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High prevalence of anti-cardiolipin and other autoantibodies in a healthy elderly population

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SUMMARY

Serum samples from 64 apparently healthy individuals (32 men and 32 women, mean age 81·0 years) were examined for the prevalence of several autoantibodies, including rheumatoid factor (RF), antinuclear antibodies (ANA), antibodies to extracted cellular antigens Ro (SSA), La (SSB), Sm, U_1nRNP and Scl-70. IgG and IgM isotype-specific ELISA methods were applied for the detection of antibodies to ssDNA (anti-ssDNA), to dsDNA (anti-dsDNA) and to cardiolipin (anti-CL). The sera of this elderly population were found to contain a plethora of autoantibodies; RF was detected in 14·1%, ANA in 31·3% and anti-Ro (SSA) in 1·6% of the individuals. Precipitating antibodies to La (SSB), Sm, U_1nRNP and Scl-70 were absent, while 15·6% of the sera displayed precipitating antibodies to a common undefined human spleen antigen. ELISA methods revealed anti-ssDNA in 17·2% of the individuals, anti-dsDNA in 14·1% and anti-CL in an extremely high incidence (51·6%). Notably, the above autoantibodies were exclusively of IgG isotype. Tests of 261 sera from healthy non-elderly individuals disclosed only anti-CL (IgG and IgM isotypes) in 2·3% of them. The levels of IgA and IgG immunoglobulins were increased in 23·4% and 29·7% of the elderly subjects, respectively. IgM was elevated in 3·1%, but it was also found decreased in 9·4%. This study documents the high incidence of autoantibodies in the healthy elderly, including for the first time, anti-CL antibodies. Furthermore, the relative impairment in IgM autoantibody production observed, possibly indicates the involution of the senescent immune system.

Keywords anticardiolipin autoantibodies elderly

INTRODUCTION

The ageing process in humans and experimental animals has been associated with several cellular and humoral immunological aberrations (Makidonan & Kay, 1980; Weksler, 1982). It is well documented that alterations in the humoral immune response during ageing include a high incidence of autoantibodies like rheumatoid factor (RF), antinuclear antibodies (ANA) and antibodies to DNA and thyroid tissue antigens (Hackett, Beech & Forbes, 1960; Seligmann, Cannat

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& Hamard, 1965; Cammarata, Rodnan & Fennell, 1967; Mackay, 1972; Hallgren *et al.*, 1973; Diaz-Jouanen, Strickland & Williams, 1975; Delespesse *et al.*, 1980; Schuller *et al.*, 1981; Goodwin, Searles & Tung, 1982; Kasjanov, Cebecaner & Balaz, 1984; Hijmans *et al.*, 1984). This overt autoimmune phenomenon has been linked with the ageing process itself, since it is usually unassociated with clinical disease (Makidonan & Kay, 1980). Therefore, it is suggested that a correction for age should be considered in the evaluation of the autoantibody profiles. However, other reports have not confirmed the prevalence of autoantibodies in the elderly individuals (Pandey *et al.*, 1979; Gordon & Rosenthal, 1984), with the exception perhaps, of RF (Silvestris *et al.*, 1985).

The purpose of the present study was to examine the concomitant expression of several serum autoantibodies in a healthy elderly population, including for the first time, anticardiolipin (anti-CL) antibodies. In the recent years, anti-CL antibodies have received an increased interest because of studies that have shown a correlation between the presence of these antibodies and vascular thrombosis, neurological disease and recurrent fetal loss in patients with autoimmune disorders (Harris, Gharavi & Hughes, 1985a). On the other hand, vascular thrombotic events contribute significantly to the morbidity and mortality rates of the elderly. Thus, in this study we examined the autoantibody profile of a healthy elderly population and whether the humoral autoimmune response in the elderly involves the production of anti-CL antibodies.

