Clin. exp. Immunol. (1979) 35, 96–100.

Levels of the serum amyloid A protein (SAA) in normal persons of different age groups

W. HIJMANS* & JEAN D. SIPE *The Laboratory of Experimental Pathology, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland* 20014, *USA and the Institute for Experimental Gerontology TNO, Rijswijk, The Netherlands*

(*Received* 29 *August* 1978)

SUMMARY

Serum amyloid A (SAA) has been implicated by three independent studies to increase in concentration with ageing. The present study measured SAA concentration in 395 samples from 302 healthy individuals ranging in age from 21 to 100 years. The average SAA concentration was 20 μg/ml, with only five serum samples falling below 5 μg/ml. SAA concentrations are expressed in terms of cross-reactivity of purified, denatured SAA with anti-AA antibodies, rather than the purified, denatured amyloid fibril protein AA from tissues, which has been used in the past. No age-related increase in SAA concentration was observed in the present study. The average SAA concentration in these normal, healthy individuals was almost a hundred-fold less than values measured in acute phase human serum in a separate study with the same reagents.

INTRODUCTION

Amyloid deposits can be found in a great majority of aged individuals and these deposits may be of clinical significance. Their chemical composition is still unknown. Possibilities for further analysis of their aetiology and pathogenesis have been provided by recent advances in the knowledge of the different forms of amyloid and amyloidosis. It is now recognized that their common characteristics, such as Congo-red binding and birefringence, their appearance in the electron-microscope and their resistance to digestion are the result of their physical chemical structure, a β-pleated sheet fibril (Glenner & Page, 1976). From a chemical point of view different forms can be distinguished. Some are composed of immunoglobulin-light chains, or parts thereof. The amyloid deposits associated with the neuroendocrine system have been termed apudamyloid. An example is the amyloid, found in conjunction with the medullary carcinoma of the thyroid, which shows a large degree of homology with calcitonin. A third type is the classical secondary amyloid, which contains the so-called AA protein. This material cross-reacts with a serum α_1-globulin of a molecular weight of about 160,000 Daltons. It is called the SAA protein, it behaves as an acute-phase reactant and it can be regarded, if not as the precursor (Linder, Anders & Natvig, 1976), then certainly as closely related to the polypeptide precursor of the amyloid fibril protein AA.

The relationship of the SAA protein to ageing has been reported by several authors. Benson *et al.* (1975) mention positive precipitin reactions in agar in twelve out of twenty persons, aged 71 to 96 years, screened for the absence of chronic inflammatory disease. The reaction was only positive in a low percentage of blood donors under 65 years of age. A comparable technique gave positive results in half the twenty aged, but healthy individuals and in 3% of blood donors investigated by Husby & Natvig

* On leave of absence from the Institute for Experimental Gerontology TNO, Rijswijk and the University of Leiden, The Netherlands.

Correspondence: Dr W. Hijmans, Institute for Experimental Gerontology TNO, 151 Lange Kleiweg, Rijswijk (ZH), The Netherlands.

0099-9104/79/0010-0096$02.00

(1974). The latter figure was increased to 10% when the more sensitive technique of immunoelectro-osmophoresis was applied. Rosenthal & Franklin (1975) confirmed the suggestion by Husby & Natvig (1974) that SAA was a normal serum component. In a radioimmunoassay, using the radioactively-labelled alkaline degraded acid-soluble fraction of amyloid ([^{125}J] DAA), they showed its presence in all human sera. Its concentration increased significantly with age, reaching very high levels in the eighth and ninth decade. Normal values averaged 94 ng/ml of DAA equivalent.

A major problem with the assessment of the results is the absence of a standard and the need to express the values in relative and arbitrary units, which are determined by the analytical technique and the antiserum. Under the conditions of our tests normal values were in the microgram range. This discrepancy and the availability of selected and well-controlled sera prompted us to determine the normal levels in adults of all age groups.

It has been noted (Gorevic, Rosenthal & Franklin, 1976; Sipe *et al.*, 1976a) that SAA values expressed in AA equivalent are much too low. This can be related to the suggestion that the conformation of SAA may interfere with its immunoassay, unless serum samples are denaturated prior to assay (Sipe *et al.*, 1976b). It has been shown that denaturated and purified SAA (SAAL) is approximately ten times less immunoreactive than AA on a molar basis (Sipe *et al.*, 1976b) and hence it is desirable to use SAAL for radioimmunoassay determinations of SAA concentration in serum.

