

## INTRACELLULAR CAROTENOID LEVELS MEASURED BY RAMAN MICROSCOPY: COMPARISON OF LYMPHOCYTES FROM LUNG CANCER PATIENTS AND HEALTHY INDIVIDUALS

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**Most studies concerning a possible protective role of carotenoids against cancer focus on serum carotenoid levels. We have used Raman microspectroscopy to study the intracellular amounts of carotenoids in lymphocytes of lung cancer patients and of healthy individuals. Our results indicate a significant decrease of carotenoids in lung carcinoma patients compared with healthy individuals, particularly in adenocarcinoma patients. Carotenoid supplementation raised the serum concentration in 2 lung cancer patients up to normal levels, whereas intracellular content remained significantly lower. This indicates that carotenoid uptake by lymphocytes is not only dependent on serum carotenoid concentration. Our findings indicate that Raman microspectroscopy, a recently developed technique to measure intracellular levels of drugs, is also well suited to obtain quantitative data on carotenoid amounts inside cells. *Int. J. Cancer* 74:20–25.**

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Numerous studies indicate that low serum carotenoid concentration and a low dietary intake of carotenoids are related to an increased risk of cancer, especially lung and stomach cancer (Peto *et al.*, 1981; Ziegler, 1989; Bendich, 1990; van Poppel, 1993; Krinsky, 1993). Three different mechanisms have been suggested to explain the protective effect of carotenoids: provitamin A activity (Lotan, 1980; Lupulescu, 1993), singlet oxygen and radical quenching (Burton and Ingold, 1984; Krinsky, 1989) and modulation of the immune system (Alexander *et al.*, 1985; Jyonouchi *et al.*, 1991).

Some of the problems that arise when assessing the relationships between cancer risk in humans and serum carotenoid concentrations are related to the large fluctuations of the latter, which depend on intake, and also the fact that dietary intake does not reflect the actual uptake in serum. Observations that carotenoids can modulate the immune system prompted us to investigate whether differences might exist between the carotenoid contents of lymphocytes of healthy individuals and of cancer patients and how this relates to the concentration of carotenoid in serum.

We have shown that Raman microspectroscopy is a sensitive technique for analysis of the carotenoid content of human lymphocytes (Puppels *et al.*, 1993). We found that the highest carotenoid concentration for healthy donors was observed in the Gall body, a cytoplasmic (lipid) spherule, present in some CD4<sup>+</sup> and CD8<sup>+</sup> cells (Gall, 1936; Bessis, 1973). The carotenoid concentration inside the Gall body was found to be about 1,000 times higher than the serum concentration. Such high carotenoid concentration in the Gall bodies of healthy donors indicates that Gall bodies are storage sites for carotenoids. In the present study we used Raman microspectroscopy to investigate whether differences exist in Gall body carotenoid concentration in lymphocytes of lung cancer patients and healthy donors. The effect of dietary  $\beta$ -carotene supplementation on serum and intracellular carotenoids was investigated in a healthy individual and in 2 lung cancer patients.

### MATERIAL AND METHODS

#### Raman microspectroscopy

Raman microspectroscopy is a spectroscopic technique that can be used to obtain information about the molecular composition of a

sample with a spatial resolution equal to that of a normal optical microscope. Raman scattering is a process in which there is energy transfer between the incident light and the scattering molecules. In most cases, the photons transfer some energy to the scattering molecule, which brings the molecule to one of the higher vibrational energy levels. The energy loss of the scattered photons results in a wavelength shift that is characteristic for that vibration of the molecule. The Raman spectrum therefore gives a fingerprint of (all excitable vibrational levels of) the molecules in the measuring volume. The intensity of a Raman line is linearly dependent on the concentration of molecules (with that particular vibration) in the sample. The wavelength shifts of a Raman line, relative to the laser line, are expressed in  $\text{cm}^{-1}$ :  $\Delta\text{cm}^{-1} = [1/\lambda_{\text{in}} - 1/\lambda_{\text{out}}] \times 10^7$  (with  $\lambda$  in nm). Carotenoids are strong Raman scatterers, especially the single and double conjugated carbon bond-stretches, which give rise to large Raman peaks at about 1,150 and 1,525  $\text{cm}^{-1}$ , respectively. All carotenoids present in humans give similar spectra, with small changes due to differences in structure. The peak at about 1,150  $\text{cm}^{-1}$  is present for all carotenoids.

