An altered (impaired) immune response has previously been detected in elderly individuals [1, 2]. Several investigators have suggested that this impaired immune function is caused by a basic defect in receptor signalling of immune cells and an altered capacity to respond to antigen-dependent activation signals [3].

In fact, any immune response implies the activation and expansion (proliferation) of antigen-specific cells, and it has been reported that with aging a smaller number of immune cells enters into the cell cycle upon antigen or mitogen stimulation [3]. Indeed the ability to produce antibodies and undergo T cell proliferation has become the paradigm of an impaired immune responsiveness with age [4]. Two major mechanisms could be responsible for this observation: (i) a different distribution of lymphocyte subsets with the aging process owing to an abnormal lymphopoietic function, including cellular maturation, and (ii) an intrinsic cellular deficiency resulting in an abnormal response to activation signals. A major participant in T cell activation and proliferation is the interleukin (IL)-2 pathway, which involves the production of IL-2 and IL-2 receptor (IL-2R) expression at the cell surface.

The impaired immune response present in elderly people may be associated with the normal aging process, with chronic pathological conditions, or with malnutrition. Malnutrition is a rather frequent finding in the elderly population (15–60%) in hospitals or living in nursing homes [5]. Therefore the early detection of malnutrition and its correction could be a useful means of preventing intercurrent and terminal infectious events in the aging population.

The Mini Nutritional Assessment (MNA) is a valuable recently developed tool to assess the risk of malnutrition in the elderly [5]. Our aim in this study was to
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determine whether there is a correlation between the MNA score, other nutritional markers, and defined immunologic indices in the elderly. These immunologic variables included the assessment of major peripheral blood lymphocyte subsets, and the capacity to respond in vitro to polyclonal mitogenic stimulation.

Materials and Methods

Subjects
Eighty-eight nursing home patients were classified according to the MNA score into three groups: group 1 (MNA ≥ 24, adequate nutritional status); group 2 (MNA between 17 and 23.5, at risk of malnutrition), and group 3 (MNA score < 17, frank malnutrition). Seventy-nine patients completed the immunological study, which took place in Toulouse (Centre de Médecine Gériatrique).

The patients were also grouped according to two other criteria: (i) plasma albumin concentrations, and (ii) the presence of anemia. Based on the first of these criteria, three groups were defined: patients with plasma albumin concentrations of < 35 g/l; patients with values between 35 and 40 g/l, and a third group with values of > 40 g/l. These albumin ranges were established according to previous nutritional studies [6].

Anemia was defined as a hemoglobin concentration of < 12 g/dl for females and < 13 g/dl for males [7]. Hematological determinations included total and differential leukocyte count and hematocrit.

Determination of Serum Immunoglobulins
Immunoglobulin isotype (IgG, IgM, IgA) determination was performed by nephelometry (ARRAY 360, Beckman).

Lymphocyte Subsets in Peripheral Blood
Blood samples were collected in sterile EDTA blood collection tubes, and flow cytometric analysis of human leukocyte subsets was performed. A one-step staining procedure was performed with FITC-labeled monoclonal antibodies against T cells (CD3+), B cells (CD19), helper T (CD4+), and suppressor/cytotoxic T cells (CD8+; Immunotech).

The proportion of CD45-RO and CD45-RA subsets within the CD4+ lymphocyte subpopulation was determined by double-staining techniques in flow cytometry (Beckton Dickinson).

In vitro Lymphocyte Functional Assays
Proliferation Assay
Peripheral blood leukocytes (PBL) were resuspended in medium consisting of RPMI 1640, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20% heat-inactivated fetal calf serum or autologous plasma (complete medium).
PBL (10^5/well) were cultured in triplicate in 96-well flat-bottomed microculture plates (Costar) in a total volume of 0.2 ml. Cell cultures were stimulated with three different concentrations of phytohemagglutinin (PHA)-A (0.5, 1, and 5 µg/ml) and with a combination of PHA/PMA (PHA 0.5 µg/ml and PMA 1 ng/ml). Cell cultures were incubated at 37°C for 72 h in a humidified atmosphere containing 5% CO2 in air. Proliferation was assessed by adding [3H]-thymidine (1 µCi/well) during the last 18 h of the cell culture.

