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Human B cell function in normal individuals of various ages

I. *IN VITRO* ENUMERATION OF POKEWEED-INDUCED PERIPHERAL BLOOD LYMPHOCYTE IMMUNOGLOBULIN-SYNTHESIZING CELLS AND THE COMPARISON OF THE RESULTS WITH NUMBERS OF PERIPHERAL B AND T CELLS, MITOGEN RESPONSES, AND LEVELS OF SERUM IMMUNOGLOBULINS

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SUMMARY

The effect of age on the *in vitro* generation of immunoglobulin-secreting cells in pokeweed mitogen-stimulated cultures was examined using a staphylococcal protein A plaque assay. Although there was no statistically significant decrease with age in the numbers of plaque-forming cells, subjects whose cells failed to produce immunoglobulin were four times more common amongst individuals over 55 years of age. Simultaneously-measured T and B lymphocyte numbers, ^{3}H-thymidine incorporation by mitogen-stimulated cultures, and serum immunoglobulins were comparable in both the young and the aged.

INTRODUCTION

The laboratory investigation of ageing humans has documented many alterations in immune function, the most widely accepted of which is the dysfunction of thymus-derived or T lymphocytes (Weksler & Hutteroth, 1974; Pisciotta *et al.*, 1967; Foad *et al.*, 1974; Hallgren *et al.*, 1978). To date, most studies concerned with the effect of ageing on human B lymphocytes have been centred in three areas: the morphological identification and enumeration of B lymphocytes; measurement of immunoglobulin or antibody levels in serum or cell culture supernatants; and the quantitation of DNA synthesis following cellular activation by B cell mitogens. On the basis of these investigations the number of peripheral blood B lymphocytes has been reported to increase (Augener *et al.*, 1974), decrease (Gajl-Peczalska *et al.*, 1974; Clot, Charmasson & Brochier, 1978; Cobleigh, Braun & Harris, 1980), or to remain unchanged (Weksler & Hutteroth, 1974; Diaz-Jouanen, Strickland & Williams, 1975a; Diaz-Jouanen, Williams & Strickland, 1975b; Barrett *et al.*, 1980) as a function of age. Measurement of serum immunoglobulin or antibody values has also produced unclear patterns of age-associated change (Buckley & Dorsey, 1971; Stoica, Samborschi & Michiu, 1978; Radl *et al.*, 1975). Although not T cell-independent, the measurement of ^{3}H-thymidine incorporation following cellular activation by pokeweed lectin has been generally considered to quantitate the DNA-synthesizing capabilities of human B lymphocytes. Responses in this assay have indicated that B lymphocyte responses, unlike T lymphocyte responses to other mitogens, do not remarkably decline with advancing age (Kishimoto *et al.*, 1978). In 1976, Gronowicz, Coutinho & Melchers described a modification of the indirect localized haemolysis-in-gel plaque assay using staphylococcal protein A

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(SpA) which permitted direct quantitation of the numbers of individual immunoprotein-synthesizing B lymphocytes irrespective of immunoglobulin class. Using a slide modification of this SpA plaque assay, the present study examined the *in vitro* proliferative and immunoglobulin-synthesizing capacities of pokeweed lectin-activated peripheral blood mononuclear cells from 126 individuals, 26 to 95 years of age. The ability of their cells to be activated, secrete immunoglobulin and form haemolytic plaques was examined as a function of age. The results were evaluated and compared with other conventional assays of T and B lymphocyte function simultaneously conducted on cells from these same individuals.

MATERIALS AND METHODS

Study population. This consisted of 126 randomly selected individuals participating in the Baltimore Longitudinal study of Aging (BLSA). They ranged in age from 26 to 95 years (mean 57 years) and included 100 males and 26 females. The BLSA, an ongoing investigation by the Gerontology Research Center of the National Institute on Aging, NIH, is a longitudinal study of normative human ageing which began in 1958. The male volunteers in this programme have undergone periodic reassessments, approximately every 2 years, of physical, biomedical and psychological factors. The female cohort of the study, initiated in 1978, has had similar parameters measured. Demographic features of the male participants have previously been published (Stone & Norris, 1966).

BLSA participants were excluded from this study if they were known to have cancer, alcoholism, renal, hepatic or haematological diseases, or were taking any drug known to influence immune function. The group does contain individuals with diseases commonly found in the elderly, such as benign prostatic hypertrophy, osteoarthritis, and various arteriosclerotic cardiovascular diseases.