For determination of the carotenoid content of Gall bodies, we used a normal (non-confocal) Raman microscope with 63 $\times$  water immersion objective. Figure 1a shows a schematic drawing of the set-up used in our study. Red light (647 nm) was used to minimize bleaching of the carotenoids. All measurements were carried out using the same conditions: 4 mW laser light on the sample and 15 sec photon collection time on the camera. Figure 1b shows a photograph of a lymphocyte containing a Gall body. In Figure 1c a typical spectrum obtained from a Gall body of a healthy individual is shown.

Calibration of the peak height vs. the concentration was done using solutions of  $\beta$ -carotene in tetrahydrofuran (THF) with different molarities (data not shown). From the background subtracted peak heights at 1,150  $\text{cm}^{-1}$ , it was calculated that the peak intensity is about 1,600 counts/mM  $\beta$ -carotene for the conditions used in our study, the detection limit being about 5 mM.

#### Preparation of samples

Peripheral blood of healthy volunteers and of lung cancer patients was collected in the morning by venipuncture in the arm, using a siliconized Vacutainer to collect serum and a heparinized

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Vacutainer to collect white blood cells. Serum was isolated after centrifugation, and lymphocytes were isolated using the Ficoll-Paque method (Böyum, 1968). Serum was collected simultaneously and stored in the dark at  $-80^{\circ}\text{C}$  until analysis using high-performance liquid chromatography (HPLC). Part of the isolated lymphocytes was immuno-stained with CD4-PE and CD8-FITC (Becton Dickinson, San Jose, CA) and analyzed with a fluorescence microscope.

#### Fluorescence microscopy

A fluorescence microscope (Nikon, type Optiphot EF) with blue light excitation was used to study the immuno-labeled lymphocytes; 100 cells of each sample were counted to determine the relative amounts of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes. In addition, the number of Gall bodies was counted and their diameter was measured in all cells, using a calibrated eyepiece micrometer.

#### HPLC

Serum samples were analyzed for the presence of carotenoids, using HPLC, at the T.N.O. Research Laboratory, Zeist, The Netherlands, as described previously (van Vliet *et al.*, 1991). Amounts of the 5 major carotenoids present in humans ( $\beta$ -carotene,  $\alpha$ -carotene, lycopene,  $\beta$ -cryptoxanthine and lutein/zeaxanthin) were determined.

#### $\beta$ -carotene supplementation

Dietary supplementation was given in the form of capsules with 25 mg crystalline  $\beta$ -carotene, generously provided by Merck (Darmstadt, Germany). Because the actual uptake of crystalline  $\beta$ -carotene is low, a high dose of 100 mg/day (intake at one meal) was chosen to obtain clear responses compared with the normal dietary intake of  $\beta$ -carotene. Cells and serum of all individuals were measured prior to the study to establish baseline levels. Blood samples were drawn on days 1, 2, 3, 5, 8, 13 and 17 after the first supplementation.

#### Statistical analysis

The significance of differences between the groups of healthy donors and lung cancer patients was calculated for all measured parameters using an unpaired double-tailed *t*-test.

## RESULTS

The first part of this pilot study focused on possible differences in the carotenoid content of Gall bodies comparing healthy persons with lung cancer patients. From 23 healthy donors, and 19 donors with different types of lung cancer, peripheral blood was collected as described above; lymphocytes were isolated and measured within 24 hr. The results are summarized in Table I. The differences between the 2 groups are clear and significant. The 2 groups were not age matched: if the age-matched part of the group of healthy donors ( $n = 11$ ) is compared with the lung cancer group, only the difference in average Raman intensities remains ( $p < 0.05$ ). These findings suggest that carotenoid concentrations decrease with age.

The individual values of the total serum carotenoid concentrations, measured with HPLC, are shown in Figure 2a. This clearly shows that the differences between the "all healthy" and "lung cancer" groups are significant, whereas the difference between the age-matched groups "healthy old" and "lung cancer" is not. The individual values of the average Gall body carotenoid concentrations, measured with Raman microspectroscopy, are shown in Figure 2b. Here also, the differences between "healthy old" and "lung cancer" are significant.