Cytokine Assay

One-milliliter aliquots of cell suspension containing 2 × 10^6 viable PBL were distributed in 24-well microplates and stimulated by adding 1 ml of complete medium containing PHA at a final concentration of 0.5 µg/ml, or PHA plus PMA (PMA final concentration 1 ng/ml). Nonstimulated control PBL were prepared in parallel. Two replicates were prepared for subject and treatment. Cultures were incubated at 37°C in a humidified atmosphere of CO2 and 95% air.

Analysis of IL-2 mRNA Expression

After 72 h of stimulation with PHA or PHA plus PMA, PBLs were collected and washed with ice-cold PBS (pH 7.4). Cells were lysed in denaturation buffer and total RNA was extracted using the Stratagene RNA isolation kit. RNA concentrations were adjusted to 1 µg/µl. Reverse transcriptase (RTP) was performed with oligo d(T)16 priming (60 min at 37°C, 10 min at 95°C). Polymerase chain reaction (PCR) was conducted following the manufacturer’s instructions (Gene Amp Kit, Perkin Elmer). Thus, 2 µl sample cDNA and 2 µl BD1 (internal standard for IL-2) were used as templates. Amplification of IL-2 transcripts was performed as follows: 45 s at 94°C, 1 min at 60°C, 1.5 min at 72°C, 30 cycles; 45 s at 94°C, 1 min at 60°C, 7 min at 72°C, one cycle. For each PCR a serial dilution of BD1 (10 to 10^-5 amol) was co-amplified to establish a standard curve for IL-2-specific transcripts. PCR products were analyzed on a 2% agarose gel containing ethidium bromide.

For the dot-blot analysis, the PCR products of the samples and standard curve (BD1) were diluted 1:10 in TE buffer (pH 8.0), loaded in duplicates on nitrocellulose (Genescreen, NEN Dupont), and fixed under UV radiation. Hybridization was performed under standard conditions with a specific 33P--IL-2 antisense probe which was end-labeled by T4 kinase (Stratagene) before use. The nitrocellulose was exposed to an X-ray film (in the presence of Stratagene reference). Spots were cut from the nitrocellulose and radioactivity was determined by liquid scintillation counting.

Statistics

Statistics were performed using the Number Cruncher Statistical Systems software (NCSS, Kaysville, Utah 84037). The influence of single independent factors was assessed by classic one-way analysis of variance (ANOVA). When the as-
sumption of normality was not valid, the Kruskal-Wallis one-way ANOVA on ranks was used as a nonparametric alternative.

**Results**

*Peripheral Blood Lymphocyte Subsets*

Total lymphocyte counts showed a trend of lower values in the group at risk of malnutrition compared with the well-nourished group, while the malnourished group had lower values when compared with the group at risk of malnutrition (Fig. 1a). However, this trend did not reach statistical significance. No real lymphopenia was observed in any of the groups. No differences were observed for CD3+ lymphocytes (total T cells; Fig. 1b), or for CD4+ or CD45RA/RO CD4+ T cell subsets (data not shown). B cell (CD19+) counts were similar for the three MNA categories and fell within the normal range (data not shown). CD8+ T cells followed a similar pattern to total lymphocytes and were lower in the malnourished group. Again, statistical significance was not reached (Fig. 1c).

A lymphocyte subpopulation like natural killer (NK) cells, which is in part CD3−, was not specifically characterized in the present study. One could speculate that this particular subset is reduced in the malnourished group and could contribute to the changes observed in the total lymphocyte counts. However, no abnormal values in the other leukocyte populations were observed.

The red blood cell compartment showed a significant difference between the well-nourished and malnourished groups ($p < 0.05$ ANOVA and Kruskal-Wallis one-way ANOVA on ranks tests), as shown by the hemoglobin and hematocrit levels (Fig. 2). When the lymphocyte subpopulations were compared between the anemic and nonanemic groups, fewer CD8+ cells were observed in the former (Fig. 3).

When groups were defined on the basis of serum albumin concentrations (group 1 < 35 g/l, group 2 between 35 and 40 g/l, and group 3 > 40 g/l) the differences in total lymphocyte counts and in the different lymphocyte subsets were still more subtle than for the MNA categories (data not shown).

In conclusion, in this trial we could not show an association between nutritional status – as defined by MNA, albumin, or anemia – and lymphocyte counts. Only a subtle change was observed for total lymphocytes and for CD8+ cells. As mentioned above, it is possible that a specific lymphocyte subset, such as NK, is more sensitive to nutritional status but this was not determined in the present study.