Isolation of mononuclear cells. Heparinized peripheral blood (20 ml) was diluted 1:4 with RPMI 1640 and separated by centrifugation on a Ficoll–sodium diatrizoate (LSM, Litton Bionetics, Kensington, Maryland) gradient. The interface cells were carefully removed, washed twice with RPMI 1640, counted and their density adjusted to $2{\cdot}5 \times 10^5$ mononuclear cells/ml in RPMI 1640 containing 10% heat-inactivated FCS (North American Biologicals, Miami, Florida, penicillin, 100 units/ml, streptomycin, 100 μg/ml, and 2 mM L-glutamine (complete medium).

Cells at a density of 5×10^4/0·2 ml of complete medium were cultured in flat-bottomed microtitre plates (COSTAR, Cambridge Massachusetts) at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Cultures were grown in triplicate with several concentrations of each mitogen except staphylococcal protein A which was found to have a wide dose–response curve. Phytohaemagglutinin (PHA) (Burroughs Wellcome Co., Research Triangle Park, North Carolina) was used at concentrations of 0·1, 0·2 and 0·3 μg/culture; pokeweed (PWM) (Sigma Chemical Co., St Louis, Missouri), 0·1, 0·2 and 0·3 μg/culture, concanavalin A (Con A) (Miles–Yeda Ltd, Rehovot, Israel), 1, 2 and 3 μg/culture and staphylococcal protein A (SpA) (Pharmacia, Piscataway, New Jersey) at 10 μg/culture. All mitogens were added at the start of culture (day 0). On day 4, each culture was pulsed with 1 μCi of ^{3}H-thymidine (Schwarz–Mann, Orangeburg, New York; sp. act. 1·9 Ci/mmol) and after a 16-hr additional incubation, collected onto glass-fibre filters with a semiautomatic cell culture harvester (BRANDL, Bethesda, Maryland). The dried trichloro acetic acid-precipitated DNA was solubilized with 0·1 ml of NCS (Amersham Corp., Arlington Heights, Illinois) and counted using a POP-POPOP toluene-based scintillation cocktail in a Beckman LS250 spectrometer (Beckman Instruments, Irvine, California).

Plaque assay. For the plaque-forming cell (PFC) assay, $2{\cdot}5 \times 10^6$ cells in 10 ml of complete medium were grown at 37°C in 17 × 100 mm round-bottomed plastic tubes (No. 2057, Falcon, Oxnard, California) in a humidified atmosphere containing 95% air and 5% CO_2. Unstimulated cultures were grown without the addition of pokeweed lectin while activated cultures were incubated with PWM at a concentration of 0·2 μg/ml. On day 6 the viability of the cultures was determined by fluorescence microscopy using both ethidium bromide and fluorescein diacetate

(Rotman & Papermaster, 1966; Edidin, 1970) and then 6×10^6 viable lymphocytes were removed for use in the plaque assay.

Immunoglobulin-secreting cells were quantitated by a slide modification of the staphylococcal protein A haemolytic plaque assay (Gronowicz *et al.*, 1976; Nagel & Chrest, 1981). Briefly the procedure was as follows: 6×10^5 viable lymphocytes were washed and resuspended in 0·3 ml of Hanks'-based Eagle's minimal essential medium (MEM) with $NaHCO_3$ and 20 mM HEPES. SpA-coupled SRBC (20 μl), 0·4 ml of melted agarose and 2×10^5 viable lymphocytes in MEM were combined in a 12×75 mm tube, mixed and then the mixture spread evenly on a warm 50×76 mm glass slide. The slides were floated, cell side down, on HEPES-buffered MEM and incubated at 37°C in a humidified 5% CO_2 and 95% air atmosphere. After 1 hr, the slides were rinsed with saline and incubated for an additional 60 min with polyvalent rabbit anti-human immunoglobulin antibody. This was followed by another saline rinse and incubation with appropriately diluted guinea-pig complement for 30–60 min. The slides were then rinsed in saline, dehydrated and fixed in acetone–ethanol, and dried. Lytic areas (plaques) were enumerated with the aid of a stereozoom microscope.

