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Carotenoids Located in Human Lymphocyte Subpopulations and Natural Killer Cells by Raman Microspectroscopy

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The presence and subcellular location of carotenoids in human lymphocyte subpopulations (CD4+, CD8+, T-cell receptor-γδ+, and CD19+) and natural killer cells (CD16+) were studied by means of Raman microspectroscopy. In CD4+ lymphocytes a high concentration (~10^{-3} M) of carotenoids was found in the Gall body. In CD8+ lymphocytes, T-cell-receptor-γδ+ lymphocytes and in natural killer cells carotenoids appeared to be concentrated (~10^{-4} M) in the Golgi complex. The concentration of carotenoids in CD19+ lymphocytes was found to be below the present detection limit, which is ~10^{-6} to 10^{-5} M. The results provide new possibilities to investigate the mechanism(s) behind the suggested protective role of carotenoids against the development of cancers. © 1993 Wiley-Liss, Inc.

Key terms: Fluorescence activated cell sorting, cancer, Gall body, Golgi complex, fluorescence microscopy

A high dietary intake of carotenoids has been found to be inversely related to the risk of many types of cancer (29,41). In laboratory animals the development of tumors can be slowed down, prevented, or reversed by carotenoid administration (reviewed in 6). The mechanism(s) involved in this apparently carotenoid mediated protection against cancer still remain to be elucidated. Some 10% of all known carotenoids are precursors of vitamin A (retinol). The anti-cancer properties of retinoids, which can modulate cell proliferation and differentiation, were recognized before those of carotenoids and are the subject of intensive investigation (21,25). However, in several studies the anti-cancer effect of carotenoids has been shown not to depend on provitamin A activity (6). Carotenoids are known as very effective quenchers of singlet oxygen and radicals, which are important in cancer etiology (11,18). Furthermore, immunomodulatory effects of carotenoids have been reported, such as the enhancement of cell surface activation marker expression on natural killer (NK) cells and lymphocytes, enhancement of NK-cell mediated cytotoxicity, and an increase in the number of helper/inducer T-lymphocytes in peripheral blood (1,16,19,30). A direct link with cell regulatory functions in the form of specific carotenoid receptors, such as described for retinoids (10,28), has not yet been established, however.

A straightforward approach to obtaining information about the role of carotenoids is first to determine their location in the cells of the immune system. The work presented here shows that Raman microspectroscopy is especially suited for this task. Raman spectroscopy has been extensively applied in studies of biological molecules. It is a versatile vibrational spectroscopic technique which yields information about molecular conformation, interactions between molecules, and the molecular composition of a sample, without the need of introducing, e.g., fluorescent or radioactive labels. A brief phenomenological description of the Raman scattering process is given in Fig. 1.

Carotenoids are very strong Raman scatterers, especially when resonantly excited (i.e., 450–500 nm), and were among the first biomolecules to be studied by Raman spectroscopy. In studies of biological membranes, carotenoid signal contributions were found in Raman spectra of pellets of isolated erythrocyte membranes (38), blood platelet membranes (2), and thymocyte endoplasmic reticulum membranes (39). Also in the spec-

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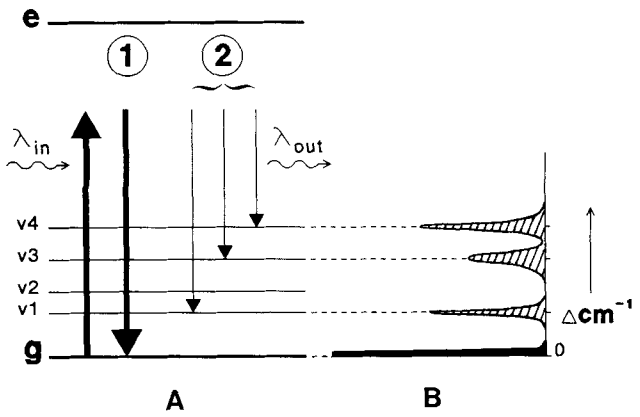