MATERIALS AND METHODS

Participants. Serum samples from 64 Greek ambulatory, apparently healthy, elderly individuals (32 men and 32 women, with age ranging from 67 to 95 years, mean age 81·0) were included in the present study. These 64 elderly individuals were selected out of the 103 residents of the Ioannina public nursing home, according to the following criteria: (1) age $\geqslant$65 years, (2) no evidence of serious medical problems, including connective tissue diseases, on the basis of complete medical history, physical examination and routine laboratory tests, (3) no prescription medication or daily non-prescription medication.

Serological studies. Coded sera were stored at $-30°C$ until testing. Rheumatoid factors (RF) activity was detected by the latex fixation test (positive RF titre $\geqslant$1:40) and antinuclear antibodies (ANA) by the indirect immunofluorescence technique using Hep-2 epithelial cells as substrate (positive ANA titre $\geqslant$1:80). The presence of precipitating antibodies to extracted cellular antigens Ro (SSA), La (SSB), Sm, U_1nRNP and Scl-70 was determined by counterimmunoelectrophoresis (Kurata & Tan, 1976) using human spleen, calf thymus and rabbit thymus extracts.

The levels of IgG and IgM antibodies to cardiolipin (anti-CL), single-stranded DNA (anti-ssDNA) and double-stranded DNA (anti-dsDNA) were determined by quantitative isotype-specific solid phase enzyme-linked immunosorbent assays (ELISA), as previously described (Gharavi *et al.*, 1987; Manoussakis *et al.*, 1987; Tzioufas *et al.*, 1987). Briefly, for anti-CL ELISA, cardiolipin (Sigma, St Louis, Mo) in ethanol (50 μg/ml) was absorbed to the surface of polystyrene microtitre plates (Nunc, Denmark) after evaporation of ethanol by overnight incubation in the dark, at 4°C. For anti-dsDNA and anti-ssDNA ELISA methods, the plates, before the antigen-coating step, were incubated with poly-L-lysine (Sigma, 50 μg/ml in PBS). The plates were then coated with dsDNA (Sigma) or ssDNA preparations (50 μg/ml in PBS). Single-stranded DNA was prepared by heat-denaturation of dsDNA, as previously described (Harris *et al.*, 1985b). An additional treatment with S_1-nuclease (Sigma, 50 IU/ml) was performed on the dsDNA-coated plates for the digestion of any ssDNA regions. The antigen-coated plates were blocked with ox serum 10% in PBS for 1 h at room temperature and 50 μl of serum samples diluted 1:50 in ox serum 10%, were added to the wells in duplicates. The plates were incubated for 3 h at room temperature and the goat anti-human IgG (or IgM respectively) conjugated to alkaline phosphatase (Sigma) was added in a dilution 1:1,000 in ox serum 10%. After 90 min at room temperature, the plates were incubated with alkaline phosphatase substrate solution for 20–30 min at 37°C. The reaction was stopped with 3 M NaOH and the optical density was read at 405 nm in a microplate reader (Dynatech, England). Between each step, the plates were washed five times with PBS (100 μl/well).

In each assay, the between day variation of optical density (OD) values was eliminated by running serial dilutions of a positive control serum (standard curve) on each plate, as previously (Manoussakis *et al.*, 1987). Therefore, the OD values of every tested sample were referred to as binding units (BU) according to the standard curve of the plate. Finally, the results were expressed as binding index (BI) calculated by dividing the BU of every sample by the mean BU of the control group plus four standard deviations (for the anti-CL assays) or three standard deviations (for the anti-ssDNA and anti-dsDNA assays), multiplied by 100. According to this formula, BI of 100 was defined as the cut-off point.

Serum immunoglobulin IgA, IgG and IgM levels were determined by single radial immunodiffusion (Behringwerke, W. Germany). The normal range of values for IgA (90–450 mg/dl), IgG (800–1800 mg/dl) and IgM (65–280 mg/dl) were those used in the laboratory.

