plate reader (Menlo Park, CA). Serum concentrations of total AGP were measured by rocket immunoelectrophoresis using goat anti-human specific AGP (Atlantic Antibodies, Stillwater MN). As a standard for AGP, a human serum calibrator kit (Atlantic Antibodies) was used. All determinations were done in duplicate.

The glycosylation microheterogeneity of AGP is based on the structure of the protein's five complex heteroglycan side chains, each of which may exist in a bi-antennary, tri-antennary, or tetra-antennary configuration. Various glycosylation patterns of AGP can be detected by reactivity of the protein with concanavalin A (con A), a lectin that preferentially binds to bi-antennary carbohydrate side chains (as opposed to tri-antennary or tetra-antennary configurations). Glycosylation patterns of AGP were studied by two-dimensional crossed-affinity immunoelectrophoresis in agarose using con A (type IV, Sigma, St. Louis MO), as previously described [17]. Briefly, in the first dimension, electrophoresis of undiluted serum samples was carried out on 1% agarose gels containing $50 \mu\text{M}$ con A for 1 h at 10 V/cm. Slabs of these gels, each containing one sample, were transferred to second-dimension plates and two gels adjacent to the first dimension gel were cast; the intermediate gel contained 7.5% α -methyl-D-mannoside (Sigma) (to dissolve con A-AGP complexes) and the second contained specific anti-AGP polyclonal antiserum (Atlantic Antibodies). Electrophoresis in the second dimension was carried out for 18 h at 2 V/cm. Gels were dried and stained with Coomassie Brilliant Blue R-250. This method reveals three or four distinct microheterogeneous variants of AGP according to con A reactivity: variant 0 (nonreactive with Con A), variant 1 (weakly reactive), variant 2 (reactive) and variant 3 (strongly reactive). The area under each precipitate is measured by planimetry and the relative amounts of the microheterogeneous variants expressed as a percentage of the total. For each sample, a reactivity coefficient (RC) was calculated according to the formula: sum of the con A reactive variants (i.e. 1, 2 and 3)/con A non-reactive variant. Figure 1 illustrates the microheterogeneous AGP variants of serum samples from an older individual (AGP-RC = 0.96; 1A) and from a young subject (AGP-RC = 1.40; 1B). All determinations were carried out in duplicate.

Statistical analysis: Since the concentrations of CRP and SAA were not normally distributed, data concerning these proteins were analysed by non-parametric tests: the Kolmogorov-Smirnov test for between-group comparisons and the Spearman rank-correlation for associations. Data for concentrations of AGP and AGP-RC, which were normally distributed, were analysed for group differences by two-tailed t test and for association by Pearson product-moment correlation. Statistical significance was defined as $p < 0.05$.