FIG. 1. Raman scattering. Energy level diagram of a molecule. g = ground state; e = excited electronic energy level; v_i = excited vibrational energy level. In a Rayleigh scattering process (1), no energy transfer takes place between the photon and the molecule. After the interaction the molecule is back in the ground state. In a Raman scattering process (2), energy transfer does occur. After a (Stokes-) Raman process the molecule ends up in a higher vibrational energy level. The energy of the photon is lowered by an equal amount, which is reflected in a shift to a higher wavelength. The wavelength shift of the Raman lines is expressed in cm^{-1}:

$$\Delta cm^{-1} = [1/\lambda_{in} - 1/\lambda_{out}].10^7 \ (\lambda \text{ in nm}).$$

The position of a Raman line depends on the masses of the atoms involved in the corresponding vibration and on the type of chemical bonds. A Raman spectrum can therefore be considered as a "fingerprint," which can be used to identify molecules in a sample, such as carotenoids in this communication. The intensity of a Raman line depends linearly on the concentration of molecules in a sample. Textbooks on Raman theory have been written by Lung (20) and Konigstein (17), and reviews of biological applications of Raman scattering can be found in refs. 12, 13, and 36.

tra of pelleted avian lymphocytes, strong carotenoid signal contributions were present (14). Working under resonance conditions, Barry and Mathies (3,4) were able to record the Raman spectra of retinal in single, frozen, photoreceptor cells.

We have recently described the possibility of performing nonresonant Raman microspectroscopic studies of single cells and chromosomes with a spatial resolution of 0.45 × 0.45 × 1.3 μm^3 (31–33) and the possibility of combining fluorescence activated cell sorting and Raman microspectroscopy (34) (see also Materials and Methods). Here these techniques were applied to investigate the presence and subcellular location of carotenoids in B-lymphocytes (CD19+), helper/inducer T-lymphocytes (CD4+), cytotoxic/suppressor T-lymphocytes (CD8+), Tγδ-lymphocytes [T-cell receptor (TCR) γδ+], and NK cells (CD16+) isolated from samples of human peripheral blood obtained from healthy individuals.

MATERIALS AND METHODS

Raman Measurements

Raman spectra of human lymphocytes and NK cells were measured using the confocal Raman microspectrometer described previously (31–33). The main features of this instrument are summarized in Fig. 2. Experimental conditions for the measurements are described under Fig. 3. The results that are presented are based on over 1,500 single cell measurements, and the spectra of Fig. 3 are typical examples. The blood of 4 different donors was used and yielded identical results.

Determination of Subcellular Carotenoid Concentration

In order to obtain information about subcellular carotenoid concentrations, a Raman spectrum of a 0.5 mg/ml β-carotene (Sigma Chemie, type III-C9875, Netherlands) solution in chloroform was recorded. The solution was contained in a glass cuvette sealed with a microscope cover glass. A ×63 Zeiss Plan Neofluar water immersion objective (numerical aperture: 1.2) with cover glass correction was used, instead of the same type of objective without cover glass correction used in the cell measurements (see the legend to Fig. 2). The experimental conditions (laser power, signal integration time, solid angle of detection, and confocal pinhole diameter (and therefore the effective scattering volume) for the cell and solution measurements were identical.

Comparison of the carotenoid Raman line intensities in the solution (known concentration) and cell (unknown concentration) spectra, therefore, provides information about subcellular carotenoid concentrations. Uncertainty is introduced by the fact that whereas in the solution measurements the whole scattering volume is filled with carotenoid solution, in the cell measurements the carotenoid containing organelle may only partly fill the scattering volume. Also cell-to-cell variations in Raman signal intensity, which may be (partly) due to this effect, were large. Line intensities ranged from about 30% to 200% of the intensities shown in Fig. 3. Therefore, the quantitative interpretation of the results was limited to an order-of-magnitude determination of the subcellular carotenoid concentration.