There is a positive correlation between the individual serum carotenoid and Gall body concentrations, although the correlation coefficients are low (total 0.47; healthy 0.36; lung cancer 0.25).

The second part of our study focused on the influence of short-term high-dose  $\beta$ -carotene supplementation on the carot-

TABLE I – MAIN DIFFERENCES BETWEEN HEALTHY PERSONS AND LUNG CANCER PATIENTS FOR THE PARAMETERS MEASURED

	Healthy (n = 23) x $\pm$ SD	Lung cancer (n = 19) x $\pm$ SD	<i>p</i> <sup>1</sup>
% cells with GBs	22.0 $\pm$ 12.6	16.0 $\pm$ 6.2	<0.05
Average number of GBs/cell	1.11 $\pm$ 0.15	1.00 $\pm$ 0.21	<0.05
Average GB Raman intensity	101.7 $\pm$ 62.9	50.3 $\pm$ 31.3	<0.005
Total GB carotenoid content <sup>2</sup>	2.88 $\pm$ 3.5	0.63 $\pm$ 0.7	<0.05
Serum lutein	0.33 $\pm$ 0.14	0.25 $\pm$ 0.12	<0.05
Serum $\beta$ -cryptoxanthine	0.28 $\pm$ 0.24	0.24 $\pm$ 0.20	NS
Serum lycopene	0.42 $\pm$ 0.41	0.13 $\pm$ 0.10	<0.01
Serum $\alpha$ -carotene	0.06 $\pm$ 0.05	0.03 $\pm$ 0.02	<0.05
Serum $\beta$ -carotene	0.36 $\pm$ 0.30	0.21 $\pm$ 0.12	NS
Total serum carotenoids	1.45 $\pm$ 0.81	0.86 $\pm$ 0.40	<0.01

<sup>1</sup>*p* values denote the chance that values from both groups have the same origin (calculated with unpaired two tailed *t*-test); differences are considered significant if  $p < 0.05$ . Raman intensity in counts/(15 sec). GB, Gall body; x, mean; SD, standard deviation; NS, not significant.<sup>2</sup>In arbitrary units.

enoid amounts present in Gall bodies in lymphocytes of healthy individuals and lung cancer patients. This was performed on 3 individuals, one healthy person and 2 advanced lung cancer patients (lung adenocarcinoma and squamous cell lung cancer). Figure 3a shows the serum carotenoid concentration response following supplementation with 100 mg  $\beta$ -carotene/day, as a function of time. The results clearly show that all serum carotenoid concentration values rise immediately after supplementation and continue thereafter. These values in the lung cancer patients were lower at the start than those in the healthy volunteer but increased to the same values as those in the healthy volunteer after 8 days of supplementation, the relative increase in the lung cancer patients thus being 2 to 3 times more marked than in the healthy individual.

The effects of supplementation on carotenoid content in the Gall bodies is shown as a function of time in Figure 3. This figure shows that the response is comparable in all cases. All baseline levels increase by about 50%. Such an increase was observed during the first 5 days of supplementation, while the carotenoid concentration remained constant thereafter. The amount of carotenoids in Gall bodies in both lung cancer patients did not reach the values of the healthy individual. This is in contrast with the findings in serum. This suggests that carotenoids are not taken up by simple diffusion.

