Pergamon

Oral Oncol, Eur J Cancer, Vol. 31B, No. 2, pp. 144–150, 1995

0964–1955/95 $9.50 + 0.00

0964–1955(94)00042–5

Influence of Aging and Chemoradiotherapy on Leucocyte Function in Oral Cancer Patients

K. Yoneda, T. Yamamoto, E. Ueta, K. Nakao and T. Osaki

Leucocyte functions and the influence of chemoradiotherapy were examined in three age groups of patients with oral cancer. The groups consisted of 66 patients below 65 years old (group A): 40 patients between 65 and 80 years old (group B); 20 patients over 80 years old (group C). 20 healthy individuals (45.8 ± 9.6 years old) were chosen as controls. Originally, no significant differences in leucocyte count, CD3 population, CD4/CD8 ratio, natural killer activity or phagocytosis of polymorphonuclear leucocytes (PMNL) were found in the patients. However, T cell blastogenesis, lymphokine-activated killer cell activity and superoxide production of PMNL were all suppressed. These functions were further suppressed by cancer therapy, the greatest suppression being seen in group C. Compared to controls and group A, the generation of interleukin-1, interleukin-6, tumour necrosis factor-alpha and granulocyte–macrophage colony-stimulating factor were markedly suppressed in group C. These results show that very old cancer patients are already in an immunologically suppressed condition and that the leucocyte functions of these patients are further impaired by cancer therapy.

Keywords: leucocyte function, aging, chemoradiotherapy, oral cancer

Oral Oncol, Eur J Cancer, Vol. 31B, No. 2, pp. 144–150, 1995.

INTRODUCTION

THE NUMBER of very old patients with malignant tumour has been increasing in parallel with the increase in life span. In these patients, physiologic hypofunction of the bone marrow and other organs, as well as the mental distress associated with aging, make radical cancer treatment difficult [1]. Immunosuppression due to aging [2–5] is one of these factors. Furthermore, anti-cancer drugs and radiation markedly suppress leucocyte function [6], and may make these patients even more susceptible to tumour cell spread and microbial invasion. Therefore, leucocyte functions and adverse effect of cancer treatment in aged patients need detailed examination in order to achieve safe and successful treatment.

Monocytes/macrophages recognize and digest foreign bodies, and offer the recognition to T cells. Besides killing by T cells, natural killer (NK) cells and lymphokine-activated killer (LAK) cells also attack tumour cells without any restriction. Furthermore, polymorphonuclear leucocytes (PMNL), as well as other phagocytic cells, kill tumour cells and microorganisms [7–9]. These killing activities were largely regulated by cytokines. Among the cytokines, interleukin-1 beta (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α) and granulocyte–macrophage colony-stimulating factor (GM–CSF) are very important [10–16].

Some lymphocyte and macrophage functions, such as NK activity and phagocytosis, are not affected by aging [17, 18]. However, cytokine production by leucocytes and reactive oxygen generation by PMNL have not been evaluated. In this study, we examine the differences in leucocyte functions and the influence of cancer treatment in three age groups of patients with oral cancer and healthy controls.

MATERIALS AND METHODS

Patients and healthy controls

126 untreated patients with oral squamous cell carcinoma were included in this study and divided into three age groups; 66 patients younger than 65 years old (group A), 40 patients from 65 to 80 years old (group B) and 20 patients over 80 years old (group C). There were no significant differences in tumour T-stage and site among the three groups (Table 1). All patients received concomitant chemoradiotherapy, and the majority (61, 35 and 13 patients of groups A–C, respectively) underwent surgery after inductive chemoradiotherapy. Between groups A and B, there were no large differences in the doses of cobalt 60 (^{60}Co) and peplomycin (PLM) with 5-fluorouracil (5-FU) given. However, patients in group C received somewhat lower doses of PLM and 5-FU; the controls consisted of 10 healthy males and females ranging in age from 38 to 54 years old. Smoking was prohibited after hospitalisation in 49 male and 3 female patients. No healthy controls had a smoking habit.

Correspondence to K. Yoneda.
The authors are at the Department of Oral Surgery, Kochi Medical School, Kohasu, Oko-cho, Nankoku-city, Kochi 783, Japan.
Received 19 Sep. 1994; accepted 10 Oct. 1994.