Sample Preparations

Peripheral blood from healthy individuals was collected in heparinized Vacutainers by venipuncture. The lymphocyte and NK cell fraction was isolated using standard density centrifugation and monocyte depletion methods, and then was incubated with either of the following fluorescein or phycoerythrine conjugated monoclonal antibodies: CD4 for helper/inducer T-lymphocytes, CD16 for NK cells, CD8 for cytotoxic/suppressor T-lymphocytes, CD19 for B-lymphocytes, and TCR-γδ-1 for $T_{\gamma\delta}$-lymphocytes (Becton Dickinson, San Jose, CA). By flow sorting on the basis of a positive fluorescence signal (excited by the 488 nm line of an Ar^+-laser, Coherent, Palo Alto, CA) on a laboratory-made flow cytometer, purified cell subpopulations were obtained. In the CRM the use of 660-nm laser light avoids the

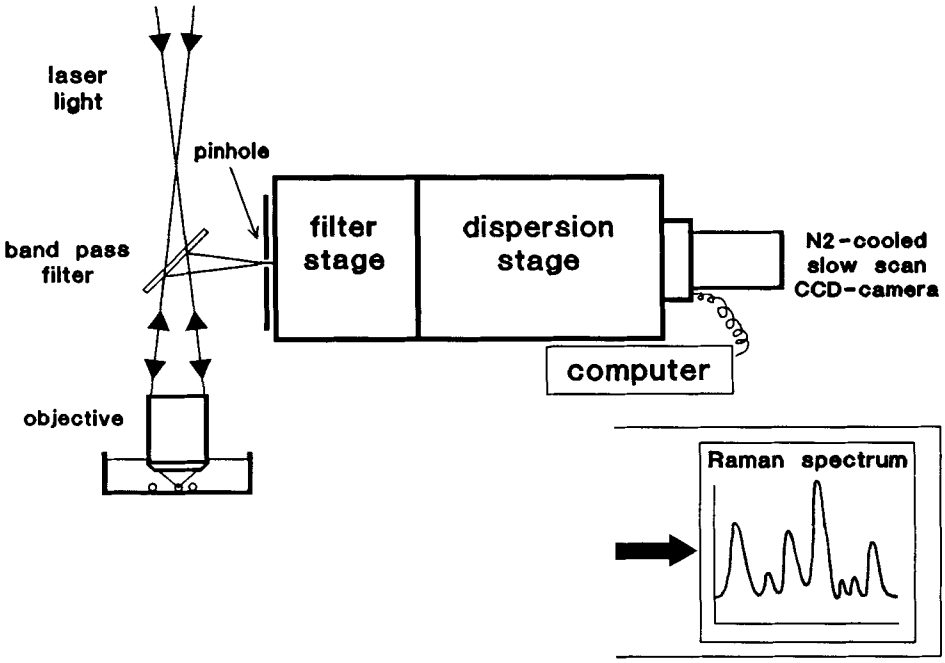

FIG. 2. Schematic of the confocal Raman microspectrometer. Laser light of 660 nm from a DCM operated model 375 B Spectra Physics dye laser (San Jose, CA) passes through a narrow band-pass filter (FWHM ~8 nm) and is focused on the object under investigation by a high numerical aperture microscope objective (×63 Zeiss Plan Neofluar, water immersion, NA 1.2). Scattered light is collected by the same objective. Raman scattered light, shifted in wavelength, is reflected by the band-pass filter and coupled into the spectrometer through a 100 μm pinhole, which enables confocal detection. Spatial resolution of the CRM was experimentally determined to be 0.45 × 0.45 × 1.3 μm^3 (33). In the filter stage the intensity of the Rayleigh scattered and reflected laser light, which is many orders of magnitude higher than that of the Raman scattered light, is suppressed. For this either a chevron-type band-pass filter set, developed in our laboratory (35), or a Raman holographic edge filter (Physical Optics Corp., Torrence, CA) is used. The dispersion stage consists of a single grating and focusing optics, which image the Raman spectrum onto a liquid-nitrogen-cooled, slow-scan CCD camera (Wright Instruments, Ltd, England). Cells are positioned in the laser focus with the CRM in the normal light microscopic mode, using the in-base illumination system of the microscope frame (Nikon Optiphot; Nippon Kogaku K.K., Tokyo, Japan). The CRM thus enables the recording of Raman spectra (i.e., information about molecular composition and structure) from precisely defined positions in a cell, as illustrated by the insert.

excitation of the fluorescent labels [34], which therefore do not interfere with the Raman measurements.