Cell characterization by immune markers. Spontaneous sheep erythrocyte rosette formation was studied using Ficoll–sodium diatrizoate-separated washed peripheral blood mononuclear cells using the method of Pang, Baguley & Wilson (1974) modified by overnight incubation at 4°C. Briefly, the method was as follows: SRBC in Alsever's solution (Colorado Serum Co. Laboratories, Denver, Colorado) were washed three times in saline and resuspended at 2% in RPMI 1640. A lymphocyte suspension (1×10^6 cells in 0·25 ml RPMI 1640) and 0·25 ml of the SRBC suspension were incubated for 5 min in a 37°C water bath, centrifuged at 50 ***g*** for 5 min and then incubated at 4°C overnight (18–20 hr). The supernatant was carefully removed without disrupting the cell pellet and 0·5 ml of freshly prepared ice-cold 1% glutaraldehyde in RPMI 1640 added. The suspension was allowed to incubate for 15 min, then gently mixed and 0·5 ml of a 1% trypan blue in saline solution added. At least 200 lymphocytes were counted to determine the percentage of rosetting cells.

Aggregate-free polyvalent fluorescein-conjugated rabbit anti-human immunoglobulin (IgG, IgA, IgM) (Miles-Yeda, Rehovot, Israel) was used to detect surface immunoglobulin-bearing cells. Lymphocyte concentration was adjusted to 2×10^6 per ml and 0·1 ml of this suspension mixed with an equal volume of the antisera previously diluted with phosphate-buffered saline to yield optimum fluorescence. After 30 min of incubation at 4°C the cells were washed three times with RPMI 1640, mounted on slides with 40% buffered glycerol and examined microscopically for membrane fluorescence. The number of cells exhibiting membrane fluorescence was counted and expressed as a percentage of the mononuclear cells in several microscope fields. A total of at least 200 lymphocytes were counted to determine the percentage with surface membrane fluorescence.

Serum immunoglobulin concentrations. Serum concentrations of IgG, IgA and IgM were measured by single end-point radial immunodiffusion using commercial plates (Tri-Partigen, Calbiochem–Behring Corp., La Jolla, California). The diameter of the precipitation rings was measured to the nearest 0·01 mm with a micrometer optic. A least squares linear regression line was then computed using the squared diameters of the precipitation rings of the secondary standards. This equation was then used to estimate the immunoglobulin concentrations of the other samples on the immunodiffusion plate.

RESULTS

Technical considerations and expression of PFC assay results

The number of peripheral blood lymphocytes producing immunoglobulin was assayed following 6 days of culture. As shown in Fig. 1, maximal numbers of pokeweed-stimulated immunoglobulin-secreting cells occurred on day 6 of culture while DNA synthesis, as assessed by the ^{3}H-thymidine incorporation by pokeweed-activated cells, occurred on day 5. In preliminary experiments to ensure maximal plaque development, various dilutions of the rabbit anti-human immunoglobulin antibody were evaluated. The optimal dilution of antisera which consistently led to the development of the greatest number of plaques was found to be a 1:200 dilution which was then routinely used in

each experiment. We have chosen to express the results of the PFC assay as the number of plaque-forming cells per 10^6 lymphocytes initially cultured. This was done in order to have the results accurately reflect the responses found in both stimulated and unstimulated cultures and does not require the use of 'compensation' factors to adjust for variations in either cell number or viability. On day 6, unstimulated control lymphocyte cultures had a mean cellular viability of 93·6% and a mean cell count of $1{\cdot}81 \times 10^5$ lymphocytes per ml. This latter figure represented about 72% of the number of cells initially cultured. In contrast, the pokeweed-stimulated cultures on day 6 had a lower mean viability of 80·5% and a higher mean cell density of $4{\cdot}31 \times 10^5$ lymphocytes per ml. This level was about 172% of the cells originally cultured.

