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Clinical report

In vitro chemosensitivity testing of hematological cancers: immunohistochemical detection of ornithine decarboxylase

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A new method for *in vitro* chemosensitivity testing of human lymphoma and leukemia patients has been developed. The method is based on the use of ornithine decarboxylase (ODC), a universal marker of proliferation, which is expressed early during the cell cycle and has a short half-life. This marker was detected by a quantitative immunohistochemical analysis, using an ODC antibody and a FITC-linked second antibody. The *in vitro* chemosensitivity of lymphocytes from four normal individuals was tested by the immunohistochemical method. Lymphocytes from 25 cancer patients were also examined. In drug-sensitive cells, the intensity of the marker declined in the presence of the drug, whereas resistance to the drug was demonstrated by the presence of the marker. A good correlation was found between the predicted chemosensitivity and the outcome of the therapy. It has been suggested that this approach could be used for *in vitro* chemosensitivity testing of hematological cancers and most likely also for other malignancies. [© 1999 Lippincott Williams & Wilkins.]

Key words: Anti-cancer drugs, chemosensitivity, lymphoma, leukemia, ornithine decarboxylase, polyamines.

Introduction

Chemotherapy, along with radiotherapy and surgery, continues to be an effective weapon to combat cancer. In most cases, the treatment of cancer patients is based on the identification of the tumor by histological examinations rather than on the determination of the sensitivity of the tumor to a given agent. Unfortunately, individual patients with apparently identical tumor histologies do not always respond identically to the same drug regimen. It is therefore not surprising that the development of reliable methods for the *in vitro* testing of sensitivity of cancer cells to various drugs has been a long-standing objective in cancer research and treatment. As in the treatment of infectious diseases, an *in vitro* chemosensitivity test for cancer could increase the chance of recovery, reduce undesired side effects and minimize the emergence of multi-drug resistance (MDR) variants. Early attempts to determine *in vitro* chemosensitivity were based on the assumption that anti-cancer activities can be determined by the formation of foci in the presence and absence of the drugs.[1,2] Soon it became evident that this assay is not practical because of the low plating efficiency; only 1–2% of the cells grew in the culture.[3] Attempts to determine the growth of the cancer cells by measuring the incorporation of radioactive precursors into cellular macromolecules remained elusive because of damage induced during isolation.[4] Methods based on the measurement of cell metabolism, such as the use of the MTT dye (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)[5] or ATP bioluminescence,[6] did not predict the chemosensitivity of primary human tumors to given anti-neoplastic agents. It therefore appears that the available methods are not satisfactory and that new approaches should be explored. The ideal approach should be based on the monitoring of a *marker* for proliferation rather than *growing* cells in culture. It would also be of advantage if the marker could be detected in individual cells, so that tumor cells could be identified and distinguished from normal cells, such as fibroblasts.

An ideal marker should meet the following requirements. (i) It should be a universal marker, present in all cells. (ii) It should be expressed during the early phases of the cell cycle. (iii) It should have a short half-

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life, so that it would decay rapidly when cell proliferation is arrested.

Ornithine decarboxylase (ODC, EC 4.1.1.17) can serve as a marker for proliferation.[7,8] This enzyme catalyzes the conversion of ornithine into the diamine putrescince, which is the precursor for the synthesis of the naturally occurring polyamines.[9,10] The polyamines, spermidine and spermine, play an essential role in growth and proliferation processes. ODC is present in all cells studied and its induction is mediated by hormones, lectins, antigens, etc., which induce transmembrane signals.[9,10] Additionally, it is expressed early in the cell cycle and has an extremely short half-life (15–20 min, the shortest of any eukaryotic enzyme).