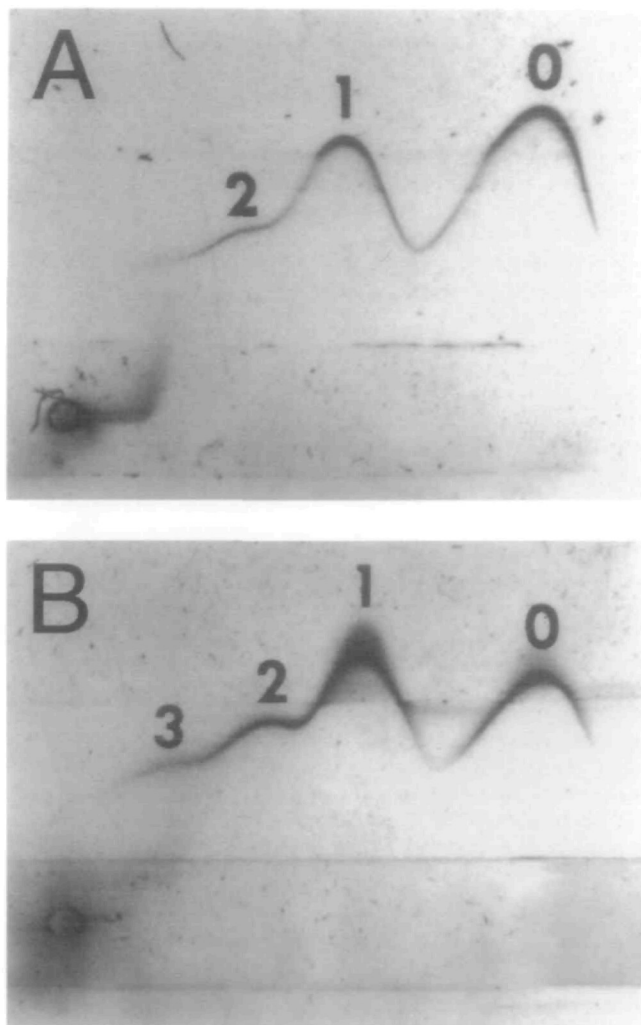


Figure 1. Crossed affinity immunoelectrophoresis of serum samples from an older patient (A) and a young subject (B). The microheterogeneous variants, related to reactivity with concanavalin A, are marked as follows: 0 = non-reactive; 1 = weakly reactive; 2 = reactive; 3 = strongly reactive.

The reactivity coefficient (AGP-RC), which is equal to the sum of the reactive variants over the non-reactive variant, is 0.96 in A and 1.40 in B.

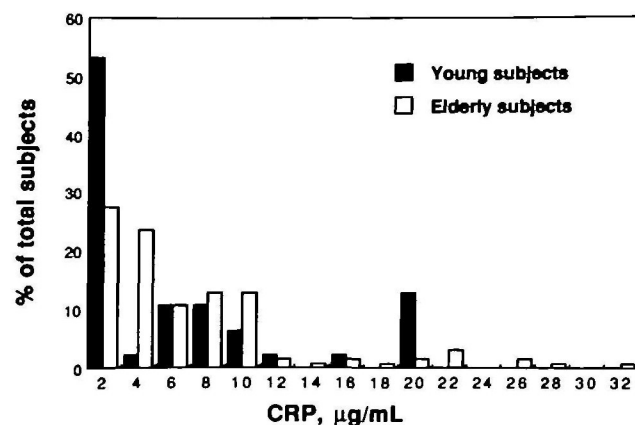


Figure 2. Distribution of serum CRP concentrations in 131 healthy elderly individuals and 47 young subjects as quantified by ELISA. The population distributions are significantly different, $p = 0.0003$.

Results

C-reactive protein: The distributions of serum concentrations of CRP as tested by ELISA in the two age groups are shown in Figure 2. The median CRP concentration in the 131 elderly individuals was significantly greater ($p = 0.0003$) than that in the group of 47 young subjects (Table III).

Serum Amyloid A: The distributions of serum SAA concentrations in the 131 elderly and 47 young subjects are depicted in Figure 3. The median SAA concentrations were similar in the two groups (Table III).

Levels and glycosylation of AGP: The mean serum concentration of AGP in the elderly subjects ($939 \mu\text{g/ml}$) was similar to that measured in the group of young individuals ($911 \mu\text{g/ml}$, Figure 4). The elderly subjects, however, had significantly lower AGP-RC values ($\text{RC} = 1.14$) than did the young controls ($\text{RC} = 1.33$, $p < 0.0001$, Figure 4), indicating an increase in the relative amounts of AGP forms with reduced binding affinity for con A (Figure 1).

Other analyses: The results of acute-phase protein determinations in the older individuals were separately analysed according to sex and race; the smaller number of younger subjects precluded valid comparisons within this group. Among the elderly individuals, serum

Table III. Comparison of acute protein concentrations in sera from 131 elderly and 47 young subjects

Test	Elderly subjects		Young subjects		p
	Mean (SD)	Median	Mean (SD)	Median	
CRP ($\mu\text{g/ml}$)	5.5 (6.0)	3.0	4.8 (6.2)	0.9	0.0003
SAA ($\mu\text{g/ml}$)	9.1 (6.1)	8	15.6 (20.4)	9	NS
AGP ($\mu\text{g/ml}$)	939 (308)	945	911 (542)	775	NS
AGP-RC	1.14 (0.29)	1.15	1.33 (0.27)	1.31	0.0001

NS = not significant.