A Golgi specific fluorescent probe (C_5-DMB-Cer, Molecular Probes, Eugene, OR) was used to identify, by means of fluorescence microscopy, the carotenoid-containing cytoplasmic structure of CD8+, CD16+, and TCR-γδ-1+ cells. The protocol of Pagano et al. (27) was used. The photographs in figure 4A–E and 4G are light microscopic images obtained with the condensor diaphragm closed to a minimum in order to enhance the contrast.

Photographs

The photographs in figure 4A,B were taken on a Nikon Optiphot microscope equipped with a classical photocamera. The photographs in figure 4C,D are photographs of video recordings of cells that were used in Raman measurements. The photographs in figure 4E–H were taken with an Argus PC imaging system (based on a model C2400-80 intensified CCD camera, Hamamatsu Photonics GmbH, Herrsching, Germany), attached to an Olympus IMT-2 inverted microscope.

RESULTS

The Raman spectrum of carotenoids is very characteristic (Fig. 3A). It contains 2 intense lines around 1157 cm^{-1} and 1525 cm^{-1} (due to in-phase vibrations of the conjugated =C-C= and -C=C- bonds, respectively). None of the Raman spectra obtained in the nucleus of cells contained these lines (neither B- nor T-lymphocytes, nor NK-cells). But in T-lymphocytes and NK-cells, high concentrations of carotenoids were found at specific sites in the cytoplasm. Identification of the specific types of carotenoids present in the cells is not possible on the basis of Raman spectra.

In helper/inducer T-lymphocytes and occasionally in a cytotoxic/suppressor T-lymphocyte, the focal point of carotenoid concentration is the Gall body (Figs. 3B and 4A,B). This not-so-well-known, usually solitary, cytoplasmic spherule, first described in 1936, is best observed in unstained cells by bright field or phase-contrast microscopy (7,15). It is very rich in lipids, often surrounded by clustered primary lysozomes, reported to segregate mainly with helper/inducer T-lymphocytes, and is also the focal point of acid hydrolase ac-