Recent studies from our laboratory demonstrated that ODC can indeed serve as a marker for proliferation.[11] Moreover, ODC was used to determine the chemosensitivity of cultured murine leukemia cells to various anti-cancer drugs.[12,13] In those studies and in others, in which the chemosensitivity of cultured human epithelial carcinoma cells was examined, the enzymatic activity of ODC was determined. This assay required at least 10^6 cells and obviously does not permit the study of individual cells. In order to detect ODC in individual cells, we developed an immunohistochemical method for staining ODC. This method permitted the detection of ODC in individual cells and thus the determination of *in vitro* sensitivity at the cellular level.[14]

In the present study we tried to apply the immunohistochemical method for the quantitative *in vitro* chemosensitivity assay of individual cells taken from hematological cancer patients.

Materials and methods

Patients

Cancer patients were treated in the Department of Hematology, Shaare Zedek Medical Center, Jerusalem, Israel. Blood samples were obtained from 25 cancer patients and from four healthy individuals, after informed consent. Eleven of the cancer patients were females and 14 were males. Their age ranged from 43 to 87 (average 61.5) years.

Preparation of ODC antibodies

Escherichia coli JM109 cells which carry plasmid pGEM-1, containing a 1.8 kb *Bam*HI-*Eco*RI fragment of mouse ODC cDNA were kindly provided by Dr C Kahana (Weizmann Institute of Science, Rehovot, Israel). Bacteria were grown in M9 medium containing ampicillin (200 μg/ml) at 37°C, and ODC protein was extracted and purified. ODC-rich fractions were injected i.v. and s.c. into rabbits (approximately 500 μg ODC protein per rabbit) in the presence of complete Freund's adjuvant (Sigma, St Louis, MO). Immunization was repeated twice by injecting the protein in the presence of complete Freund's adjuvant in 3-week intervals. Rabbits were then bled and sera were tested for ODC neutralization activities.

In vitro chemosensitivity assay

Heparinized fresh blood was mixed with an equal volume of DMEM medium (Beit Haemek, Israel) and

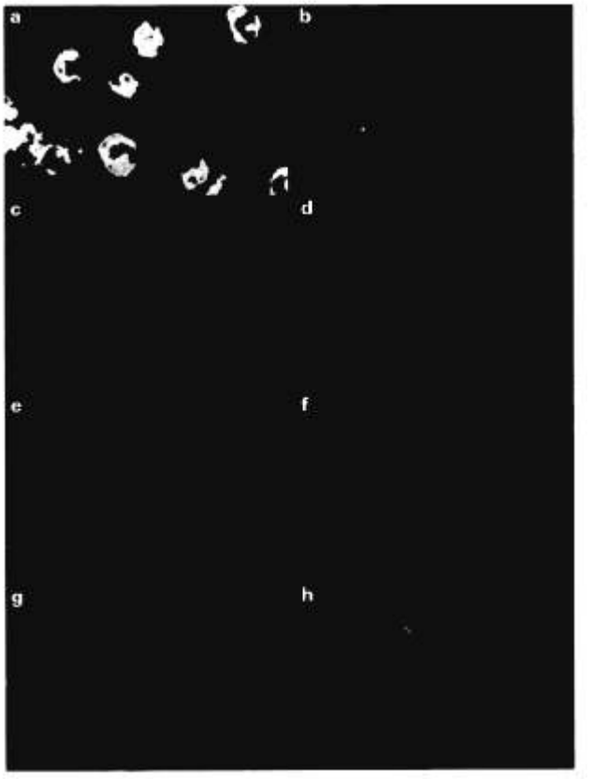

Figure 1. Detection of ODC by the immunohistochemical method. Treatment: (a) 2 μg/ml PHA-stimulated lymphocytes, (b) non-stimulated controls, (c) stimulated lymphocytes in the presence of 3×10^{-8} adriamycin, (d) stimulated lymphocytes in the presence of 10^{-5} adriamycin, (e) stimulated lymphocytes in the presence of 10^{-9} methotrexate, (f) stimulated lymphocytes in the presence of 10^{-6} methotrexate, (g) stimulated lymphocytes in the presence of 2×10^{-8} vincristine and (h) stimulated lymphocytes in the presence of 5×10^{-8} vincristine.