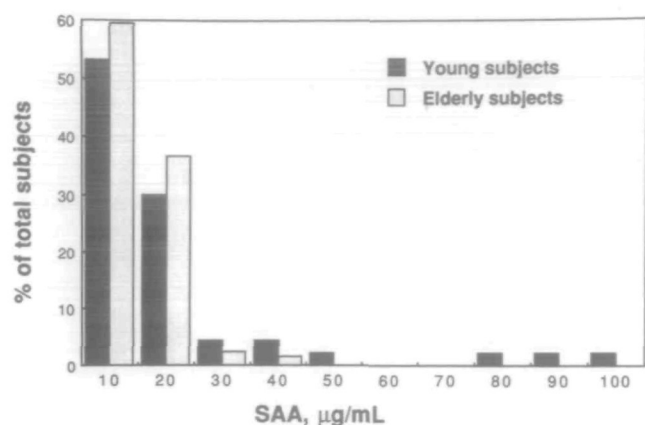


Figure 3. Distribution of serum SAA concentrations in 131 healthy elderly individuals and 47 young subjects quantitated by ELISA. There is no difference in the population distributions.

concentrations of CRP and SAA were similar in the 64 women and 67 men. Concentrations of AGP were slightly higher in women than in men (995 vs. 886 $\mu\text{g}/\text{mL}$, $p = 0.044$), and values for AGP-RC somewhat lower (1.08 vs. 1.19, $p = 0.027$). Comparison of results between the 94 white and 37 black subjects disclosed no difference in AGP concentrations or AGP-RC values. However, the median serum concentrations of CRP and SAA were slightly higher in black than in white subjects (CRP: 7.0 vs. 3.0 $\mu\text{g}/\text{mL}$, $p = 0.019$; SAA: 11.4 vs. 8.1 $\mu\text{g}/\text{mL}$, $p = 0.016$).

Within the elderly sample there was no correlation of CRP, SAA, AGP, or AGP-RC with age (range 65–94) (data not shown). Concentrations of CRP were significantly correlated with levels of the acute-phase proteins SAA and AGP (Table IV). The glycosylation patterns of AGP were not correlated with concentrations of any of the acute-phase proteins.

Discussion

The results of this study provide support for the hypothesis that both quantitative and qualitative alterations of acute-phase protein production occur

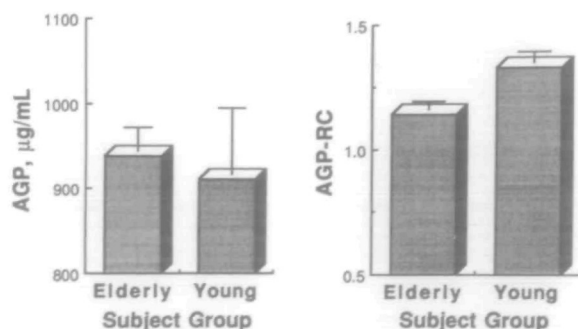


Figure 4. Serum concentrations [mean (SEM)] of AGP and AGP-RC in the two subject groups. AGP concentrations are similar, but AGP-RC is significantly reduced in the elderly group ($p < 0.0001$).

Table IV. Correlations between acute-phase protein measurements in 178 subjects

	CRP	SAA	AGP	AGP-RC
CRP	1.0			
SAA	0.37*	1.0		
AGP	0.32*	0.04	1.0	
AGP-RC	-0.06	0.03	0.11	1.0

Values represent Spearman rank-correlation coefficients.