Table 1. Clinical profiles of cancer patients submitted

Item	Controls	Group A	Group B	Group C
Male/female	10/10	48/18	23/17	8/12
Age (mean years)	45.8±9.6	53.4±7.9	70.8±4.1	86.0±4.2
Tumour stage (case number)				
T1	—	10	4	3
T2	—	30	17	8
T3	—	15	13	5
T4	—	11	6	4
Treatment				
^{60}CO (Gy)	—	32.3±11.0	31.3±8.6	34.0±11.9
PLM (mg)	—	52.0±22.1	42.3±19.6	30.2±18.8
5-FU (mg)	—	3520±1520	3200±1390	2760±1540
Surgery (case number)		61	35	13

Controls: healthy persons.

Blood sampling

Peripheral venous blood samples were obtained from controls, and each patient before treatment, about a week after the completion of chemoradiotheraphy and just before discharge.

Separation of lymphocytes, monocytes and PMNL

Mononuclear cells and PMNL in the peripheral venous blood were separated by Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, New Jersey, U.S.A.) density gradient centrifugation at 1500 rpm for 30 min. The PMNL, lying just above the red blood cell layer, were collected, and contaminating erythrocytes removed by dextran sedimentation and hypotonic shock. The PMNL were then washed twice with Ca- and Mg-free phosphate-buffered saline (PBS, pH 7.2), and adjusted to the required concentration. Lymphocytes and monocytes were collected from the top layer, and washed three times. These cells were resuspended in RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum. Non-adherent lymphocytes were separated from monocytes by two plastic-adherence steps. PMNL and lymphocytes both showed a purity of over 97% and a viability of over 98% on microscopy using Giemsa staining and trypan blue exclusion, respectively. Monocyte purity and viability were more than 95% as determined by phagocytosis of yeast particles and trypan blue exclusion, respectively.

Flow cytometry

The lymphocyte surface phenotypes were determined using monoclonal fluorescein isothiocyanate (FITC)-conjugated antibodies (anti-CD3: Leu4, anti-CD4: Leu3a, and anti-CD8: Leu2a, Becton Dickinson Immunocytometry Systems, Mountain View, California, U.S.A.). Isolated lymphocytes (5×10^5 cells) were resuspended in 100 µl PBS containing 0.1% NaN_3 and packed into culture tubes. After saturation with three fold-diluted FITC-conjugated mAb solution, the tubes were incubated for 30 min on ice in the dark. Cells were then washed twice, resuspended in a 0.1% NaN_3 solution, and analysed using an EPICS V flow cytometer (Coulter, Hialeah, Florida, U.S.A.).

Blastogenesis

Lymphocytes (1×10^5 cells) in 0.1 ml RPMI1640 were seeded into round-bottomed microwells, and 0.1 ml of 1 µg/ml phytohaemagglutinin-P (PHA, Difco, Detroit, Michigan, U.S.A.) or 10 µg/ml concanavalin A (Con A, Difco) solution was added. Blastogenesis was quantitated by adding [^{3}H]-thymidine (New England Nuclear, Boston, Massachusetts, U.S.A.) during the final 6 h of 72 h incubation, and measuring the amount incorporated.

NK and LAK activity

Both NK and LAK activities were measured by a ^{51}Cr release assay using K562 and Daudi cells as targets for NK and LAK cells, respectively. LAK cells were induced from lymphocytes by culturing in medium containing 100 U/ml human recombinant IL-2 (rIL-2, Shionogi Pharmaceutical Co., Osaka, Japan) for 3 days. Effectors and $Na_2{}^{51}CrO_4$-labelled targets (200 µCi/ml, New England Nuclear) were co-cultured for 4 h at a ratio of 25:1. The percentage of specific tumour cell lysis was calculated by the formula:

$$\% \text{ specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

where spontaneous release is that observed in the absence of effector cells, and maximal release is that found in the supernatant after treatment of the target cells with 1% Triton X-100.

Phagocytosis

PMNL were co-incubated in HBSS with 1 µm latex particles at a ratio of 1:100 for 1 h at 37°C. Phagocytosed particles were observed microscopically, and PMNL containing more than five particles were considered to be phagocytic cells. Phagocytic activity was expressed as the percentage of phagocytic cells in 400 cells counted.