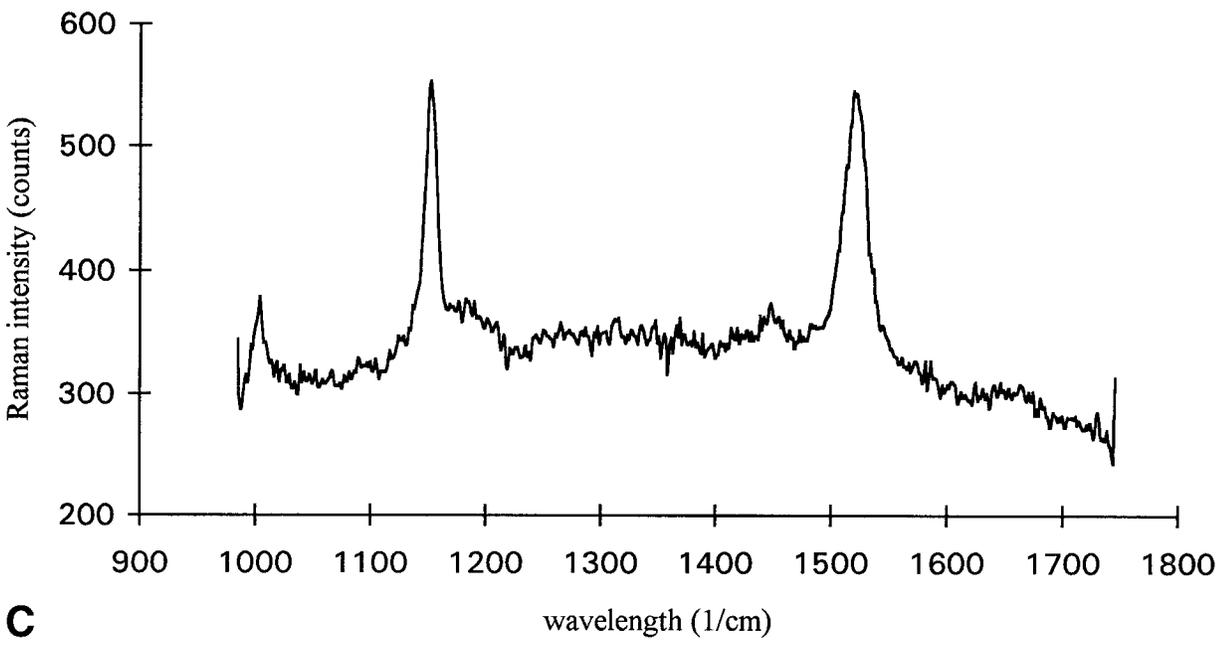
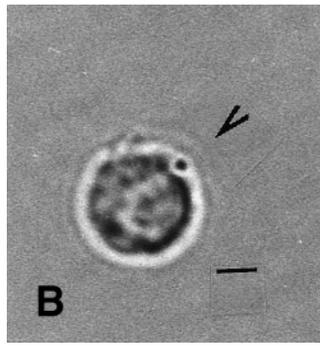
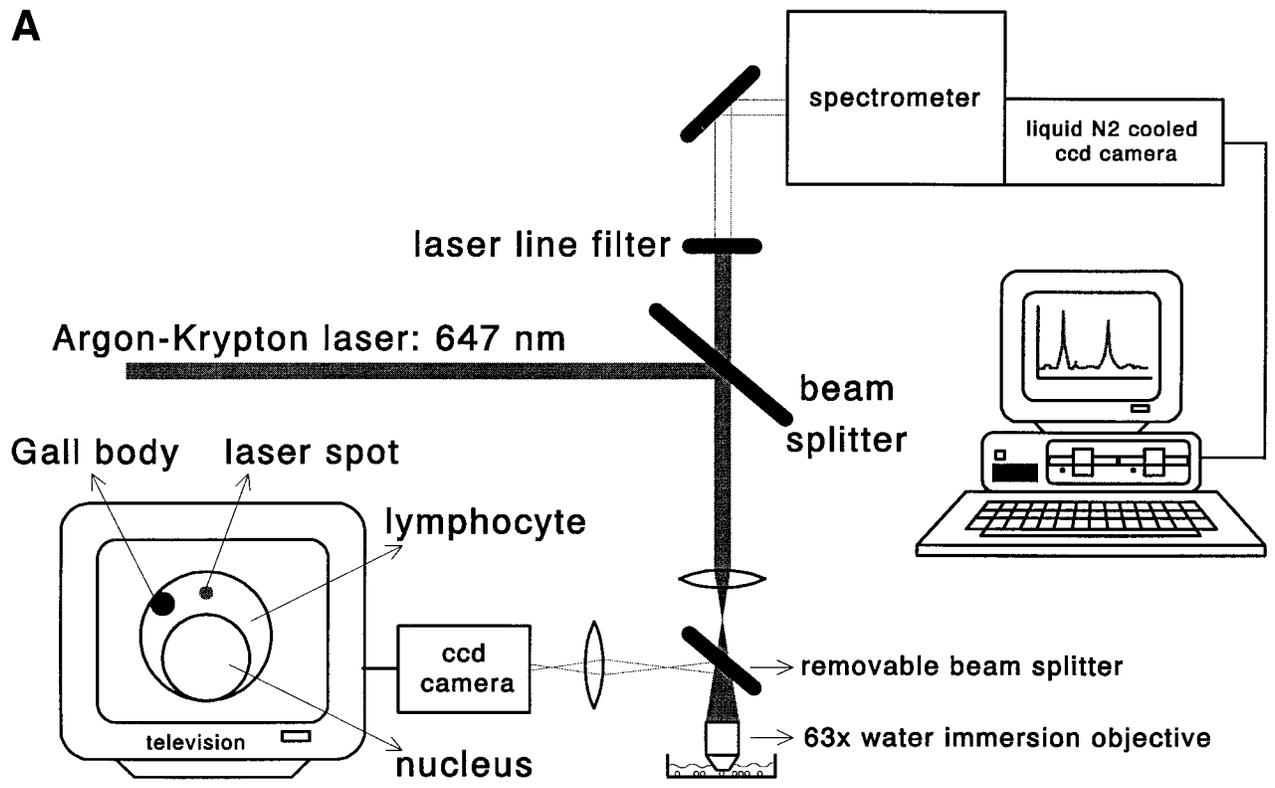
## DISCUSSION