*Serum Immunoglobulins*

No differences were observed for serum IgG and IgM concentrations among the three MNA categories. In contrast, a higher concentration of serum IgA (Fig. 4) was detected in the malnourished group ($p < 0.05$ by ANOVA and Krus-
Fig. 1. Total lymphocyte counts ($10^3$/ml) were higher in group 1 but the difference did not reach statistical significance (a); a similar pattern was observed for CD8+ cells (c), whereas similar counts for CD3+ cells were found in all three groups (b).
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Fig. 2. Hemoglobin (g/dl) and hematocrit (%) in the three MNA categories. Significant differences for hemoglobin ($p = 0.02$) and hematocrit ($p = 0.018$) were observed between the well-nourished and malnourished groups according to the Kruskal-Wallis one-way ANOVA on ranks test.

When patients were divided into anemic and nonanemic groups, a nonsignificant trend showing higher IgA concentrations in the anemic group was also observed (data not shown). In contrast, no difference in immunoglobulin levels for any of the isotypes was observed when groups were defined according to albumin concentrations.
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Fig. 3. Lymphocyte counts ($\times 10^3$); $p = 0.07$ between groups.

Fig. 4. Comparison of serum IgA concentrations; $p < 0.05$ between malnourished and at-risk groups by Kruskal-Wallis one-way ANOVA on ranks test.
Proliferation and Activation of Mitogen-Treated PBL

A clear stimulation of T cell proliferation was achieved with PHA, at all doses tested, and with PHA/PMA combined treatments. However, [3H]-thymidine incorporation was similar for the three categories defined by the MNA score in all conditions tested (data not shown). In agreement with the proliferative activity, no differences in CD25 expression (marker of activation) on CD3+ T cells were observed comparing the different MNA groups 72 h after in vitro stimulation.

When groups were defined according to albumin levels, [3H]-thymidine incorporation was lower (Fig. 5) in the group with a plasma albumin of <35 g/l (p < 0.05 by Kruskal-Wallis one-way ANOVA on ranks).

IL-2-Specific mRNA

IL-2-specific mRNA was determined by a semiquantitative method combining RT-PCR and a dot-blot technique. IL-2 transcription was low to undetectable in nonstimulated control cells and PHA-treated PBL. In contrast, IL-2-specific mRNA expression in PBL was clearly higher, compared with the controls, after the combined treatment using PHA/PMA. Higher quantities of specific IL-2 mRNA were detected in the MNA “well-nourished” category compared with the other categories. Although this difference did not reach statistical significance (p = 0.06), it shows an obvious trend (Fig. 6).
**Discussion**

The aim of our study was to establish a correlation between a clinical tool, such as the MNA, for screening for malnutrition in the elderly, and some biological immune markers. This appeared to be of major interest since impaired immune function in the elderly population often leads to a fatal outcome. We and others have hypothesized that a part of the immune deficit observed in elderly people is caused by nutritional deficiencies and not exclusively by an aging genetic program [8, 9]. In the past it has also been shown that malnutrition is a common subclinical underlying condition in this population [5].

In this study we explored two major sets of indices reflecting the cellular basis of immune dysfunction and its potential association with malnutrition. The first included the determination of serum levels of different antibody isotypes and blood lymphocyte populations; the second was related to the capacity of immune cells to react in vitro to activation signals such as polyclonal mitogens. IL-2 induction is closely related to lymphocyte proliferation and reflects the capacity of T lymphocytes to enter cell cycle upon (antigen) mitogen stimulation. This is a basic and crucial event in any specific immune response.

In this study good proliferation activity was observed for the three doses of PHA tested and also for the combination of PHA/PMA. No clear differences or dose-response type of pattern were observed among the different treatments (data not shown). However, IL-2-specific mRNA was only clearly upregulated by the...
combined PHA/PMA stimulus, while no clear specific message induction was observed above the resting levels with single PHA treatment at whatever dose tested.

Comparing the levels of IL-2 mRNA expression after 72 h of PHA/PMA stimulation among different MNA categories revealed a higher level of IL-2 transcripts in the well-nourished group (Fig. 6). This may suggest that the diminished IL-2 production previously reported in the elderly [2] could in part be linked to nutritional status. In addition, since PMA interacts directly with protein kinase C (PKC), a defect in T cell activation in the elderly may rather be associated with an altered expression of receptors (receptor densities) or with the physical status of plasma membrane than with the distal events in the genetic activation by antigens.