layered on Ficoll-paque (Pharmacia Biotech, Uppsala, Sweden) at a ratio of 4:1. After centrifugation at 1500 r.p.m. for 25 min, lymphocytes were collected and washed twice with DMEM medium by centrifugation at 1200 r.p.m. for 10 min. Lymphocytes were then suspended in complete RPMI 1640 medium (containing 50 U/ml penicillin, 50 μg/ml streptomycin and 15% autologous plasma) supplemented with 2 μg/ml phytohemagglutinin (PHA; Sigma). The PHA-stimulated lymphocytes were incubated at 37°C in a 5% CO_2 incubator, with or without anti-cancer drugs at different concentrations. After 24 h, lymphocytes were washed with phosphate-buffered saline (PBS; 0.8% NaCl, 0.115% Na_2HPO_4, 0.2% KH_2PO_4 and 0.02% KCl) by centrifugation at 1200 r.p.m. for 10 min. Slides were prepared either by dropping the cells on slides coated with L-polylysine (Sigma; 1:10 in double-distilled water) or by using a cytospin at 1500 r.p.m. for 5 min. Slides were dried at room temperature for 2 h and then fixed with cold (−20°C) methanol for 2.5 min. Slides were next rinsed twice with PBS and incubated with 1% bovine serum albumin (BSA in PBS) for 30 min at room temperature in a humidified box. Slides were then incubated overnight with rabbit anti-human ODC antibodies (1:400 in 1% BSA/PBS) at 4°C. After repeated rinsing, the slides were exposed for 1 h at 37°C to FITC-conjugated affinipure goat anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove PA; 1:100). Finally, the slides were washed 4 times with PBS and covered with a mounting solution (90% glycerol, 10% PBS, pH 10, 0.1% sodium azide and 5% 3,3'-diaminobenzidine). Specimens were examined by a Confocal Laser Scanning Microscope, Phoibos 1000 (Sarastro, Sweden) at 730 mV. The value of each section is the average of fluorescent activities in cells from two areas. Results are expressed as arbitary units per μm^2 area of scanned cells.

Results

Normal individuals

A typical example of an *in vitro* chemosensitivity test is illustrated in Figure 1. It may be seen that ODC protein was detected at high amounts in PHA-