Superoxide (O_2^-) generation

O_2^- was spectrophotometrically assayed by a cytochrome *c* (type VI, Sigma Chemical Co., St. Louis, Missouri, U.S.A.) reduction method using a double wavelength spectrophotometer (UV-300, Shimadzu Ltd, Kyoto, Japan) equipped with a thermostatted cuvette holder. The reaction mixtures (1×10^6/ml PMNL and 100 µM cytochrome *c* in HBSS) in the cuvette were preincubated at 37°C for 1 min, and 50 ng/ml

Table 2. Cell counts, blastogenesis, NK activity, and phagocytosis of PMNL in age groups before cancer therapy

Cell counts, subset proportion, NK activity and others	Healthy persons (control group)	Cancer patients		
		Group A	Group B	Group C
PMNL	3660±1170	3710±1230	3690±1225	3560±1133
Lymphocytes	1770±460	1750±510	1780±530	1520±560
CD3 (%)	60.6±7.7	63.8±12.6	61.8±12.6	61.5±9.4
CD4/CD8	2.1±0.3	2.2±0.5	2.0±0.5	2.0±0.3
Blastogenesis (Con A, $\times 10^4$ dpm)	15.2±5.0	8.8±7.1*	8.7±6.8*	6.0±3.5*
NK activity (%)	39.0±11.4	37.3±16.2	35.3±17.7	38.1±18.9
Phagocytosis (%)	57.5±3.3	56.1±5.0	56.0±3.8	55.4±4.1

*Significantly lower than the control value ($P<0.001$, t-test).

phorbol 12-myristate 13-acetate (PMA, Sigma) was added. The kinetics of cytochrome *c* reduction were measured by the absorbance change at 550–540 nm. O_2^- concentration was calculated from the linear portion of the cytochrome *c* reduction curve.

Assay of cytokines

PMNL (2×10^6 cells), lymphocytes (1×10^6 cells) or monocytes (1×10^5 cells) were incubated in 1 ml of RPMI1640 medium containing 10% autologous serum in the presence or absence of LPS (100 ng/ml), IL-2 (10 U/ml) or TNF-α (50 U/ml), respectively. After 48 h incubation, the culture supernatants were stored at −80°C until cytokine titration. IL-1β and GM–CSF levels were determined by enzyme-amplified sensitivity immunoassay (EASIA), using IL-1 β-EASIA (Medgenix Diagnostics, Brussels, Belgium) and GM-CSF-EASIA (Medgenix Diagnostics). IL-6 and TNF-α levels were determined by enzyme-linked immunosorbent assay (ELISA) using Quantikine™ (Research and Diagnostic Systems, Minneapolis, Minnesota, U.S.A.) and BIOKINE™ (T Cell Sciences Inc., Cambridge, Massachusetts), respectively.

RESULTS

Cell counts and proportion of T cell subsets

There were no differences in PMNL and lymphocyte counts among the groups. The counts of PMNL and lymphocytes were about 3600 cells/μl and 1700 cells/μl, respectively, being slightly decreased in group C (Table 2). After the completion of cancer therapy, leucocyte counts decreased in all patient groups, but a significant decrease in PMNL and lymphocytes was observed only in group C (data not shown).

The CD3 population was similar between the control and patient groups, and the proportions of CD4 and CD8 were almost the same in all groups (data not shown) with a CD4/CD8 ratio of about 2:1. Cancer therapy did not affect the CD3 population or the CD4/CD8 ratio (Table 2).

Blastogenesis

PHA-induced blastogenesis was originally suppressed in group A ($17.2\pm9.9\times10^4$ dpm), group B ($15.0\pm9.0\times10^4$ dpm) and group C ($9.7\pm5.5\times10^4$ dpm), compared with the control group ($24.5\pm8.2\times10^4$ dpm) (Fig. 1). After inductive chemo-radiotherapy, lymphoblastogenesis decreased slightly and was followed by a further significant decrease in DNA synthesis in groups B and C. ConA-induced blastogenesis was also significantly decreased, especially in group C (Table 2).

NK and LAK activity

No difference in NK activity was observed among the groups examined (Table 2), and no change in NK activity was induced by cancer therapy (data not shown). However, a marked difference in LAK activity was observed between the control and patient groups (Fig. 2). The original LAK activities of groups A, B and C were 55.3±16.6%, 58.1±16.8% and 56.8±17.1%, respectively, these activities being significantly lower than that of the controls (72.8±9.6%). The killing activity gradually decreased during cancer therapy, and significantly decreased after completion of cancer therapy in groups B and C (48.0±15.1% and 42.9±11.3%, respectively).