The following findings emerge from this pilot study:

- Raman microspectroscopy is well suited for determining carotenoid concentrations inside cells.
- Intracellular carotenoid amounts in lymphocytes decrease with age; in age-matched groups, the carotenoid content of lymphocytes is significantly lower in the lung cancer patients.
- A very weak correlation only exists between the carotenoid content in lymphocytes and the serum carotenoid concentration.
- Carotenoid supplementation restores normal carotenoid concentration in the serum, but intracellular values remain significantly low (*i.e.*, 50% increase in lymphocyte carotenoid levels and 3- to 5-fold increase in serum concentration).

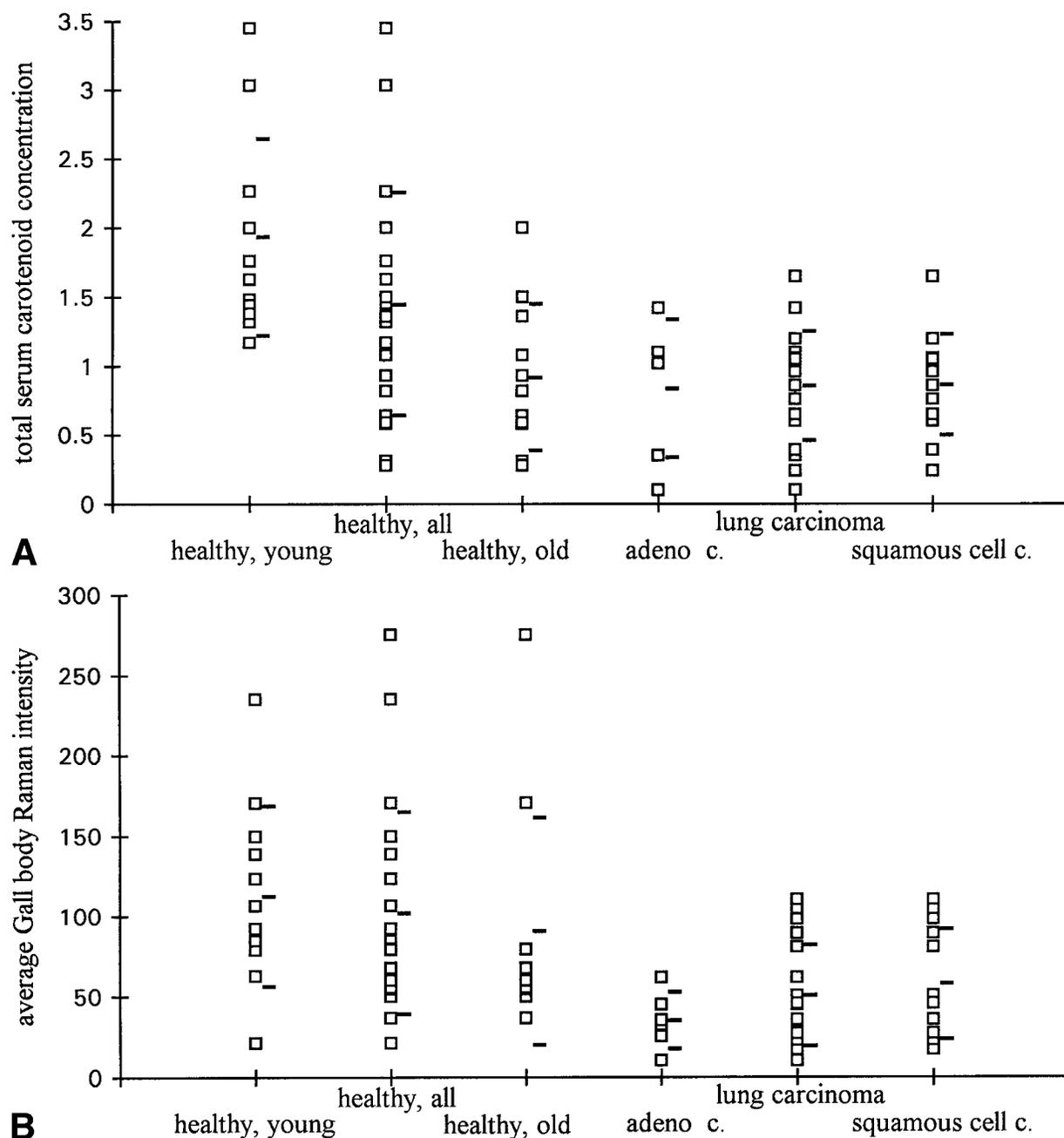
The intracellular carotenoid concentration may thus be a more accurate parameter than that in the serum.