MATERIALS AND METHODS

Sera. All sera had been stored at −20°C for a maximum of 10 years. They were obtained from five different sources. (1) Sera from ninety-two NIH blood donors of both sexes, aged 21–60 years. They were selected to cover this age group. (2) Sera from seventy-three volunteers (twenty males and fifty-three females) of 95 years and older, living in homes for the aged in The Hague, The Netherlands. This group constituted about one third of the total population of this age group living in this area. Information on the socio-economic level and the physical health did not indicate any substantial difference between this group of volunteers and others living at home. They did not suffer from any overt disease and on physical examination no enlarged lymph nodes or hepatosplenomegaly were found. Data on their immunoglobulins have been published (Radl *et al.*, 1975). (3) The sera from thirteen male and sixteen female volunteers of the age group 76–90 years were obtained in the same homes as those mentioned in group 2. Their health status was comparable. (4) A total of 116 samples from twenty-two male, white Americans, who participated in the longitudinal follow-up study, as carried out by the National Institute for Aging in Baltimore, Maryland, were made available to us by Dr R. Andres. The average number of samples per individual was 5·3 collected in an average period of almost 6 years. The ages at sampling ranged from 36 to 84 years. (5) Single samples from eighty-five individuals were selected from the same study. Ages ranged from 27 to 92 years, with an average of 53 years.

Radioimmunoassay. A solid-phase radioimmunoassay was used, as described recently (Sipe *et al.*, 1976a). The serum samples were incubated at 37°C for 24 hr in 10% formic acid and the formic acid was removed by lyophilyzation before assay in plastic microtitration plates coated with anti-AA immunoglobulin. The antibody preparation was the same as had been used for the last few years in our laboratory. It was derived from a pool of antisera, which had been purified by affinity chromatography on highly purified immobilized tissue amyloid protein.

All tests were done in duplicate plates, each of which contained a dilution series of a purified and formic acid-treated SAA preparation (SAAL) from a sample with a high SAA level, in addition to a standard which consisted of a serum sample from one normal individual and four 'blank' wells with only the 1·6% casein solution in the 100 mM pH 8·6 barbital buffer used as a diluent.

Results were expressed in μg/ml SAAL. Levels below 5·0 μg/ml could not be detected accurately and were rated at a level of 2·0 μg/ml for the statistical evaluation.

RESULTS

The combined results are shown in Fig. 1. They are presented as geometric averages per decade with 95% confidence limits. It is clear that there was no increase of these values with age, the average being just under 20 μg/ml.

Values below 5·0 μg/ml could not be estimated with any degree of reliability. These low values were found in only twelve samples, all of which were obtained from persons between 60 and 90 years of age. They did not have any significant effect on the overall results. The same can be said about the abnormally high levels. A value of over 80 μg/ml was classified as such, because all five samples of one series which

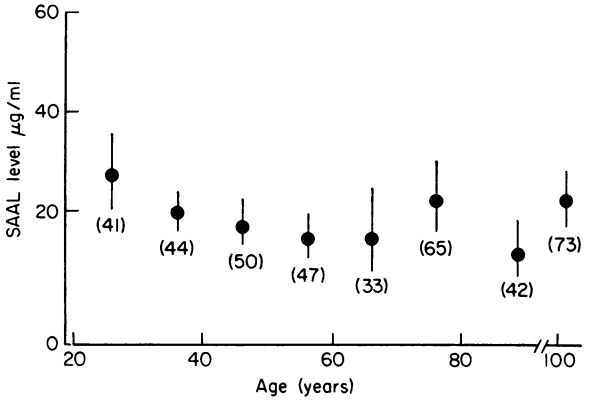

FIG. 1. Levels of the amyloid precursor substance in the serum of normal individuals of different age groups. The bars indicate 95% confidence levels of the mean, the dots give the geometric averages. The figures between brackets indicate the number of specimens in each age group.

were positive in the Ouchterlony technique exceeded this level. All abnormally high levels are presented in Table 1 and again it can be concluded that the age did not influence their distribution.

Multiple samples were available from twenty-two participants in the longitudinal study in Baltimore. Fig. 2 presents the curves which were obtained from these persons. These individual curves fail to indicate a general tendency to rise with age. This was observed only twice (Figs 2a and e). The majority stayed on the same level and a decrease was seen four times (Figs 2b, c and e). Two curves were erratic (Figs 2a and d).