Cytokine generation

An age-related decrease in cytokine generation was observed in lymphocytes, monocytes and PMNL. Monocytes from patients in group B, and both lymphocytes and monocytes in group C, generated about half the amount of GM–CSF in the presence or absence of IL-2 and TNF-α as compared with control cells (Fig. 3a). A decreased generation of TNF-α was observed in groups B and C, this being about 66 and 50%, respectively, of that generated in the controls (Fig. 3b). However, lymphocytes from patients in groups A and B produced IL-1β at about the same level as the control group, while the cytokine generation of lymphocytes in group C was suppressed to about 75% of the control level (Fig. 3c). In addition, an age-associated decrease of IL-1β generation was observed in both monocytes and PMNL. For example, the PMNL in group C patients produced 81.7±12.6 and 71.7±7.6 pg/ml/2×10^6 cells in the presence or absence of LPS, respectively, being about 55% of the values in the controls (148.3±32.5 and 126.7±17.6 pg/ml/2×10^6 cells, respectively).

IL-6 generation of lymphocytes and monocytes was not suppressed in groups A and B. However, a marked decrease of the cytokine generation was seen in group C (Fig. 3d). Compared to lymphocytes and monocytes, IL-6 production of PMNL showed a slightly age-related suppression. In group C, IL-6 production by PMNL was decreased by about 50%.

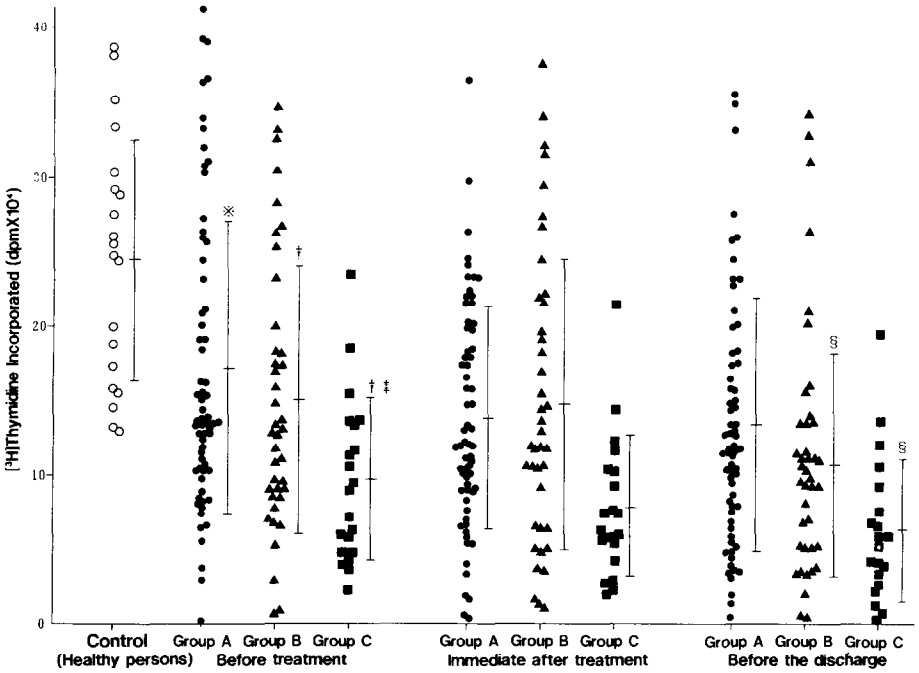

Fig. 1. Lymphocyte blastogenesis by PHA in age groups. Lymphocytes (1×10^5 cells/well) from healthy controls (○), group A (●), group B (▲) and group C patients (■) were stimulated with PHA (0.5 μg/ml) for 3 days, and then pulsed with [³H]thymidine and incorporated isotopes were counted. Each bar indicates mean ± 1 S.D. *$P<0.01$, and †$P<0.001$ (versus control, by *t*-test). ‡$P<0.01$ (versus group A, by *t*-test), §$P<0.01$ (versus before treatment, by paired *t*-test).