Statistical analysis. The data were analysed by determining (BMDP programs) the parametric Pearson correlation coefficients, the non-parametric Spearman correlation coefficients and by using linear regression analysis, Student's *t*-test and chi-square test with Yate's correction, where applicable.

RESULTS

Controls

Previous studies in our laboratory of 170 non-elderly healthy blood donors (25 to 48 years of age) had revealed no evidence of positive RF (titre $\geqslant$1:40), ANA (titre $\geqslant$1:80) or antibodies to extracted cellular antigens. In this study, the results of serum samples from non-elderly healthy blood donors (12 to 53 years of age), tested for anti-CL (261 sera), anti-ssDNA (40 sera) and anti-dsDNA activity (119 sera), were included. These studies disclosed positivity only for anti-CL antibodies in six young healthy individuals (2·3%, three with IgG and three with IgM anti-CL antibodies in low levels, Figs 1 and 2).

Elderly individuals

Rheumatoid factor. RF activity was observed in 14·1% of the sera (9/64, four men and five women). Positive RF titres ranged from 1:40 to 1:320 (mean 1:156) and were more frequent in

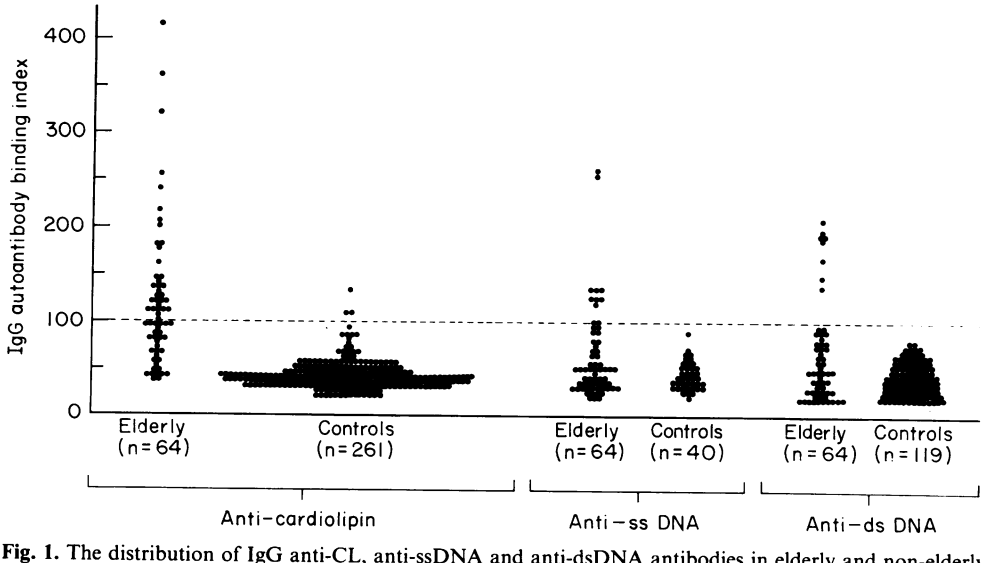

Fig. 1. The distribution of IgG anti-CL, anti-ssDNA and anti-dsDNA antibodies in elderly and non-elderly (control) groups. The horizontal broken line represents the upper limit of normal values.

elderly individuals with age <81 years (6/29, 20·7%) compared to those with age $\geqslant 81$ years (3/35, 8·6%).

Antinuclear antibodies. Using Hep-2 cells as substrate, ANA were detected in 31·3% of the sera (20/64, eight men and 12 women). Several immunofluorescence patterns were observed, including fine speckled (in 15 sera), nucleolar and cytoplasmic (in three sera each), and homogeneous and discrete speckled (i.e. anti-centromere antibody (Tan *et al.*, 1980)) (in one serum each). Positive ANA titres ranged from 1:80 to 1:640 (mean 1:184). Neither ANA positivity nor titres correlated with age or sex of the elderly studied.