**A**



**C**

FIGURE 1



**FIG. 2** – (a) Concentration of total serum carotenoids ( $\mu\text{moles/l}$ ), measured with HPLC. Bars, average  $\pm$  standard deviation. Difference between “all healthy” group and “lung cancer” group is significant ( $p = 0.006$ ). Difference between “healthy, old” group and “lung cancer” group is not significant ( $p = 0.72$ ). (b) Concentration of total carotenoids in the Gall body of lymphocytes, measured as Raman intensity [counts/(15 sec); every point is an average of 25 measurements in different cells]. Bars, average  $\pm$  standard deviation. Difference between “all healthy” group and “lung cancer” group is significant ( $p = 0.003$ ). Difference between “healthy, old” group and “lung cancer” group is significant ( $p = 0.04$ ).

**FIG. 1** – (a) Schematic representation of the Raman microspectroscopy used in our study. The laser is focused to a spot ( $0.5 \mu\text{m}$  in diameter) in the cell, using a normal CCD camera and TV for determining the exact place. After rejection of the laser line with a filter, the Raman scattered light is coupled into a spectrometer, and the spectrum of the dispersed light is projected on a liquid  $\text{N}_2$ -cooled camera. The spectra are stored using a normal personal computer. (b) Photograph of a lymphocyte containing a Gall body (arrow). Scale bar =  $2 \mu\text{m}$ . (c) Typical spectrum from a Gall body of a healthy individual. The peak height at  $1,150 \text{ cm}^{-1}$  (background subtracted) is proportional to the carotenoid concentration inside the Gall body (see text).

The results of short-term  $\beta$ -carotene supplementation show that there is no simple relation between serum and intracellularlymphocyte carotenoid content. During  $\beta$ -carotene supplementation the serum carotenoid concentration rises continuously, while there is only a small increase in the Gall body carotenoid content during the first days. Our observation that Gall body carotenoid concentration rises during the first days only is not related to saturation of the Gall bodies with carotenoids, since the variation in individual Gall body concentrations was not significantly decreased (data not shown). In addition, even higher carotenoid contents have been observed in other healthy individuals.