TABLE 1. Abnormally high levels of SAAL (µg/ml)

Age	Level	Age group	Total in group	Percentage with abnormally high levels
25	120			
26	100	20–29	41	7
28	200			
—	—	30–39	44	0
44	85	40–49	50	2
—	—	50–59	47	0
63	90			
64	87	60–69	33	9
67	130			
70	140			
71	84			
71	360	70–79	65	6
78	85			
87	85	80–94	42	2
95	260			
96	102			
96	195	⩾95	73	5
98	400			
Total	16		395	4

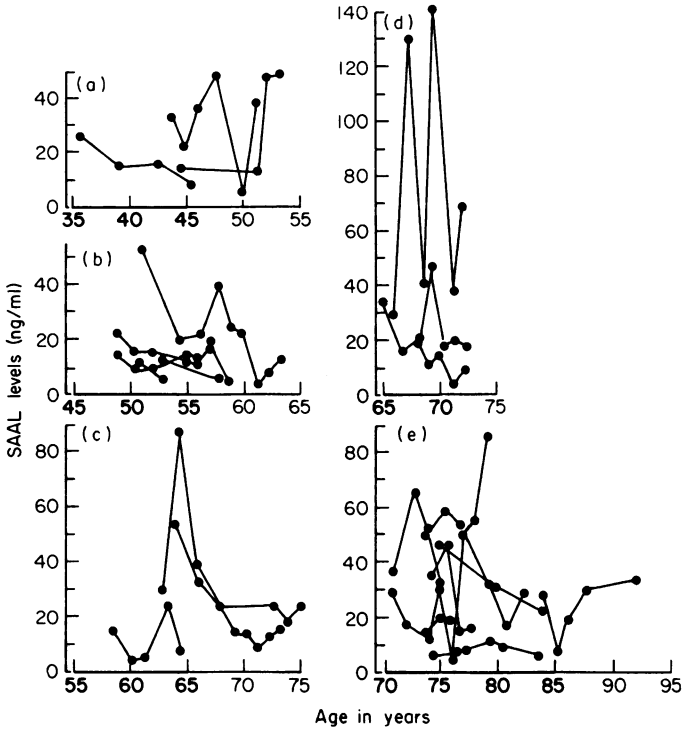

FIG. 2. Levels of the amyloid precursor substance in the sera of participants of the Baltimore longitudinal study. The age period in which a given individual entered the study is indicated in bold type on the abscissa.

DISCUSSION

During the 5 years in which the structure and properties of SAA have been studied intensively, it has become apparent that SAA exhibits great variability with respect to concentration, immunoreactivity and conformation (Gorevic *et al.*, 1976; Sipe *et al.*, 1976b). The observations that SAA is associated with the HDL_3 subclass of lipoproteins in human serum (Benditt & Eriksen, 1977) and that it is a normal acute phase reactant (Rosenthal & Franklin, 1975; McAdam *et al.*, 1975; 1978) provide reasonable explanations for what had seemed aberrant behaviour. Several studies (Sipe *et al.*, 1976a, b; 1977) of both human and murine SAA have shown that formic acid denaturation can increase the immunoreactivity of a given serum sample as much as 100-fold, and that reproducible values for the SAA concentration of a given sample can be obtained after formic acid denaturation. This is consistent with earlier observations that amyloid fibrils require denaturation in order to be solubilized and to be immunogenic and strongly antigenic (Isersky *et al.*, 1971). To date, there have been no reports that amyloid A fibrils, once denatured and dissociated, can be reassembled into beta pleated sheet fibrils. SAA appears to be similar to AA in that denaturation and exposure of its AA antigenic determinants are not readily reversed upon the return of SAA-containing samples to physiological conditions.

The difficulties in assessing the normal baseline concentration of SAA have already been discussed (Ignaczak *et al.*, 1977). Without denaturation of serum samples, the association of SAA with serum lipoproteins can mask a variable portion of the AA determinants available in SAA and can result in as much as a 100-fold variation in SAA concentration. An acute phase response to infection and inflammation can bring about 1000-fold increases in SAA concentration over a 24 hr period. Studies of SAA employing the mouse model have frequently noted fluctuations of ten- to twenty-fold in baseline concentrations of SAA brought about by fighting among male mice (K. P. W. J. McAdam & J. D. Sipe, unpublished observations). The fluctuation between 5·0 and 150 μg/ml SAA with time for various individuals, as shown in Fig. 2, may be a reflection of subclinical infection or inflammation. A single dose

of the inflammatory agent etiocholanolone increases the SAA concentration to more than 1000 μg/ml at the height of the acute phase response (McAdam *et al.*, 1978), as determined with the same reagents and conditions employed in this study.

Our data fail to indicate any increase in SAA concentration with ageing as had been observed by three groups of investigators. These diverging results cannot be satisfactorily explained on the basis of the selection of the serum samples. Although differences between our group of test subjects and those of the American and Norwegian investigators may exist, these are not likely to be of that order of magnitude, especially since all others emphasize that their aged subjects were free from chronic inflammatory disease.

