Pure & Appl. Chem., Vol. 69, No. 10, pp. 2131–2134, 1997.
Printed in Great Britain.

Carotenoid levels in human lymphocytes, measured by Raman microspectroscopy

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Abstract: Carotenoid levels in lymphocytes obtained from peripheral blood of healthy people have been investigated by Raman microspectroscopy. We observed that carotenoids are concentrated in so-called "Gall bodies". The level of carotenoids in living human lymphocytes was found to be age-dependent and to decrease with age. We performed carotenoid uptake experiments using liposomes containing beta-carotene (egg phosphatidylcholine and egg phosphatidylglycerol, 10/1 molar ratio). We observed that beta-carotene is rapidly taken up *in vitro*, and transported to the Gall body. From these findings we conclude that Raman microspectroscopy is a sensitive method to determine carotenoid levels in single living cells.

INTRODUCTION

It is thought from epidemiological studies, as well as from experimental and clinical data, that carotenoids are cancer chemopreventive agents (refs 1, 2). Despite recent advances, the anticarcinogenic mechanisms remain as yet unknown. All carotenoids, including beta-carotene which can be converted into retinol *in vivo*, and others, like canthaxanthin, which cannot, have immunomodulating activity at physiological concentrations. Modulation of immunoreactivity by carotenoids might contribute to the anti-cancer effect of these compounds (ref 3). For this reason we investigated the carotenoid content in human lymphocytes. Up till now most studies have used high performance liquid chromatography to determine carotenoid levels in serum. However, this technique does not allow sensitive measurement of carotenoid levels in single living blood cells. Recently, G.J.Puppels et al., 1993 have shown, by using Raman microspectroscopy, that carotenoids are not only present in serum, but can be accumulated in human lymphocytes, and are concentrated in Gall bodies (ref 4).

MATERIALS AND METHODS

Donors

Healthy volunteers were sampled at random. All donors had no major health problems, and were neither smoking nor taking any medication. The average age of the younger donors was 27±3 years and of the older donors was 53±4 years.

Isolation of peripheral blood lymphocytes and culturing conditions

Human lymphocytes have been collected from freshly isolated heparinized peripheral blood according to standard methods (ref 5). Cell viability was determined by use of ethidium/acridine orange, and was no less than 95 %. Lymphocytes were incubated *in vitro* at 37^{0}C in an atmosphere of humidified CO_2/air (5/95 %). Standard RPMI-1640 medium supplemented by 2 mM of L-glutamine, 3 % of heat-inactivated fetal calf serum, 100 U/ml of penicillin, 100 mg/ml of streptomycin, 25 mM of hepes was used for culture.

Isolation of $CD4^+$ and $CD8^+$ lymphocyte subsets

Lymphocyte subsets were isolated by negative selection using immunomagnetic beads (Dynabeads M-450 Sheep anti-Mouse IgG , Dynal, Oslo, Norway) (ref 6). Lymphocytes collected from peripheral blood were first incubated with a cocktail of $CD19^+$, $CD16^+$, and $CD4^+$ or $CD8^+$ mouse antibodies. Thereafter, the target cells were rosetted with Dynabeads M-450 Sheep anti-Mouse IgG and could easily be isolated by holding a magnet on the wall of the test tube for 3 minutes. The cells that are not caught by the magnet are $CD4^+$ or $CD8^+$ lymphocytes (ref 7).

Beta-carotene used and preparation of liposomes

Beta-carotene was purchased from Sigma Chemical Co. The liposome form of beta-carotene was kindly provided by Department of Pharmaceutics, University of Utrecht, the Netherlands. Chromatographically pure phospholipids (egg phosphatidylcholine and egg phosphatidylglycerol, 10/1 molar ratio) were used to prepare liposomes according to a standard liposome preparation procedure (8). The liposomes particles had a diameter ranging from 114 - 125 nm. The liposomes were added to cultured lymphocytes at a beta-carotene concentration of 2.6×10^{-6} mg per 10^6 cells in 0.25 ml of culture medium.

Sample preparation

Isolated lymphocytes (subsets) were in some experiments incubated with liposomes and put on a quartz glass covered by poly-L-lysine (0.01 % solution in phosphate buffer saline) for Raman microspectroscopy.