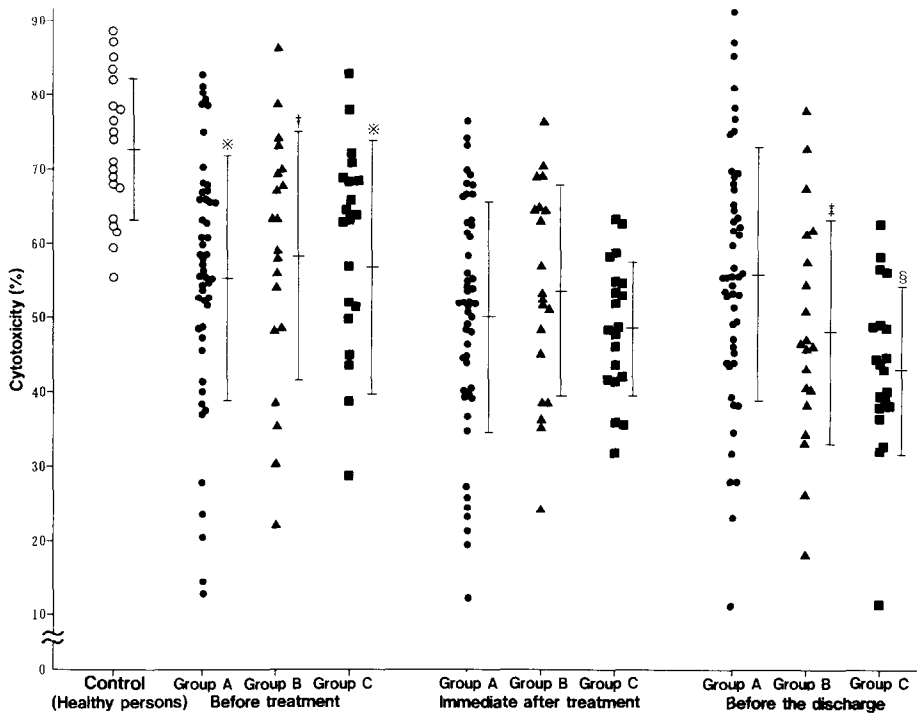

Fig. 2. Suppression of LAK activity in cancer patients. Lymphocytes were cultured with rIL-2 (100 U/ml) for 3 days, and cytotoxicity against Daudi cells was examined. Symbols are as in Fig. 1. *$P<0.001$, †$P<0.01$ (versus control, by *t*-test), ‡$P<0.05$, §$P<0.01$ (versus before treatment, by paired *t*-test).

Phagocytosis and O_2^- generation of PMNL

Phagocytic activity was normal, even in group C (Table 2), and was not affected by cancer therapy (data not shown). However, a significant decrease of O_2^- generation was seen in all patient groups. The amounts of O_2^- generated were 105.2 ± 19.4, 79.7 ± 27.4, 82.6 ± 28.4 and 79.7 ± 22.5 pmol/min/10^4 cells in the control, and groups A–C, respectively (Fig. 4). After inductive chemoradiotherapy, O_2^- generation