It seems more likely that these differing observations may derive from differences in antigens, antibodies and denaturation employed to analyse the serum samples. A quantitative comparison with the other reports (Benson *et al.*, 1975; Husby & Natvig, 1974) is not possible because their results are based on non-quantitative double diffusion tests in agar, but it is relevant that we also found a low percentage of positive results in normal donors in our Ouchterlony tests. Comparison with the quantitative radioimmunoassay data from Rosenthal & Franklin (1975) is hampered by the fact that no standards are available. They express their results as ng/ml of AA equivalent, whereas our normal values lie in the μg range, and exceed those presented by Rosenthal & Franklin by a factor of about 200. Sipe *et al.* (1976a) have presented data which show that treatment of whole serum—as used by other investigators—with formic acid, as done in this study, gives an increase of antigenic activity of the serum component of a sufficient degree to explain the differences in normal values.

This treatment with formic acid may also lead to the masking of the effect of ageing: the increased level of SAA, as detected by immunological methods. From previous studies (Sipe *et al.*, 1976a) it was concluded that the quaternary structure of SAA is such that its AA determinants are relatively inaccessible for immunoreaction. Unfolding of these determinants is promoted by dissociation of SAA to SAAL, in our procedure, by formic acid. It can also occur spontaneously and perhaps this becomes more frequent with ageing.

The authors are indebted to Dr G. G. Glenner for making available his laboratory facilities and for his advice and encouragement. They are grateful to Dr R. Andres from the National Institute of Aging, Baltimore (Maryland) for putting at their disposal selected sera from the longitudinal study. They would like to thank Pleuni van den Berg for her assistance.

REFERENCES

BENDITT, E.P. & ERIKSEN, N. (1977) Amyloid protein SAA is associated with high density lipoprotein from human serum. *Proc. nat. Acad. Sci.* (*Wash.*), **74**, 4025.

BENSON, M.D., SKINNER, M., LIAN, J. & COHEN, A.S. (1975) 'A' protein of amyloidosis. Isolation of a cross-reacting component from serum by affinity chromatography. *Arthr. and Rheum.* **18**, 315.

GLENNER, G.G. & PAGE, D.L. (1976) Amyloid, amyloidosis, and amyloidogenesis. *Int. Rev. exp. Pathol.* **15**, 1.

GOREVIC, P.D., ROSENTHAL, C.J. & FRANKLIN, E.C. (1976) Amyloid-related serum component (SAA)—studies in acute infections, medullary thyroid carcinoma, and post-surgery. *Clin. Immunol. Immunopathol.* **6**, 83.

HUSBY, G. & NATVIG, J.B. (1974) A serum component related to nonimmunoglobulin amyloid protein AS, a possible precursor for the fibrils. *J. clin. Invest.* **53**, 1.

IGNACZAK, T.F., SIPE, J.D., LINKE, R.P. & GLENNER, G.G. (1977) Immunochemical studies on the nature of the serum component (SAA) related to secondary amyloidosis. *J. Lab. clin. Med.* **89**, 1092.

ISERSKY, C., PAGE, D.L., CUATRECASAS, P., DELELLIS, R.A. & GLENNER, G.G. (1971) Murine amyloidosis: immunologic characterization of amyloid fibril protein. *J. Immunol.* **107**, 1690.

LINDER, E., ANDERS, R.F. & NATVIG, J.B. (1976) Connective tissue origin of the amyloid-related protein SAA. *J. exp. Med.* **144**, 1336.

MCADAM, K.P.W.J., ANDERS, R.F., SMITH, S.R., RUSSELL, D.S., PRICE, M.A. (1975) Association of amyloidosis with erythema nodosum leprosum reactions and recurrent neutrophil leucocytosis in leprosy. *Lancet*, **ii**, 572.

MCADAM, K.P.W.J., ELIN, R.J., SIPE, J.D. & WOLFF, S.M. (1978) Changes in human serum amyloid A and C-reactive protein after etiocholanolone-induced inflammation. *J. clin. Invest.* **61**, 390.

RADL, J., SEPERS, J.M., SKVARIL, F., MORELL, A. & HIJMANS, W. (1975) Immunoglobulin pattern in humans over ninety-five years of age. *Clin. exp. Immunol.* **22**, 84.

ROSENTHAL, C.J. & FRANKLIN, E.C. (1975) Variation with age and disease of an amyloid A protein-related serum component. *J. clin. Invest.* **55**, 746.

SIPE, J.D., IGNACZAK, T.F., POLLOCK, P.S. & GLENNER, G.G. (1976a) Amyloid fibril protein AA: purification and properties of the antigenically related serum component as determined by solid phase radioimmunoassay. *J. Immunol.* **166**, 1151.

SIPE, J.D., MCADAM, K.P.W.J., TORAIN, B.F. & GLENNER, G.G. (1976b) Conformational flexibility of the serum amyloid precursor SAA. *Brit. J. exp. Path.* **57**, 582.

SIPE, J.D., MCADAM, K.P.W.J., TORAIN, B.F. & POLLOCK, P.S. (1977) Isolation and structural properties of murine SAA—the acute phase serum precursor of amyloid AA. *Immunol. Commun.* **6**, 1.