Precipitating antibodies to extractable antigens. Precipitating antibodies were detected in 13 sera (20·3%, seven men and six women). In most of them, the procedure failed to reveal identity with any of the antibody systems under consideration, except for the serum of a female individual aged 83, where the presence of anti-Ro (SSA) antibodies was demonstrated.

It is notable however, that in 10 out of the remaining 12 sera with undefined precipitins (15·6% of the elderly), these precipitins were detected solely with human spleen extract and were found to be identical to each other. Most of these sera (8/10) were from individuals with age $\geqslant 81$ years. Two such serum samples with very strong precipitin lines were selected for further experiments. Comparison of the precipitins of these two sera with a panel of 53 sera with previously detected undefined precipitin lines from patients with autoimmune diseases (namely, systemic lupus erythematosus (SLE), primary Sjögren's syndrome, rheumatoid arthritis, and scleroderma) failed to demonstrate any identity.

Anti-cardiolipin antibodies. The elderly subjects in this study displayed a high incidence of anti-CL antibodies in their sera. Anti-CL were present in 51·6% of the sera (33/64) and were expressed solely by IgG isotype (Figs 1 & 2). Anti-CL were more frequent in men (62·5%, 20/32), than in women (40·6%, 13/32), while the mean IgG anti-CL assay levels were higher in men ($142{\cdot}2 \pm 87{\cdot}4$) than in women ($92{\cdot}7 \pm 51{\cdot}4$, $t = 2{\cdot}76$, $P < 0{\cdot}005$). In addition, the mean positive IgG anti-CL BI (BI $\geqslant 100$) was higher in men ($179{\cdot}9 \pm 90{\cdot}8$) compared to women ($143{\cdot}0 \pm 40{\cdot}7$). There was no difference in the age between elderly individuals with anti-CL antibodies and those without.

Antibodies to ssDNA and to dsDNA. Eleven individuals (17·2%, five men and six women) had anti-ssDNA antibodies in their sera, while nine (14·1%, three men and six women) displayed anti-dsDNA antibodies. Virtually all anti-ssDNA and anti-dsDNA antibodies detected were expressed by IgG isotype (Figs 1 & 2). Only one woman aged 82, had moderately low levels (BI = 120) of IgM anti-dsDNA antibodies. Most of the elderly with anti-ssDNA (10/11, 90·9%) had age $\geqslant 81$ years

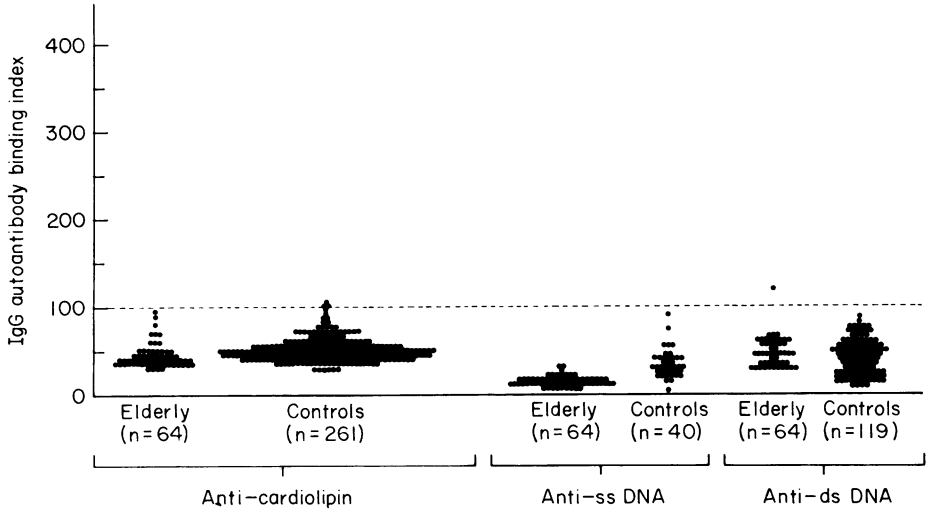

Fig. 2. The distribution of IgM anti-CL, anti-ssDNA and anti-dsDNA antibodies in elderly and non-elderly (control) groups. The horizontal broken line represents the upper limit for normal values.