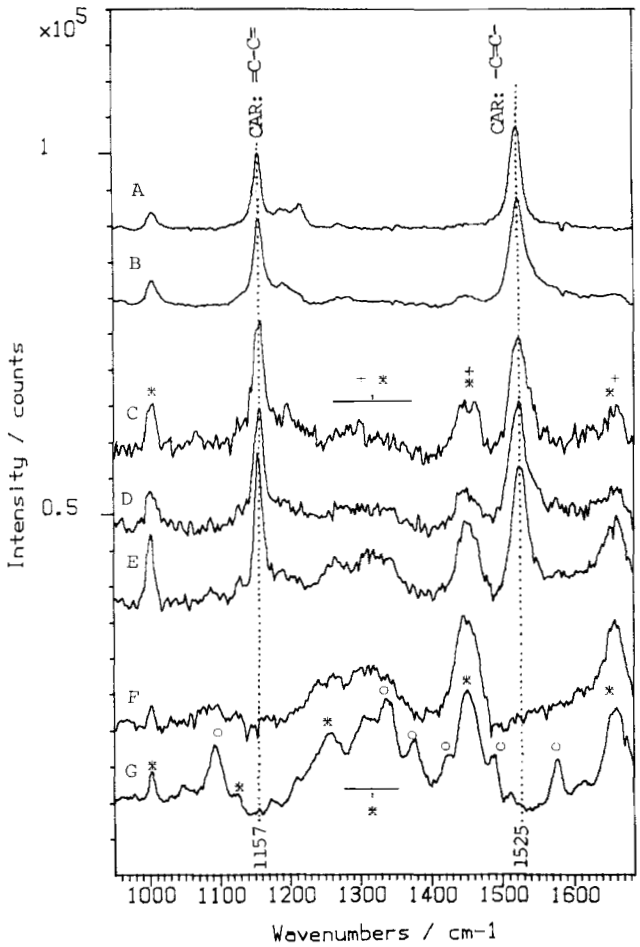

FIG. 3. Raman spectra of β-carotene, human T-lymphocytes and NK cells. **A:** β-carotene solution in chloroform (0.5 mg/ml). **B:** Helper/inducer T-lymphocyte (CD4+): spectrum obtained from Gall body (see Fig. 4). **C:** NK-cell (CD16+): spectrum obtained in the cytoplasm, apparently from the Golgi complex (see Fig. 4). **D:** Cytotoxic/suppressor T-lymphocyte (CD8+): spectrum obtained in the cytoplasm, apparently from the Golgi complex (see Fig. 4). **E:** $T_{\gamma\delta}$-lymphocyte (TCR-γδ-1+): spectrum obtained in the cytoplasm, apparently from the Golgi complex (see Fig. 4). **F:** B-lymphocyte (CD19+): characteristic cytoplasmic Raman spectrum (average of 9 measurements), containing protein and phospholipid signal contributions (31,33,34), but lacking carotenoid signal. **G:** Nuclear Raman spectrum: average of 10 measurements in different types of lymphocytes and NK cells, containing DNA and protein signal contributions (31,33,34), but lacking a carotenoid signal. CAR = carotenoid. *, protein signal contribution; +, phospholipid signal contribution; o, DNA signal contribution.

For more specific assignments of Raman lines see refs. 31, 33, and 34. Conditions: laser power: 5–6 mW (on sample); signal integration time for each measurement: 30 s, cells on poly-L-lysine-coated fused silica substrates and immersed in Hank's buffered salt solution (HBSS, according to GIBCO 041-04025, phenol red omitted). The intensity scale is for spectra A and B; the multiplication factor for spectra C-G is 8.