Immunoglobulin synthesis by pokeweed-stimulated cultures

After culture with pokeweed lectin for 6 days, peripheral blood lymphocytes were evaluated for the number of cells with the ability to synthesize immunoglobulin using the SpA plaque assay. The number of pokeweed induced immunoglobulin-secreting peripheral blood cells as a function of cell donor age is shown in Fig. 2. Although there was a decrease in the mean number of pokeweed-stimulated immunoglobulin-producing cells from older individuals, the magnitude of this difference was not statistically significant ($r = -0{\cdot}149$, $P > 0{\cdot}05$) when compared to the level found with cells from younger donors. Linear regression analysis also demonstrated that there was only a slight correlation between levels of pokeweed-activated ^{3}H-thymidine incorporation and the number of pokeweed-induced plaque-forming cells ($r = +0{\cdot}184$, $P < 0{\cdot}05$). The major factor contributing to the lesser mean number of immunoglobulin-secreting cells in the older population was the inclusion of the results from 14 subjects over age 55 who had cells which did not develop into plaques on pokeweed stimulation. By analysis of proportions, the difference in the percentage of non-responders in the young and old (> age 55 years) age groups was highly significant ($P < 0{\cdot}001$). Comparison of other characteristics of these two age groups using the paired t-test was less informative. No statistically significant difference between the groups could be demonstrated in ^{3}H-thymidine incorporation by cells activated by any of the mitogens tested, serum immunoglobulin values or numbers of peripheral blood T and B lymphocytes. The number of immunoglobulin-secreting cells was also not related to the viability or number of cells present on day 6 of culture.

Immunoglobulin synthesis by nonstimulated cultures

Cell cultures from 65 of the 126 individuals in the study (52%) failed to produce immunoglobulin-synthesizing cells when evaluated after 6 days. Those individuals who did respond produced a mean 68 PFC/10^6 lymphocytes initially cultured. Statistical comparisons of individuals producing and

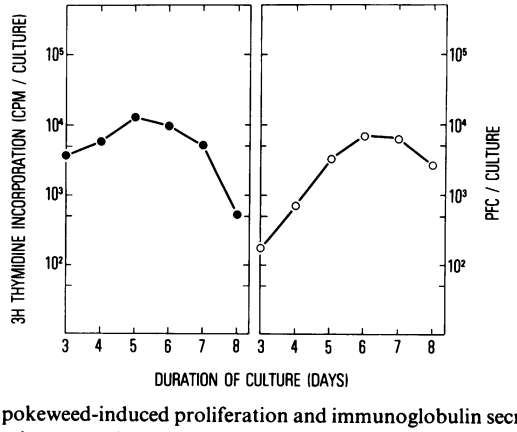

Fig. 1. Time kinetics of the pokeweed-induced proliferation and immunoglobulin secretion by peripheral blood lymphocytes. ^{3}H-thymidine incorporation of peripheral blood lymphocytes as a function of time (days) in culture (*left*). Number of immunoglobulin-secreting cells from the same individuals also as a function of the duration of culture (*right ordinate*). Each point represents the mean of seven individuals of various ages. Each individual had triplicate cultures performed on the cell samples.

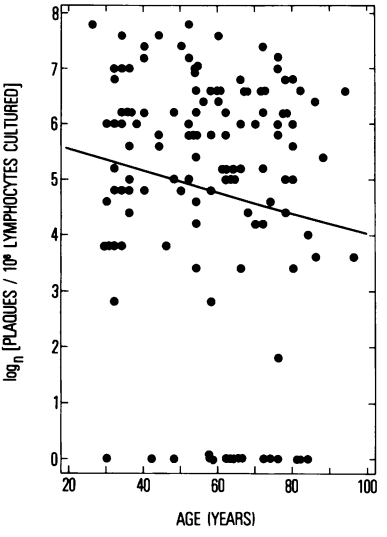

Fig. 2. Comparison of the numbers of pokeweed-activated immunoglobulin-synthesizing cells with the age of the cell donor ($r = -0{\cdot}149$, P = n.s.). Fourteen of the 17 cultures of pokeweed-stimulated cells which were non-responders were cells from individuals over 55 years of age.

not producing non-stimulated plaques did not reveal any significant differences between them in age, cell viability before or after culture, levels of mitogen-stimulated DNA synthesis, serum immunoglobulin values, percentages of peripheral blood T or B lymphocytes or numbers of PFC produced after cellular activation by pokeweed mitogen.