Raman microspectroscopy measurements

Carotenoid levels in Gall bodies of human lymphocytes were measured by using the confocal Raman microspectrometer as described by Puppels et al. (ref 4). The measurements were performed under identical experimental conditions: 10 seconds laser exposure time for Raman signal integration, laser power 4 mW on the cell sample; red laser light at wavelength 660 nm from a dye laser was used to avoid carotenoid bleaching. Raman imaging of lymphocytes was carried out to get information about the intracellular localization of carotenoids. This was done by scanning the cell surface with a laser beam at wavelength 647.1 nm in the region for single bond bond-stretch vibration of carotenoids at wavenumber 1157 cm^{-1}.

Statistical analysis of experimental data

Each lymphocyte was measured once. A set of measurements includes 20-25 spectra for every cell preparation for each time point. Raman data analysis was carried out by use of RCM-proc and TOMRAM software designed in the Department of Applied Physics, University of Twente, the Netherlands. Statistical analysis was performed with Student's test; $P<0,05$ was considered to be significant.

RESULTS

The molecular structure of carotenoids makes them very strong Raman scatterers. The single and double bond conjugated polyene carbon-carbon bond-stretch vibrations give intensive Raman peaks at about 1157 and 1525 cm^{-1}, respectively (ref 9) (Fig. 1).

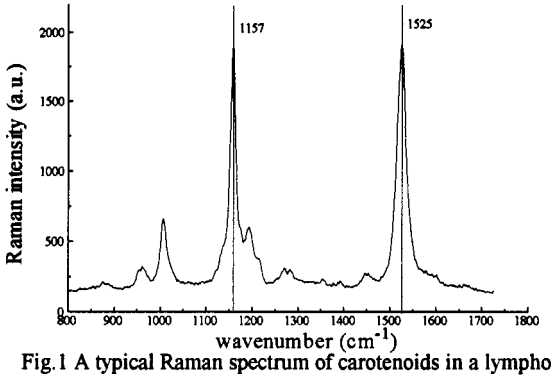

Fig.1 A typical Raman spectrum of carotenoids in a lymphocyte.

All carotenoids present in human tissues give rise to similar Raman spectra, with minor changes due to differences in structure, but the peak at about 1157 cm^{-1} is present for all carotenoids. Recently, it was found that in lymphocytes carotenoids are concentrated in non-specific lysosomal organelles termed Gall bodies (ref 4). In our experiments carotenoid Raman spectra were derived from the Gall body. This organelle is recognized in living unstained lymphocytes as a dark round spot. A bright light image of a human lymphocyte with a Gall body is presented in Fig. 2. The corresponding Raman image of a human lymphocyte is shown in Fig. 3.

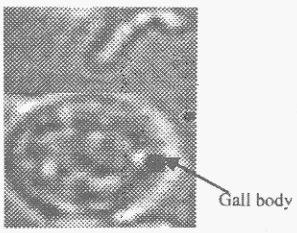

Fig. 2 Bright light image of a human lymphocyte (Zeiss Plan Neofluor water immersion objective, 63x, NA1.2).

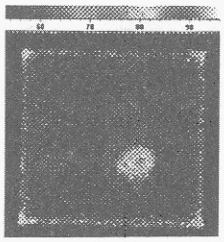

Fig. 3 Raman image of a human lymphocyte.

Next we examined a series of lymphocytes isolated from donors that differ in age. We observed a different carotenoid accumulation in human lymphocytes of young and older donors. The carotenoid level was age-dependent and was significantly lower in older people (Fig. 4).

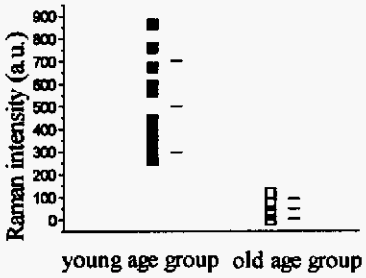

Fig. 4 Basic carotenoid levels in human lymphocytes in young and older donors. Differences are significant (P<0,05).

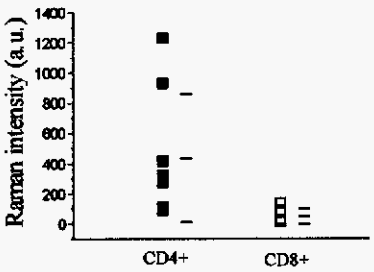

Fig. 5 Carotenoid levels in Gall bodies of $CD4^+$ and $CD8^+$ lymphocytes.