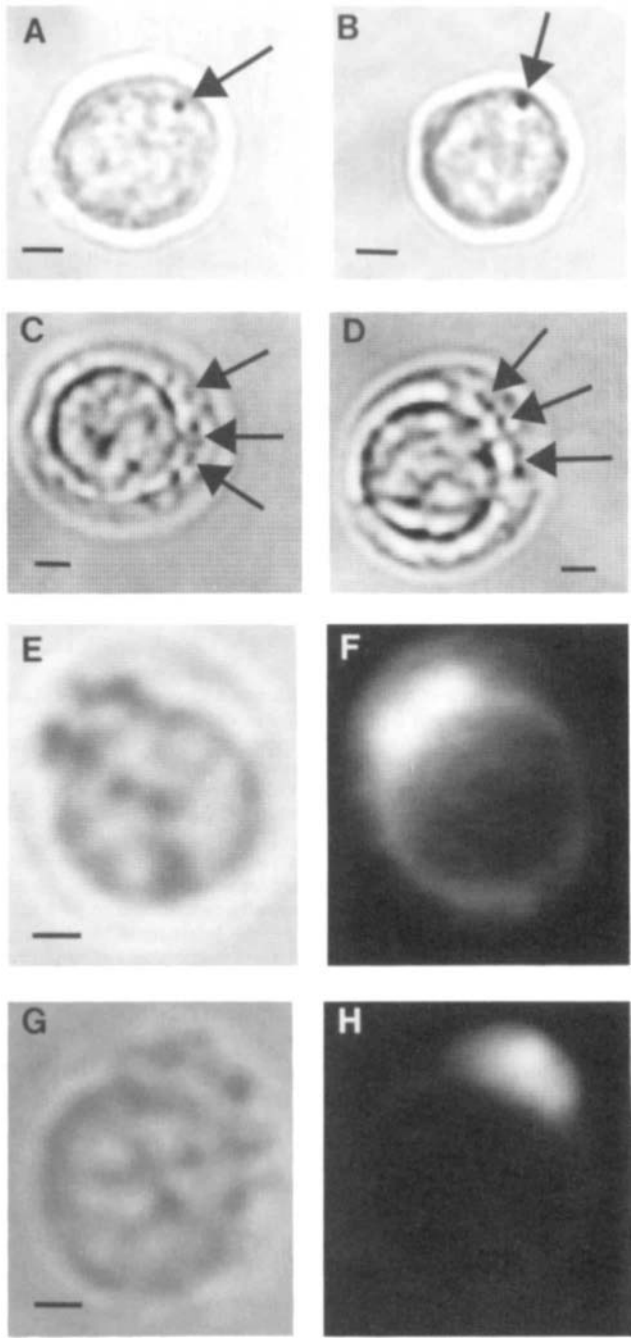

FIG. 4. Photographs illustrating the subcellular locations of carotenoids in lymphocytes and NK cells. **A,B:** Gall bodies (arrows) in helper/inducer T-lymphocytes. **C:** Cytotoxic/suppressor T-lymphocyte: arrows indicate cytoplasmic structure found to contain carotenoids. **D:** NK-cell: arrows indicate cytoplasmic structure found to contain carotenoids. **E-F,G-H:** Photographs of lymphocytes showing cytoplasmic structures identical to those in photographs C and D to be stained by the Golgi-specific probe C_5-DMB-Cer. Bars denote 2 μm.

tivity (8,22,23,25,40). Carotenoids are lipophilic, which of course facilitates their concentration in the Gall body. The function of the Gall body is unknown at this moment.

Comparison of the intensities of the carotenoid Raman signal obtained from Gall bodies (Fig. 3B) and that of a β-carotene solution (Fig. 3A) shows that in the

Gall body the carotenoid concentration is of the order of 0.5 mg/ml ($\sim 10^{-3}$ M), which is very high in comparison with the normal physiological carotenoid concentration in blood plasma of 10^{-6} M (5,37).

In cytotoxic/suppressor T-lymphocytes, $T_{\gamma\delta}$-lymphocytes, and NK-cells, there appears to be a correlation between the location of the Golgi complex and the location of carotenoids (Figs. 3C–E and 4C–F). The Golgi complex is well developed in these cells, in contrast to helper/inducer T-lymphocytes (9,24). The cytoplasmic structures containing carotenoids (Fig. 4C,D), which are of a tubulovesicular nature, as can be seen upon focusing up and down through the cells, are morphologically identical to the structures that are preferentially stained with C_5-DMB-Cer, a Golgi specific fluorescent probe (27; Fig. 4E–H). The carotenoid concentration, as determined from the intensity of the Raman signal (Fig. 3C–E; note that these spectra have been multiplied by a factor of 8 with respect to spectra 3A–B), is roughly one order of magnitude lower (i.e., 10^{-4} M) than in Gall bodies. In most measurements on B-lymphocytes, no carotenoid signal was found, although they also possess a well-developed Golgi complex. In only 5 out of a total of 120 measurements was a very faint carotenoid signal obtained, just above the noise level (corresponding to a concentration of $<10^{-5}$ M).

DISCUSSION

The results show that carotenoids are present in high concentrations at distinct sites in the cytoplasm of specific cell populations. This may reflect some form of specificity in the function of carotenoids in the immune system, which may lie in the actual nature of their function (including possible unknown functions) or in the location and/or time which they fulfill their function. Knowledge about which cells of the immune system do contain (large amounts of) carotenoids and which do not, and about the subcellular location of carotenoids, can be used to give direction to further investigations regarding the role of carotenoids.

In the light of the important role carotenoids appear to have in the immune system, their strikingly high concentration in the Gall body warrants further characterization of this cytoplasmic spherule and its until now elusive function. A role in the immune functioning of lymphocytes and NK-cells, e.g., protection against possible reactive byproducts of a cytotoxic cell-target cell interaction, or against radicals and reactive oxygen species produced by phagocytes operating in the vicinity, may be reflected in a redistribution of carotenoids upon activation of a cell. This is the subject of further investigations at this moment.

The work presented here is an example of the way in which Raman spectroscopy can be used in cell biology to investigate the molecular composition of cells and cell organelles without having to rely on the accessibility to cytochemical probes. However, in contrast to (immuno)fluorescence methods, the technique is in principle nonspecific, i.e., all molecules in the measuring volume contribute to the Raman signal. This nonspecificity is a limitation of the Raman technique, because it can make it difficult to detect small amounts of one type of molecule (or molecular conformation) against a background of other molecules (or molecular conformations), which is the strong point of immunofluorescence methods. In view of their advantages and disadvantages, Raman microspectroscopy and fluorescence methods are complementary rather than competitive techniques, and, as illustrated by the present work, can be fruitfully combined in investigations.

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