In this study, our failure to detect a clear induction of IL-2 mRNA with a single PHA treatment was probably related to the late mRNA sampling rather than to an impairment in signal transduction. The high proliferative stimulation seems to suggest this interpretation.

Concerning the lymphocyte populations, total lymphocyte counts were within the normal range for all the groups and lymphopenia was not detectable. Nevertheless, in both the malnourished group (according to the MNA category) and the anemic group, total lymphocyte and CD8+ T cell numbers were lower than in the other groups. However, this diminution was not statistically significant.

There is some discrepancy in the effect of age and malnutrition on the NK populations. Unfortunately this subset of lymphocytes could not be determined in this study. In contrast, lymphopenia has previously been described in elderly people and was associated with low CD4+ counts [10]. It is a matter of debate, though, whether low CD4+ T cell counts are related to malnutrition or to a chronic inflammatory condition [11].

**Conclusion**

In conclusion, although the population of elderly people included in this study showed markers of malnutrition, the deficiencies were not severe enough to alter the homeostasis of the immune system. In fact, MNA detection of malnutrition seems to anticipate other biochemical markers of malnutrition and indicates a population at risk, before the physiology of the immune response is profoundly altered.

**References**


**Discussion**

*Dr. Steenhout:* Dr. Schiffrin, your data on IgA are very interesting. We have recently conducted a study in infants with criteria of malnutrition, and we also found an increase of IgA in saliva. I would be interested in your opinion on the mechanism. It is a good thing to identify indicators, but we also need to know the underlying pathophysiology. Is it related to infectious disease perhaps? Have you found that patients with low MNA scores have a higher frequency of infectious disease? I'm concerned that the trials may have included patients with different pathologies, which could affect the indices that were measured.

*Dr. Schiffrin:* I think we can assume that the IgA isotype comes mainly from GALT (gut-associated lymphoid tissue). We know that elderly people have increased gut permeability – the mucosal barrier works less efficiently and there is a greater antigen and infection challenge. This is speculation, but the mucosal barrier is probably more altered in patients with a low MNA score and a low hemoglobin. We know that anemic patients have reduced gut perfusion and increased permeability, so there is increased passage of antigens and a greater IgA response. I think we can assume that the inefficient mucosal barrier allows more macromolecules to pass, leading to a greater IgA response.

*Dr. Vellas:* I have two comments. First, I believe it is important that we identify the extent to which MNA is a marker of nutrition and the extent to which it is a marker of frailty. This could be important for the future. Second, in relation to MNA and immunological function, it seemed in the study we did in Toulouse that the people with low MNA scores had malnutrition, because they had a decreased energy intake. However, they had no inflammation and no evidence of other disease. I think impairment of immune function is most likely to be a result of hypercatabolism and of malnutrition plus disease, but not of malnutrition alone. This may be an important point.

*Dr. Schiffrin:* I agree with you. We were looking specifically for inflammatory markers and evidence of a catabolic state which would result in a modification of the immune response. The Toulouse population was too healthy to resolve this issue!

*Dr. Vellas:* One of our exclusion criteria was acute disease.

*Dr. Morley:* Most immune functions show a subtle decrease with age. Malnutrition on the whole makes that worse, particularly if severe. The only immune function that I’m aware of that does not decline with age but does decline with malnutrition is the CD4 count. We have shown this in an older age group and it was also shown many years ago in a pediatric population [1]. You did not present CD4 data. Do you have such data?
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Dr. Schiffrin: CD4 didn’t change or showed only a proportional increase because CD8 and NK cells decreased.

Dr. Morley: That suggests that your population had minimal malnutrition.

Dr. Camilla: I have two questions. First, how old does one need to be to be called elderly? I’ve seen data from 55 to over 75! Second, MNA looks like any other kind of global assessment of nutritional risk. How good is it for interventional studies? Don’t we need to correlate the scores with functional variables other than fancy immunological tests?

Dr. Guesry: I’m not going to tell you how old you should be to be called elderly, but MNA has been validated in a certain age range, and outside that range it has not been validated. So for your first question the answer is in Yves Guigoz’ article!

Dr. Guigoz: Regarding interventions, we have already used MNA assessment during an intervention study [see Arnaud-Battandier et al., this volume]; we now need more intervention studies, which is the next step.

Reference