To gain more insight into the distribution of carotenoids in lymphocyte subsets, we isolated $CD4^+$ and $CD8^+$ cells by negative selection. The data shown in Fig. 5 indicate that carotenoids accumulate somewhat better in $CD4^+$ lymphocyte Gall bodies, although this was not statistically significant, probably due to the low number of cells counted. This confirms and extends previous observations made by Puppels et al (ref 4). It is well established that carotenoids present in human tissues are derived from food, absorbed via the lymphatic system, and transported by plasma lipoproteins which are the major carriers of intact beta-carotene (ref 10).

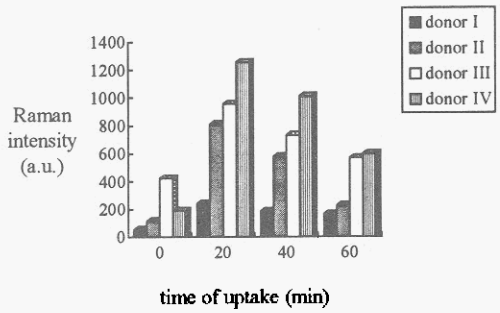

Fig. 6 Time dependence of beta-carotene uptake by human lymphocytes *in vitro*.

To obtain some insight into the uptake of carotenoids by lymphocytes and the transport to Gall bodies, we incubated lymphocytes with liposomes containing beta-carotene. As shown in Fig. 6, beta-carotene seems indeed to be incorporated into lymphocytes, and reaches the Gall body. Preliminary experiments indicate that maximum carotenoid levels are reached after 20 minutes of incubation and that this level subsequently decreases with time. In control experiments performed with liposomes not containing beta-carotene the carotenoid levels in the Gall bodies were not altered (data not shown). These initial experiments indicate that beta-carotene can be effectively transported into Gall bodies of human lymphocytes *in vitro* by means of a liposome-delivery system. A detailed study will be necessary to reveal the mechanism by which beta-carotene is taken up by human lymphocytes.

CONCLUSIONS

We have examined the carotenoid levels in Gall bodies of lymphocytes of healthy donors by means of Raman microspectroscopy.

We observed major differences in food-derived carotenoid levels in lymphocytes; these differences were dependent on age. We found that the carotenoid concentration in Gall bodies decreases with age. We also measured carotenoid levels in lymphocytes of lung cancer patients (Bakker Schut T. et al. submitted). In these patients we found similarly low or even lower levels of carotenoid concentrations in Gall bodies compared with those of healthy older people. Our findings are in agreement with some of the prospective and retrospective epidemiological studies which show that dietary carotenoid supply is negatively correlated with the incidence of certain cancers (refs 1, 2). It will be interesting to carry out a more detailed study comparing the carotenoid levels in lymphocytes (subsets) with cancer incidence.

Our findings suggest a tendency for carotenoids to accumulate in the Gall bodies of $CD4^+$ lymphocytes. Differences in carotenoid concentrations in different cell types may also affect the functional activity of mononuclear cells after supplementation with beta-carotene or other carotenoids (refs 3, 11). These initial findings should be extended by a more detailed study.

We used Raman microspectroscopy to obtain information about how beta-carotene is taken up by lymphocytes and transported to the Gall bodies *in vitro*. We used beta-carotene because it is one of the most frequently consumed carotenoids in the human diet, and the chemopreventive anti-tumour effect is supposed to be mainly mediated by this carotenoid (refs 12, 13). We used liposomes to deliver beta-carotene, instead of toxic organic solvents that are traditionally used to dissolve highly lipophilic substances. Liposomes are the carriers that enhance the bioavailability of beta-carotene for cultured lymphocytes *in vitro*. Our initial findings show that beta-carotene molecules are indeed transported from the culture medium into human lymphocytes, and that the maximum uptake is reached within 20 minutes. Beta-carotene in these lymphocytes was accumulated in the Gall bodies in these uptake experiments *in vitro*, and thus these experiments seem to mimic the situation *in vivo*. In conclusion, we show that Raman microspectroscopy is a sensitive method for determining carotenoid levels in single living cells without any further staining.

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