($\chi^2 = 5{\cdot}38$, $P < 0{\cdot}025$). The mean age of individuals with anti-ssDNA antibodies was higher ($83{\cdot}8 \pm 3{\cdot}7$), compared to those without ($80{\cdot}5 \pm 6{\cdot}2$, $t = 2{\cdot}35$, $P < 0{\cdot}025$). In addition, the mean positive IgG anti-ssDNA BI (BI $\geqslant 100$) was higher in women ($167{\cdot}8 \pm 70{\cdot}1$) than in men ($121{\cdot}4 \pm 12{\cdot}7$).

Serum concentrations of immunoglobulins. The striking prevalence of IgG isotypes among anti-CL, anti-ssDNA and anti-dsDNA antibodies directed us to examine the serum concentrations of immunoglobulins IgA, IgG and IgM, in order to investigate the relationship between the levels of serum immunoglobulins and the isotypic expression of the autoantibodies.

Elevated levels of immunoglobulins were observed in 37·5% of the sera (24/64, 10 men and 14 women). This hypergammaglobulinaemia mainly concerned the IgA (in 15/64, six men and nine women) and the IgG immunoglobulin class (in 19/64, nine men and 10 women), while IgM in excess was evident only in two sera (two men, in both together with elevated IgG levels). The presence of IgA and IgG hypergammaglobulinaemias were found to correlate to each other (coexisted in 10/24, $\chi^2 = 10{\cdot}23$, $P < 0{\cdot}002$). Hypogammaglobulinaemia was observed only in the IgM immunoglobulin class in 9·4% of the sera (6/64, four men and two women), where normal levels of IgA and IgG were found. The statistical analysis of the immunoglobulin levels of our elderly individuals disclosed a tendency of the IgA levels to increase with age ($r = 0{\cdot}24$, $P = 0{\cdot}057$) and of the IgM levels to decrease with age ($r = 0{\cdot}26$, $P = 0{\cdot}035$).

Correlation of the serological parameters. There was no correlation between the presence of anti-CL antibodies and the presence of any other autoantibody under study. More than half (17/33, 51·5%) of anti-CL positive sera had no other autoantibody. In fact, these sera were frequently negative for ANA (78·8%), for anti-ssDNA (90·9%) and for anti-dsDNA antibodies (87·9%). In this study, ANA or anti-ssDNA antibodies were less likely to be found alone (35·0% and 27·3% respectively), while RF and anti-dsDNA antibodies were always detected in the presence of other autoantibodies.

Most of the sera with IgG anti-dsDNA antibodies (5/8, 62·5%) had also IgG anti-ssDNA antibodies ($r = 0{\cdot}57$, $P = 0{\cdot}001$, $\chi^2 = 9{\cdot}80$, $P < 0{\cdot}005$). Positive ANA tests were frequently observed in sera with anti-ssDNA (7/11, $\chi^2 = 4{\cdot}79$, $P < 0{\cdot}05$) and with anti-dsDNA activity (8/9, $\chi^2 = 13{\cdot}22$, $P < 0{\cdot}001$).

No association could be demonstrated between the presence of hypergammaglobulinaemia and any particular autoantibody under consideration. However, autoantibodies of at least one specificity were present in all 19 sera with IgG hypergammaglobulinaemia ($\chi^2 = 6{\cdot}52$, $P < 0{\cdot}025$).

DISCUSSION

It is generally accepted that asymptomatic elderly individuals frequently have autoantibodies in their sera. In the present study we examined the concomitant expression of several autoantibodies in a healthy elderly population, including anti-CL antibodies.