Mitogen responsiveness

Preliminary data examination by analysis of variance revealed that the data for ^{3}H-thymidine incorporation were heteroscedastic, and therefore, if not corrected, unsuitable for analysis by techniques assuming a normal distribution of variables. Transformation to the natural logarithm [$1n$ (x)] was found to make the variance independent of its mean and therefore logarithmic values were used for subsequent calculations. As shown in Fig. 3, the incorporation of ^{3}H-thymidine into cellular DNA in 5-day cultures of peripheral blood mononuclear cells activated with PHA, Con A and SpA diminished as a function of increasing age; however, no similar change was found in PWM-stimulated cultures. Data analysis by linear regression analysis yielded coefficients (r) of $-0{\cdot}481$ for PHA ($P < 0{\cdot}001$), $-0{\cdot}341$ for Con A ($P < 0{\cdot}001$) and $-0{\cdot}190$ for SpA ($P < 0{\cdot}05$) for the cellular response to the mitogens as a function of age. The regression coefficient for the PWM response as a function of age was $-0{\cdot}053$, which was not statistically significant. Statistical comparison between ^{3}H-thymidine incorporation by peripheral blood lymphocytes activated by concanavalin A ($r = +0{\cdot}235$, $P < 0{\cdot}01$) and soluble staphylococcal protein A ($r = +0{\cdot}285$, $P < 0{\cdot}01$) correlated with pokeweed-induced PFC numbers. DNA synthesis following cellular activation by phytohaemagglutinin did not correlate with PWM-induced cellular immunoglobulin secretion ($r = +0{\cdot}124$, $P > 0{\cdot}05$).

Rosettes and membrane fluorescence

The percentage of peripheral blood lymphocytes which had surface membrane immunoglobulin (B lymphocytes) or which formed E rosettes (T lymphocytes) as a function of the age of the cell donor is shown in Fig. 4. There was no statistically significant difference in the percentage of B or T lymphocytes in the peripheral blood lymphocyte population as a function of the age of cell donor. Numbers of T and B lymphocytes were also not related to the number of background or pokeweed mitogen-stimulated plaques. Among the entire population, E rosette-forming cells represented

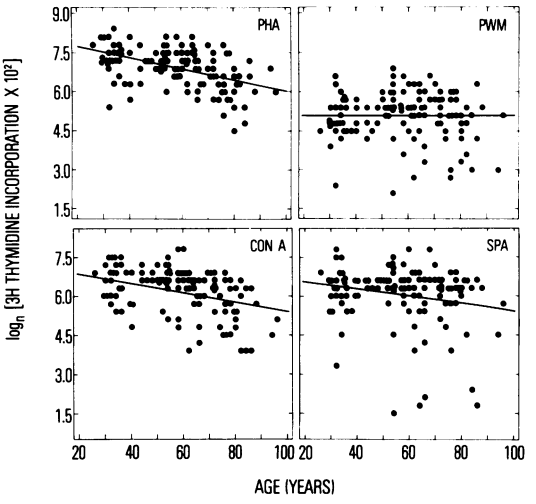

Fig. 3. Proliferative response of peripheral blood cells to different mitogens from individuals of various ages. Cellular activation was measured by ^{3}H-thymidine incorporation by the peripheral blood mononuclear cells cultured for 5 days. PHA, $r = -0{\cdot}481$, $P < 0{\cdot}001$; PWM, $r = -0{\cdot}053$, $P =$ n.s.; Con A, $r = -0{\cdot}341$, $P < 0{\cdot}001$; SpA, $r = -0{\cdot}190$, $P < 0{\cdot}05$. Each point on the figure represents the mean c.p.m. of three replicate cultures of cells from one individual.

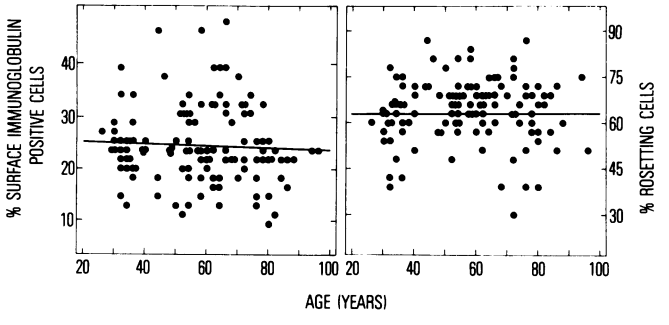

Fig. 4. Numbers of peripheral blood lymphocytic cells bearing surface membrane immunoglobulin (SIg) or forming spontaneous SRBC rosettes as a function of age. Percentages of cells with SIg detected by polyvalent anti-human (IgG, IgA, IgM) immunoglobulin, $r = +0{\cdot}064$, $P =$ n.s. (*left*). The percentage of cells forming rosettes with SRBC after 18 hr of incubation at 4°C, $r = +0{\cdot}014$, $P =$ n.s. (*right*). Each point represents the results obtained on cells from one individual. The percentage values were obtained by counting 200 cells in each sample.