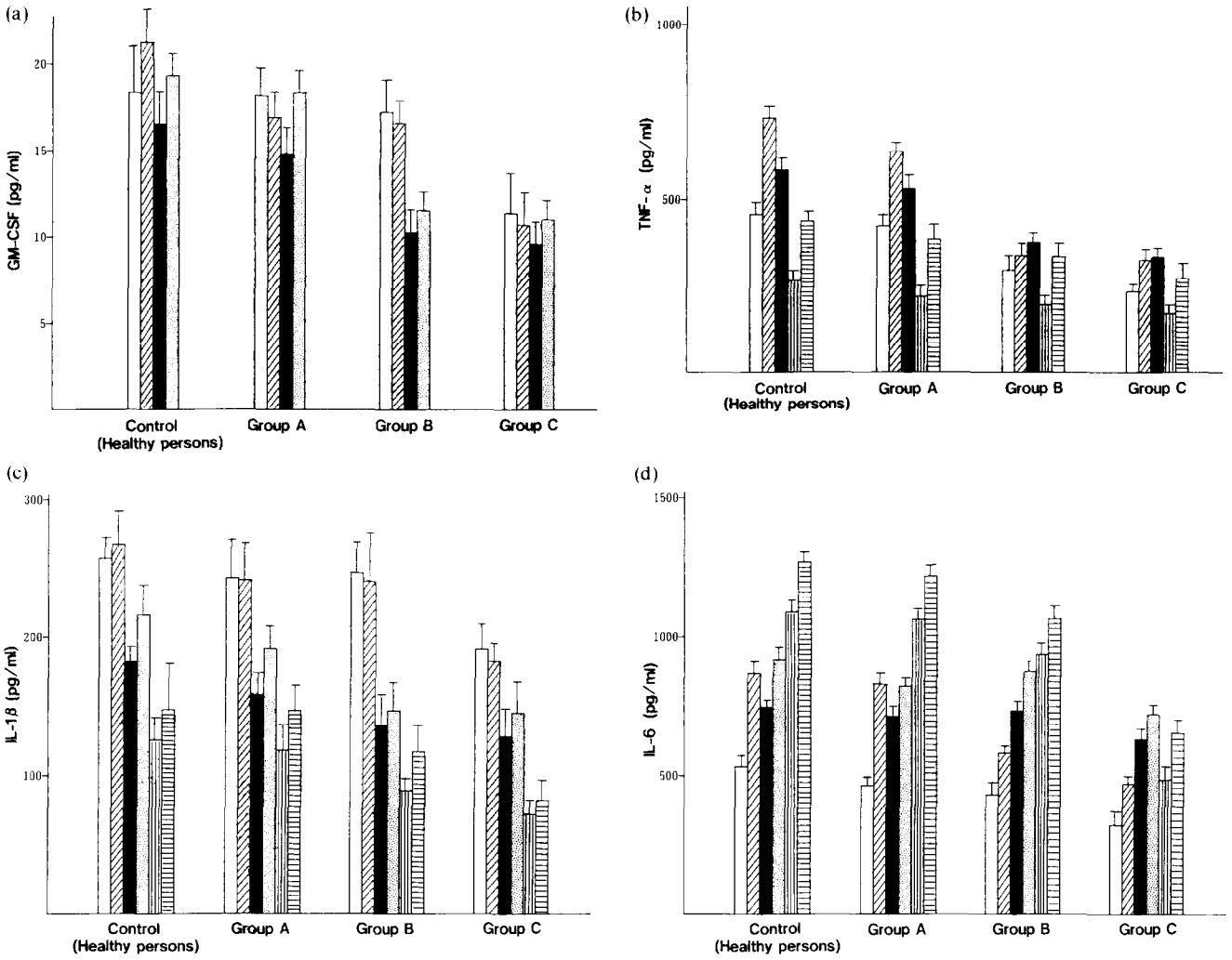

Fig. 3. Cytokine generation of lymphocytes, monocytes and PMNL in age groups of patients with oral carcinoma. Lymphocytes with (▨) or without (□) rIL-2, monocytes with (▩) or without (■) rTNF-α, PMNL with (▤) or without (▥) LPS were cultured for 48 h as described in Materials and Methods. Cytokines released into the medium were measured. (a) GM-CSF; (b) TNF-α; (c) IL-1β; (d) IL-6.

was decreased to 68.4 ± 24.9 and 64.6 ± 19.5 pmol/min/10^4 cells in groups B and C, respectively. However, group A did not exhibit any significant decrease of O_2^- generation. After these decreases, O_2^- productivity of PMNL remained constant.

DISCUSSION

Investigation of leucocyte function in cancer patients is very important because tumour cell invasion and dissemination, and also opportunistic infection may occur if lymphocyte and PMNL functions are suppressed. Leucocyte dysfunction may result from both aging and cancer therapy. For the safe treatment of highly aged patients, their leucocyte functions and the influence of cancer therapy on leucocytes should be examined.

Elderly patients originally exhibited no abnormality in leucocyte counts, CD3 proportion, CD4/CD8 ratio, NK activity or phagocytosis. These parameters, except for the cell count, remained virtually constant, even during and after treatment. However, a decrease in T cells and CD4/CD8 ratio is occasionally noted in autoimmune diseases [19] and immunosuppressive viral infection [20]. Not only the proportions of T cell subsets, but also NK and phagocytic activities, seem to be immutable in malignant tumours, and hardly affected as far as the tumour stage has not progressed.

The finding that both lymphocyte and PMNL counts decreased with advance of cancer therapy in group C might be explained by bone marrow suppression. It was expected that, in very old patients, more marked bone marrow suppression would be induced, and recovery from the suppression would be delayed. However, no such clear suppression of the bone marrow was found in group C.

T cell blastogenesis was markedly suppressed by cancer therapy. Taken together with the original suppression of the DNA synthesis of T cells in groups B and C, T cells may become too hypofunctional with age to react to mitogens and cytokines. Hypofunction of T cells from very old patients is also considered by the lymphocyte cytokine productivity. The influence of aging was observed in the production of all cytokines examined, especially in TNF-α and GM-CSF production in groups B and C. Lowered cytokine production was also observed in monocytes and PMNL of the elderly groups. The present results of cytokine generation in the presence or absence of IL-2 and TNF-α stimulation suggest a