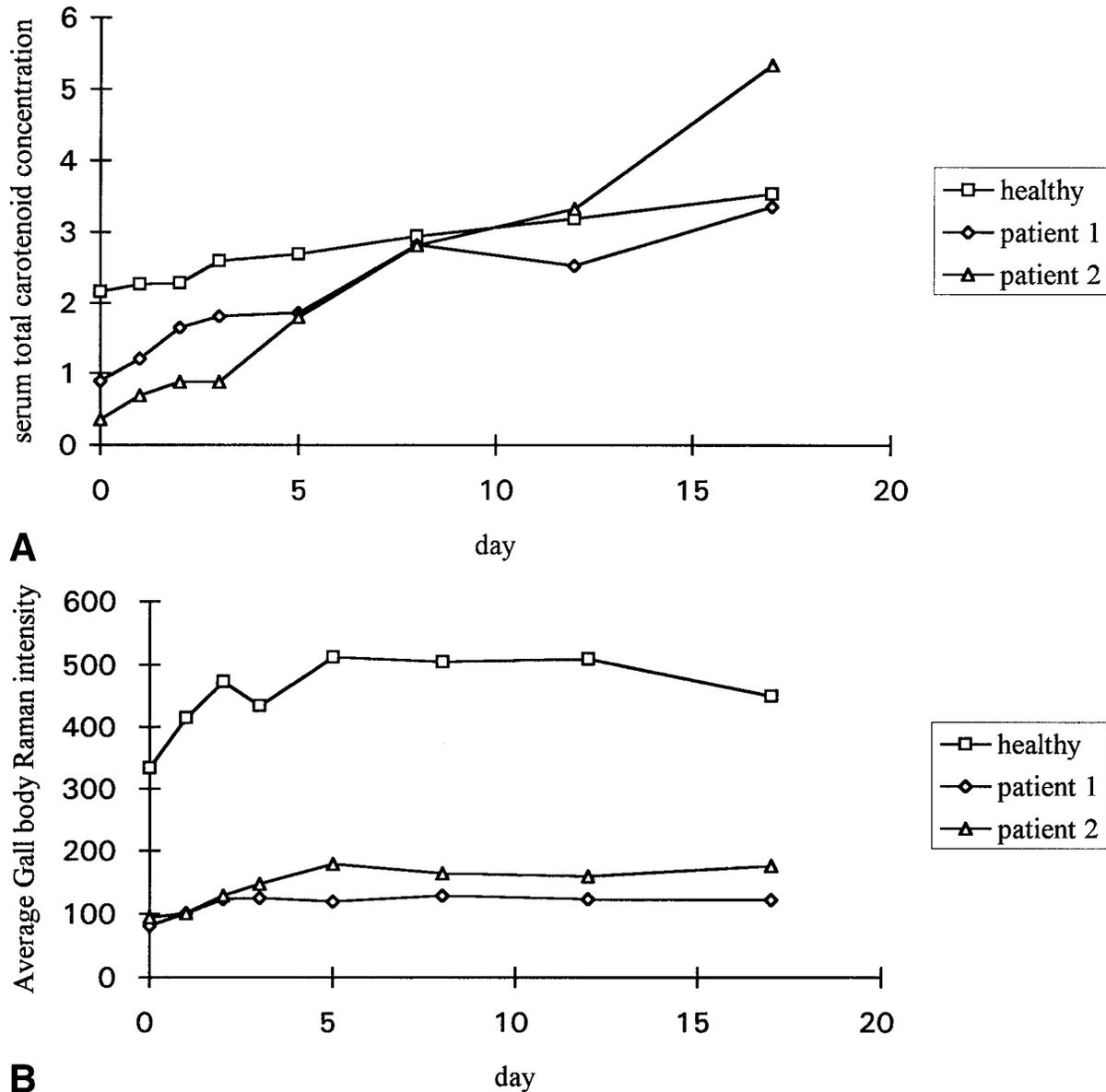


FIG. 3 – (a) Total serum carotenoid concentration response ( $\mu\text{moles/l}$ ) to supplementation with 100 mg  $\beta$ -carotene/day as a function of time. Levels on day 0 are basal levels; supplementation was started at day 0. (b) Gall body total carotenoid concentration response measured as Raman intensity [counts/(15 sec); each point in the graph is an average over 25 Gall bodies] upon supplementation with 100 mg  $\beta$ -carotene/day as a function of time. Levels on day 0 are basal levels; supplementation was started at day 0.

Our findings are based on a relatively small number of exploratory experiments. They suggest that evaluation of intracellular carotenoid concentration may provide additional and different information, compared with measurements in the serum. Our results therefore stress the need to investigate the significance of the high carotenoid amounts found in lymphocytes, and they

open novel ways to study the mechanism(s) that may quantitatively correlate carotenoids and lung cancer.

#### ACKNOWLEDGEMENTS

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