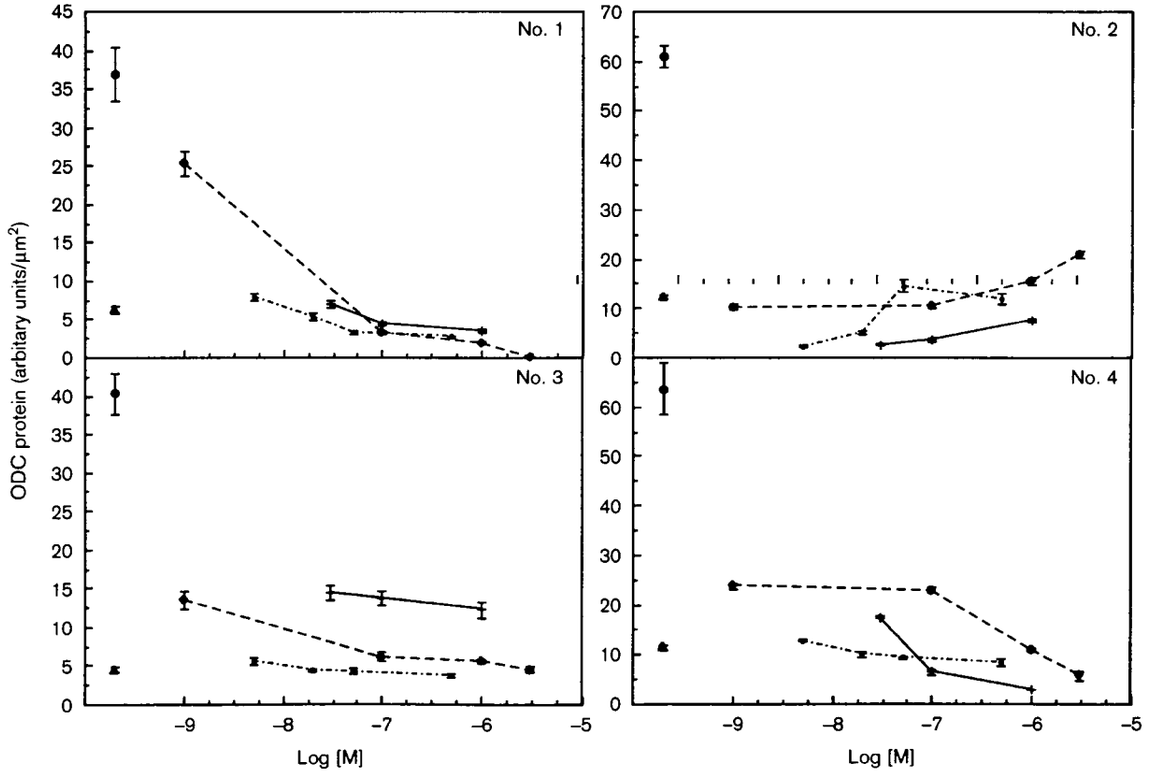

Figure 2. Detection of ODC in lymphocytes of normal individuals. Treatment: ●, 2 μg/ml PHA; ▲, non-stimulated controls; +——+, stimulated lymphocytes in the presence of adriamycin; ◆- - - -◆, stimulated lymphocytes in the presence of methotrexate; ■-···-■, stimulated lymphocytes in the presence of vincristine.

stimulated lymphocytes (Figure 1a), but was minimal in non-stimulated cells (Figure 1b) and in PHA-stimulated cells treated with anti-cancer drugs at various concentrations (Figure 1c-h). These findings can be quantitated. Results obtained with four normal individuals are shown in Figure 2. It may be seen that the ODC content of normal unstimulated lymphocytes was below 20 (4-12 arbitary units/μm^2). A 6- to 7-fold increase in ODC protein was observed after stimulating normal lymphocytes with PHA. Treating the normal cells with adriamycin (10^{-6} to 3×10^{-8} M), methotrexate (3×10^{-6} to 10^{-9} M) or vincristine (5×10^{-5} to 5×10^{-9} M) led to a significant reduction in cellular ODC. It may be concluded that normal