$63{\cdot}4 \pm 10{\cdot}9\%$ (mean $\pm$s.d.) and surface membrane Ig$^+$ cells $24{\cdot}5 \pm 7{\cdot}9\%$ of the peripheral blood mononuclear cells.

Serum immunoglobulins

Since serum immunoglobulin values do not follow a normal Gaussian distribution (Claman & Merrill, 1964), all immunoglobulin concentrations were first converted to their respective natural logarithm prior to statistical analysis. When the relationship between serum immunoglobulin concentrations and the ability of peripheral blood mononuclear cells to form PFCs following PWM activation was examined using both parametic (least squares linear regression) and non-parametic (Kendall tau) statistical methods, no discernible relationship could be found between the number of PFCs and serum values for IgG, IgA or IgM. Using regression analysis there was a statistically significant decrease only in serum IgM values as a function of age ($r = -0{\cdot}257$, $P < 0{\cdot}001$).

DISCUSSION

In these studies, the effect of age on the *in vitro* generation of immunoglobulin-secreting cells from human peripheral blood was examined. While statistical analysis of the entire group did not reveal any significant age-associated alterations in numbers of PWM-inducible plaque-forming cells produced *in vitro*, the incidence of a non-response was four times greater in individuals over 55 years of age then in younger subjects. These data on the occurrence of a non-response are similar to the results of Haynes & Fauci (1978), who found using a direct PWM-induced anti-SRBC plaque assay, that 12% of 100 'normal' subjects were low or non-responders. Although they did not specifically examine the effect of age on this assay they were also unable to find differences between responders and non-responders (defined by them as individuals producing less than 25 plaques per 10^6 cells) in the immunoglobulin levels, degree of mitogen-activated cell proliferation or number of peripheral blood B lymphocytes. They attributed the lack of plaque production to the presence of subpopulations of activated suppressor T lymphocytes in the non-responders, a conclusion which they have further investigated (Haynes & Fauci, 1979).

Using the staphylococcal protein A plaque assay, several investigators have published PFC kinetic data similar to those reported here (Hammarström *et al.*, 1979; Pryjma *et al.*, 1980; Bird & Britton, 1979). Additional variables, particularly those involving the conditions of lymphocyte culture, serum supplementation, type and source of developing antisera and methods of calculating and expressing the numbers of plaques, make detailed comparisons between this and other studies difficult. The major discrepancy between the present data and those of Hammarström *et al.* (1979) and Pryjma *et al.* (1980) is the number of plaque-forming cells reported. In our studies, 65 of 126 (52%) of the individuals tested had cells which showed no spontaneous (non-pokeweed-stimulated) immunoglobulin secretion when studied after 6 days in culture, while at the same time, pokeweed mitogen-activated cultures produced only an average of 454 plaques per 10^6 cells initially cultured. Both figures are lower than other reports. The aetiology of these differences is not readily apparent; however, the high viability and cell numbers which we found in our cultures makes the possibility of suboptimal culture conditions doubtful, but does not exclude this possibility.

Several studies have emphasized the relationship between alterations in numbers of regulatory T lymphocyte subpopulations (particularly those demonstrating suppressor activity) and age-associated declines in lymphocyte function (Gupta & Good, 1979; Kishimoto *et al.*, 1979). A recent study (Barrett *et al.*, 1980), using a co-culture technique, has reported normal helper and suppressor T lymphocyte function for pokeweed mitogen-stimulated IgG synthesis in a group of normal elderly individuals. Our findings are in general agreement with the data of Barrett *et al.* (1980); however, additional investigations are needed to examine in detail the helper and suppressor lymphocyte status of our non-responder individuals.

The changes in cell function which occur during human ageing appear to be both subtle and complex, and to be regulated by the function of various subpopulations of lymphocytes and other accessory cells. Continued investigation on the various cellular subpopulations and their functional interactions will be needed to elucidate this complex subject.

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