* $p < 0.0001$.

with physiological ageing in humans. The median concentration of the major human acute-phase protein, CRP, in a group of 131 healthy elderly persons (3.0 $\mu\text{g}/\text{mL}$) was greater than in 47 younger individuals (0.9 $\mu\text{g}/\text{mL}$), and the distribution of CRP concentrations differed significantly in non-parametric statistical testing. Population data from previous studies of CRP concentrations in sera of healthy young adults aged <63 years have disclosed median CRP amounts of 0.6–0.8 $\mu\text{g}/\text{mL}$ [18, 19], concentrations similar to that of our young subjects, but less than that of the older group. In six previous population studies [18–23], the proportions of healthy young adults with 'clinically significant' CRP elevations (defined as greater than 10 $\mu\text{g}/\text{mL}$ [24]) were 0–2%, whereas two surveys of CRP levels in elderly people have disclosed such levels (>10 $\mu\text{g}/\text{mL}$) in 25% of 45 [10] and 14% of 37 [11] healthy subjects, findings that are similar to those we observed in our elderly sample (12%). Finally, in the only other controlled survey of CRP concentrations in relation to age, Caswell *et al.* [12] reported significantly increased concentrations in 57 65–74-year-old and 32 75–84-year-old individuals compared with 19 25–34-year-old subjects by non-parametric statistical testing.

The persons who participated in the present study, which represents the largest study of acute-phase proteins in elderly subjects, were carefully selected to exclude individuals with disorders or conditions that could result in elevation of CRP. The inability to demonstrate a significant correlation of CRP concentrations (or AGP-RC values) with age within this elderly group may be related to the small number of very old individuals aged ≥ 85 years ($n = 16$), or to the rigorous selection criteria. These criteria may have limited the number of chronologically oldest individuals (≥ 85 years), and limited selection to old individuals who are biologically young for their age. It is also possible that alterations of acute-phase proteins have largely occurred prior to age 65, as suggested by others [12, 13].

Population studies of the other major acute-phase protein in humans, SAA, have not been reported previously because, until recently, rapid simple methods for reliable measurement of SAA were not available [25]. The data reported here constitute the first comparison of SAA concentrations in relation to

age in healthy individuals. As expected, CRP and SAA concentrations were significantly correlated in the young and older subjects. In contrast with our findings concerning CRP, however, the distributions of SAA concentrations were similar in the elderly and young subjects, suggesting the possibility of a discordance between CRP and SAA with ageing. It is of interest that a recent survey of 99 hospitalized elderly persons disclosed that concentrations of CRP and SAA were highly correlated (as we found), but that CRP concentrations were more strongly correlated with mortality [26]. Further studies will be necessary to resolve speculation concerning discordant CRP and SAA concentrations in the elderly.

The possibility of altered acute-phase protein regulation with ageing in humans is further supported by studies of minor acute-phase proteins. For example, concentrations of fibrinogen, haptoglobin, and α_1 -proteinase inhibitor [13] have been reported to be increased in elderly subjects. Concentrations of the 'negative' acute-phase protein, albumin, have been shown not only to decline with physiological ageing, but also to correlate with morbidity and mortality in elderly subjects [27, 28]. Taken together, these prior reports and our current study suggest the possibility of a general phenomenon of altered 'set points' for acute-phase protein concentrations in older people. None the less, the acute-phase response to tissue injury or inflammation in elderly subjects appears to be quantitatively unimpaired [29, 30].

It is of interest that median concentrations of both CRP and SAA were slightly but significantly higher in the elderly black sample than in the white. Limited data are available concerning racial distribution of acute-phase protein concentrations. However, a recent large survey disclosed small but significantly higher levels of ESR in black persons aged 18–74 than in white persons [31]. With the recent availability of reliable and sensitive assays for CRP and SAA, it should be possible to confirm whether there are racial or genetic differences in concentrations of these major acute-phase proteins in apparently healthy individuals.