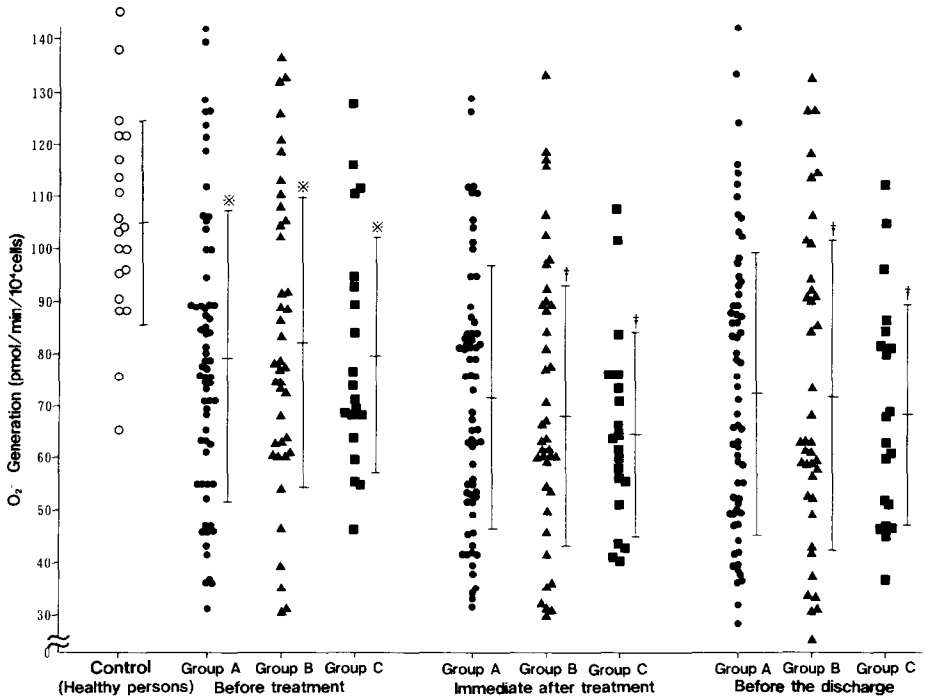

Fig. 4. Influence of aging and cancer therapy on O_2^- generation of PMNL. PMNL were stimulated with PMA, and generated O_2^- was assayed by a cytochrome *c* reduction method. Each symbol indicates the same as Fig. 1. *$P<0.001$ (versus control, by *t*-test), †$P<0.001$ (versus before treatment, by paired *t*-test).

hypofunctional cytokine network in highly aged cancer patients.

The function of NK cells seems also to be influenced by aging. However, NK activity was similar in all three groups, and was not affected by cancer therapy. The original LAK activity in aged patients was markedly lower than that in the controls. Cancer treatment induced further suppression of LAK activity in both groups B and C. T cells, especially CD8 cells, are induced to be LAK cells by appropriate stimulation with, for example, IL-2 or anti-CD3 antibody [21], but their LAK activity is much lower than that of NK cells [22–24]. Therefore, LAK activity of unfractionated lymphocytes greatly depends on NK cells, and the decreased LAK activity can be interpreted as poor LAK induction from NK cells. The immunological parameters examined in the present study may be influenced by tobacco and alcohol, but there were no differences between patients with and without these habits.

In prevention of opportunistic infection, which occasionally occurs in compromised patients, PMNL play the most important role by killing microbial pathogens by means of reactive oxygen species [7, 8, 12]. It is well known that bacterial and fungicidal activities are closely correlated with the productivity of reactive oxygen species, especially O_2^- [7]. In all the patient groups, O_2^- production was originally lower than that in the control group, and a further decrease by cancer therapy was observed. In the three patient groups, the original O_2^- production was similar, but the production was most suppressed by cancer therapy in group C. Therefore, the risk of opportunistic infections must be considered in the treatment of very old patients. From these results, it is clear that elderly patients are already immunologically suppressed, and that leucocyte functions are easily impaired by cancer therapy. Thus, the evaluation of the immunological status of patients before and during treatment is important for successful treatment, especially in aged patients.