The properties of the isotype-specific ELISA methods applied in this study were examined previously in extensive preliminary experiments, where the high antigenic specificity of the assays was demonstrated. In addition, the adoption of stringent cut-off points for positivity precluded the possibility of false-positive results (Gharavi *et al.*, 1987; Manoussakis *et al.*, 1987; Tzioufas *et al.*, 1987).

In agreement with previous reports, the serological profile of our elderly population was found to contain a plethora of autoantibodies, including RF (in 14·1% of the sera), ANA (in 31·3% of the sera, including one serum with anti-centromere antibodies) and antibodies to ssDNA (in 17·2%) and to dsDNA (in 14·1%). In addition, a remarkable incidence of anti-CL antibodies (51·6%) was observed.

The incidence of anti-CL antibodies in the elderly has not been previously reported. The presence of anti-CL antibodies in patients with autoimmune disorders has been associated with an increased incidence of vascular thrombotic events, recurrent abortions, thrombocytopenia and neurological disease (Harris, Gharavi & Hughes, 1985a). We recently described the incidence of IgG and IgM anti-CL antibodies in a large series of unselected patients with various autoimmune

rheumatic diseases, where an association of anti-CL with the presence of central nervous system involvement (epilepsy) in patients with SLE, was demonstrated. In addition, the presence of IgG and IgM anti-CL antibodies in low levels in 2·3% of 267 sera from healthy blood donors was ascertained (Manoussakis *et al.*, 1987).

Although the cross-reactive specificity of antibodies to DNA and to phospholipids has been reported (Lafer *et al.*, 1981; Shoenfeld *et al.*, 1983), this finding was not confirmed in subsequent inhibition experiments (Harris *et al.*, 1985b; Manoussakis *et al.*, 1987). This lack of cross-reactivity between anti-DNA and anti-CL antibodies is further supported by the fact that the presence of these autoantibodies in the elderly studied, could not be correlated.

Compared to the data from 86 SLE patients previously studied (Manoussakis *et al.*, 1987) positive IgG anti-CL tests in this elderly group were significantly more frequent (51·6% versus 17·4% in SLE patients, $P<0·0001$) but in comparable levels (mean positive BI in the elderly: $165·4 \pm 76·5$, range: 100–414, compared to $228·0 \pm 186·4$, range: 110–692 in the SLE patients, difference not significant). In contrast, positive IgG anti-dsDNA and anti-ssDNA tests were significantly more frequent and in higher levels ($P<0·001$) in 41 SLE patients previously studied (Tzioufas *et al.*, 1987) compared to this elderly group.

The current interest in these antibodies is due to the fact that anti-CL antibodies represent an example of an epiphenomenon whose presence has been associated with clinical manifestations, namely thromboembolism and neurological disease. On the other hand, venous and arterial thromboembolic phenomena are problems frequently encountered in the elderly. Although the advanced atherosclerotic process is primarily incriminated, the possibility of anti-CL antibodies being involved in the pathogenesis of the thromboembolism in the elderly cannot be ruled out and remains to be examined prospectively.

It is well documented that anti-dsDNA antibodies are highly specific for SLE (Swaak *et al.*, 1979; Eaton, Schnneider & Schur, 1983; Weinstein *et al.*, 1983; Tzioufas *et al.*, 1987). However, the presence of anti-dsDNA antibodies, as well as of ANA in the serum of healthy elderly individuals, suggests caution in the application of the ARA criteria for the diagnosis of SLE (Tan *et al.*, 1982) in elderly individuals.

In this study, the presence of anti-Ro (SSA) antibodies was evident in the serum of one elderly individual (1·6%). The previously reported incidence of anti-Ro (SSA) in the elderly ranges from 1·7% (Alspaugh, Buchanan & Whaley, 1978) to 6·9% (Strickland *et al.*, 1984), while the rheumatoid arthritis precipitin was observed in 8·3% (Alspaugh *et al.*, 1978). Interestingly, 10 of our elderly individuals studied (15·6%) had a common (yet undefined) precipitating antibody to extracted human spleen antigen in their sera. Further experiments are in progress to assess the nature and properties of this antigenic system.