It has recently become apparent that the acute-phase response consists of both quantitative changes in plasma acute-phase proteins, and qualitative changes involving glycoproteins. The protein most frequently studied in this context is α_1 -acid glycoprotein [32]. While concentrations of this acute-phase protein may vary to a moderate degree (increasing up to four-fold) during an acute-phase response, altered patterns of glycosylation of the five heteroglycan side chains of this protein may also occur, independently of concentrations of the protein [33]. During an acute-phase response, two types of alterations of relative amounts of the various heteroglycan forms of AGP have been distinguished [34]. Type I changes are characterized by increased reactivity with con A, and have been found in acute inflammation. In contrast, Type II changes have been seen in some chronic inflammatory states and are characterized by decreased con A reactivity. For example,

in patients with rheumatoid arthritis, increased clinical disease activity has been associated with a decrease in AGP-RC [35]. In the present study the mean value of AGP-RC in the older individuals was significantly decreased, a Type II or chronic inflammation-associated alteration. This finding is similar to that of Kawerk *et al.* [36], who observed an age-associated reduction in con A reactivity of AGP in a group of 31 healthy elderly compared with 23 younger individuals. It is of interest that AGP-RC values were also somewhat lower in women than in men. Whether such changes are related to hormonal [37] factors is unknown.

The mechanism by which ageing leads to altered glycosylation of glycoproteins and elevated concentrations of CRP is unclear. Multiple recent studies, however, have elucidated the importance of cytokines in regulation of the acute-phase response. Interleukin-6 (IL-6) and IL-1, in particular, are capable of inducing synthesis and secretion of acute-phase proteins by hepatocytes [38] and of altering the glycosylation pattern of acute-phase glycoproteins [33]. Other cytokines including tumour necrosis factor α and transforming growth factor β , as well as cytokine receptors and glucocorticoids, are also capable of influencing the acute-phase response [38]. The interplay of these various mediators constitutes a complex network that modulates acute-phase protein responses in different circumstances. Data from several recent reports of elevated levels of IL-6 with ageing in humans [39, 40] suggest that this cytokine may be responsible for altered acute-phase protein production with ageing.

It is intriguing to speculate whether altered acute-phase protein concentrations in elderly people have clinical significance beyond merely being a reflection of potential cytokine dysregulation during human ageing. For example, do these changes reflect subtle 'sub-clinical' inflammatory processes such as atherosclerosis [41]? Alternatively, such acute-phase protein alterations may represent an inevitable physiological process associated with ageing analogous to decline in bone mass. Of perhaps greater clinical relevance is the possibility that altered acute-phase protein concentrations are predictive of morbidity or mortality in elderly subjects, in a manner analogous to the predictive value of CRP and SAA levels for morbidity and mortality in patients with unstable angina [42]. The fact that infection and malignancy are increasingly common with ageing in humans is well known. Whether the occurrence of such events can be correlated with alterations of acute-phase proteins is not known. Less dramatic but equally devastating conditions in some elderly persons include disorders which have been associated with the geriatric 'failure to thrive' syndrome [43]: weight loss, decline in muscle mass, diminished mobility, pressure sores and cognitive dysfunction. It has been speculated that some of these age-associated abnormalities may be associated with altered cytokine production [44], but no consistent alterations of the cytokine network or acute-phase protein response have been clearly identified. Further studies would be useful

to address the intriguing possibility that altered production of acute-phase proteins with ageing in humans is not merely a physiological phenomenon, but truly reflects a predisposition to age-associated disorders in elderly people.

Acknowledgements

This work was supported by US PHS Grants PO1 AG04391, RO1 AG02467, KO8 AI01062, and in part by Lederle-Praxis Biologicals, Pearl River, NY.

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Author's addresses

S. P. Ballou*, G. B. Lozanski, S. Hodder, D. L. Rzewnicki, I. Kushner
Department of Medicine,

A. B. Ford
Department of Epidemiology and Biostatistics,
Case Western Reserve University, USA

L. C. Mion
Division of Patient Care Operations,
Cleveland Clinic Foundation, USA

J. D. Sipe
Department of Biochemistry, Boston University, USA

*Address correspondence to S. P. Ballou, MD,
MetroHealth Medical Center, 2500 MetroHealth Drive,
Cleveland, OH 44109, USA

Received in revised form 20 October 1995