1. Phister JE, Jue SG, Cusack BJ. Problems in the use of anticancer drugs in the elderly. *Drugs* 1989, **37**, 551–565.
2. Bloom ET, Umehara H, Bleackley RC, Okumura K, Mostowski H, Babbitt JT. Age-related decrement in cytotoxic T lymphocyte (CTL) activity is associated with decreased levels of mRNA encoded by two CTL-associated serine esterase genes and the perforin gene in mice. *Eur J Immunol* 1990, **20**, 2309–2316.
3. Fülöp T, Fóris G, Wórum I, Leövey A. Age-dependent alterations of Fcγ receptor-mediated effector functions of human polymorphonuclear leucocytes. *Clin Exp Immunol* 1985, **61**, 425–432.
4. Murasko DM, Weiner P, Kaye D. Decline in mitogen induced proliferation of lymphocytes with increasing age. *Clin Exp Immunol* 1987, **70**, 440–448.
5. Niwa Y, Kasama T, Miyachi Y, Kanoh T. Neutrophil chemotaxis, phagocytosis and parameters of reactive oxygen species in human aging: cross-sectional and longitudinal studies. *Life Sci* 1989, **44**, 1655–1664.
6. Ichinose Y, Hara N, Motohiro A, Noge S, Ohta M, Yagawa K. Influence of chemotherapy on superoxide anion-generating activity of polymorphonuclear leukocytes in patients with lung cancer. *Cancer* 1986, **58**, 1663–1667.
7. Ferrante A. Tumor necrosis factor alpha potentiates neutrophil anti-microbial activity: increased fungicidal activity against *Torulopsis glabrata* and *Candida albicans* and associated increases in oxygen radical production and lysosomal enzyme release. *Infect Immun* 1989, **57**, 2115–2122.
8. Thompson HL, Wilton JMA. Interaction and intracellular killing of *Candida albicans* blastospores by human polymorphonuclear leucocytes, monocytes and monocyte-derived macrophages in aerobic and anaerobic conditions. *Clin Exp Immunol* 1992, **87**, 316–321.
9. Umeda T, Hara T, Hayashida M, Niijima T. Role of hydroxyl radical in neutrophil-mediated cytotoxicity. *Cell Mol Biol* 1985, **31**, 229–233.

10. Borish L, Rosenbaum R, Albury L, Clark S. Activation of neutrophils by recombinant interleukin 6. *Cell Immunol* 1989, **121**, 280–289.
11. Iho S, Shau H, Golub SH. Characteristics of interleukin-6-enhanced lymphokine-activated killer cell function. *Cell Immunol* 1991, **135**, 66–77.
12. Muñoz-Fernández MA, Fernández MA, Fresno M. Synergism between tumor necrosis factor-α and interferon-γ on macrophage activation for the killing of intracellular *Trypanosoma cruzi* through a nitric oxide-dependent mechanism. *Eur J Immunol* 1992, **22**, 301–307.
13. Roilides E, Uhlig K, Venzon D, Pizzo PA, Walsh TJ. Enhancement of oxidative response and damage caused by human neutrophils to *Aspergillus fumigatus* hyphae by granulocyte colony-stimulating factor and gamma interferon. *Infect Immun* 1993, **61**, 1185–1193.
14. Shalaby MR, Aggarwal BB, Rinderknecht E, Svendersky LP, Finkle BS, Palladino MA. Activation of human polymorphonuclear neutrophil functions by interferon-γ and tumor necrosis factors. *J Immunol* 1985, **135**, 2069–2073.
15. Steinbeck MJ, Roth JA. Neutrophil activation by recombinant cytokines. *Rev Infect Dis* 1989, **11**, 549–568.
16. Suzu S, Yokota H, Yamada M, *et al.* Enhancing effect of human monocytic colony-stimulating factor on monocyte tumoricidal activity. *Cancer Res* 1989, **49**, 5913–5917.
17. Sansoni P, Cossarizza A, Brianti V. Lymphocyte subsets and natural killer cell activity in healthy old people and centenarians. *Blood* 1993, **82**, 2767–2773.
18. Vitale M, Zamai L, Neri LM, *et al.* The impairment of natural killer function in the healthy aged is due to a postbinding deficient mechanism. *Cell Immunol* 1992, **145**, 1–10.
19. Smolen JS, Chused TM, Leiserson WM, Reeves JP, Alling D, Steinberg AD. Heterogeneity of immunoregulatory T-cell subsets in systemic lupus erythematosus: correlation with clinical features. *Am J Med* 1982, **72**, 783–790.
20. Liu C, Muirhead KA, George SP, Landay AL. Flow cytometric monitoring of human immunodeficiency virus-infected patients: simultaneous enumeration of five lymphocyte subsets. *Am J Clin Pathol* 1989, **92**, 721–728.
21. Ochoa AC, Gromo G, Alter BJ, Sondel PM, Bach FH. Long-term growth of lymphokine-activated killer (LAK) cells: role of anti-CD3, β-IL1, interferon-γ and -β. *J Immunol* 1987, **138**, 2728–2733.
22. Sawada H, Abo T, Sugawara S, Kumagai K. Prerequisite for the induction of lymphokine-activated killer cells from T lymphocytes. *J Immunol* 1988, **140**, 3668–3673.
23. Damle NK, Doyle LV, Bradley EC. Interleukin 2-activated human killer cells are derived from phenotypically heterogeneous precursors. *J Immunol* 1986, **137**, 2814–2822.
24. Tilden AB, Itoh K, Balch CM. Human lymphokine-activated killer (LAK) cells: identification of two types of effector cells. *J Immunol* 1987, **138**, 1068–1073.