It is noteworthy that 76·6% (49/64) of the elderly studied had at least one of the autoantibodies under consideration in their sera. Elderly men appear to be auto-responders more often (84·4%) than women (68·8%). However, the prevalence of autoantibodies, other than anti-CL, was slightly higher in women (56·3%) compared to men (43·8%). As also previously described (Goodwin *et al.*, 1982), we could not demonstrate any particular influence of sex on the induction of any specific autoantibody in the elderly. Although several aspects of the age-dependent immunological aberrations have been related to women (Whittingham *et al.*, 1971; Hooper *et al.*, 1972; Hallgren *et al.*, 1973; Delespesse *et al.*, 1977), it has been suggested that after the age of 70 there is no difference between the two sexes (Delespesse *et al.*, 1974; Mascart-Lemone *et al.*, 1982).

Another interesting finding in this study was the striking prevalence of IgG isotype among autoantibodies detected in the sera of our elderly population by the isotype-specific enzyme-immunoassays (Figs 1 & 2). This finding, compared to the multiple-isotypic (IgG and IgM) expression of anti-DNA and anti-CL antibodies in autoimmune patients (Gharavi *et al.*, 1987; Manoussakis *et al.*, 1987; Tzioufas *et al.*, 1987), indicates that the IgM-isotypic expression of these autoantibodies is markedly impaired in the elderly; a fact possibly reflecting the involution of one or more aspects of the immune system during senescence. Several studies have implied that, in the ageing immune system, apart from T cell aberrations, B cells display intrinsic impairments (Price & Makidonan, 1972; Weiner *et al.*, 1978; Friedman & Globerson, 1978; Kishimoto *et al.*, 1980; Hollingsworth & Otto, 1981; Ceuppens & Goodwin, 1982; Ennist *et al.*, 1986), including aberrant

terminal differentiation into IgM-immunoglobulin secreting cells (Ceuppens & Goodwin, 1982; Ennist *et al.*, 1986); This age-associated decline of the intrinsic ability of B cells to produce IgM is essentially in agreement with the alterations frequently observed in the concentrations of serum immunoglobulins of the elderly; it is well documented that the concentrations of IgG and IgA immunoglobulins increase with age, while the concentration of IgM maintains or tends to decrease (Hallgren *et al.*, 1973; Buckley, Buckley & Dorsey, 1974; Radl *et al.*, 1975; Kashimoto *et al.*, 1978; Moulias *et al.*, 1984). This immunoglobulin profile is compatible with the alterations of immunoglobulin levels observed in the elderly studied.

Nevertheless, it should be noticed that, despite the impaired IgM production during ageing, IgM anti-IgG antibodies may be responsible for the RF activity which is frequently observed in the sera of the elderly (Helmer, Levin & Rudd, 1963; Hallgren *et al.*, 1973; Diaz-Jouanen, 1975; Hijmans *et al.*, 1984; Silvestris *et al.*, 1985), since the usual agglutination tests primarily detect IgM antibodies. We do not have a ready explanation for this discrepancy. However, it is interesting that RF was the sole serum autoantibody in the elderly studied, whose incidence tended to decline with age progression in individuals aged 65 and over.

Although serum IgG levels are frequently elevated in the elderly, ageing in humans has been associated with an impairment in the production of specific IgG antibodies (Roberts-Thompson *et al.*, 1974; Whittingham, Buckley & Mackay, 1978; Delfraissy *et al.*, 1980; Kishimoto *et al.*, 1980). In addition to the possibility that the impaired primary IgM response in the elderly (Delfraissy *et al.*, 1980) is crucially involved, our data indicate that a considerable amount of the increased IgG of the elderly may be attributed to the circulating IgG autoantibodies rather than to an immune response against exogenous antigens.

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