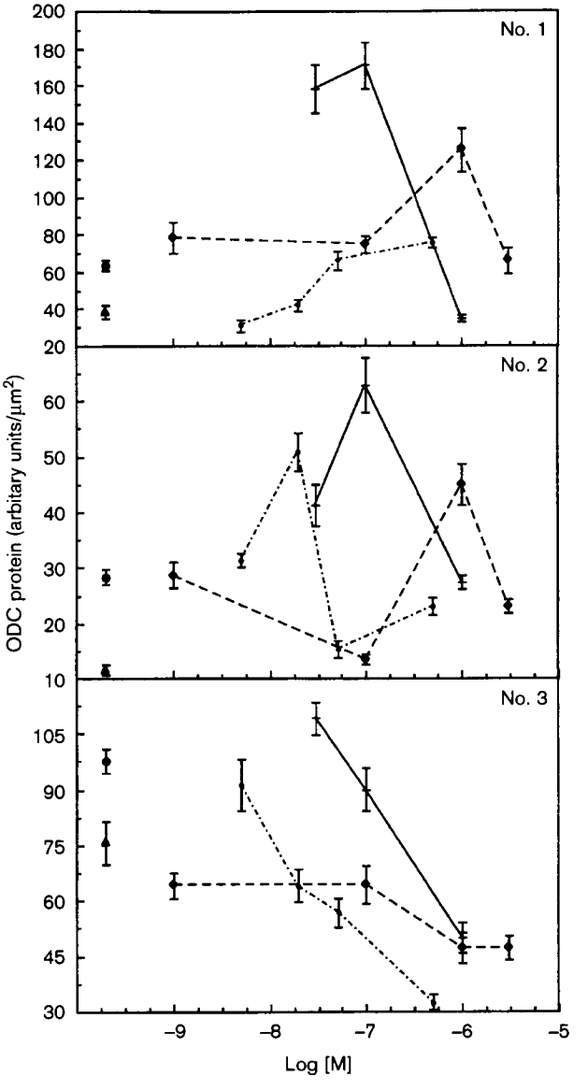

Figure 3. Detection of ODC in lymphocytes of deceased cancer patients. Treatment: ●, 2 μg/ml PHA; ▲, non-stimulated controls; +——+, stimulated lymphocytes in the presence of adriamycin; ◆- - - -◆, stimulated lymphocytes in the presence of methotrexate; ■····■, stimulated lymphocytes in the presence of vincristine. Patient no. 1 suffered from non-Hodgkin's lymphoma with secondary AML; no. 2 CLL and no. 3 multiple myeloma. The *in vitro* test showed that all of them had MDR.

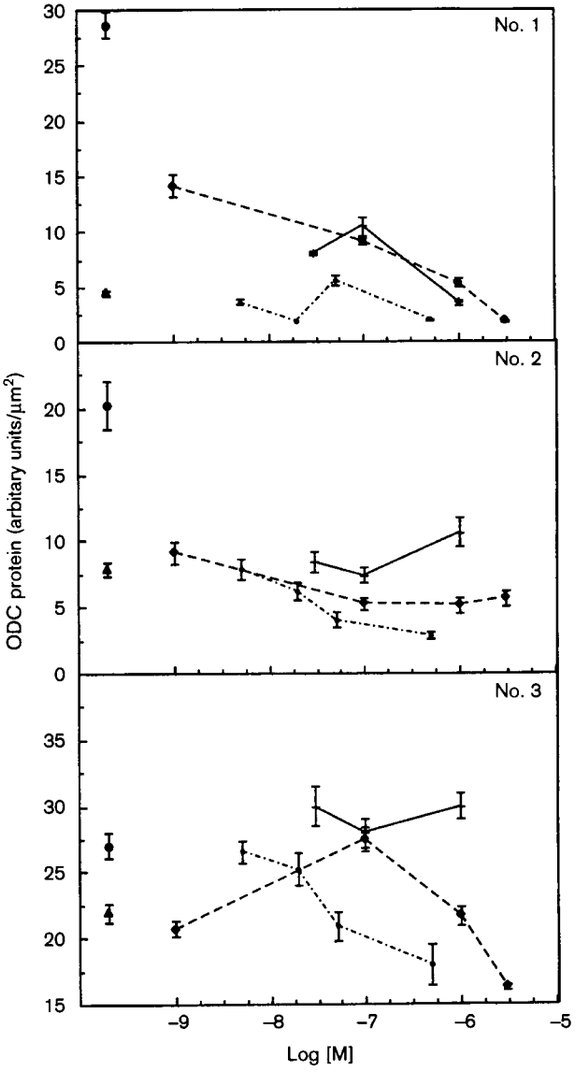

Figure 4. Detection of ODC in lymphocytes of mild cases. Treatment: ●, 2 μg/ml PHA; ▲, non-stimulated controls; +——+, stimulated lymphocytes in the presence of adriamycin; ◆- - - -◆, stimulated lymphocytes in the presence of methotrexate; ■····■, stimulated lymphocytes in the presence of vincristine. Patient nos 1 and 2 suffered from CLL and no. 3 from CML. None of them needed clinical treatment.

lymphocytes are not multidrug resistant and are sensitive to the drugs at the tested concentrations.

Deceased patients

Three of the cancer patients died as a result of their disease. In two of the patients (Figure 3, nos 1 and 3), ODC protein values were high even in the non-stimulated lymphocytes. It has been well established that ODC activity and protein concentrations are elevated in cancer cells and that this increase could reflect the severity of the disease.[9] Indeed, clinical findings confirmed that patient no. 3 suffered from an aggressive multiple myeloma. All the three patients showed multidrug resistance (Figure 3). Patient no. 1 was a lymphoma patient with a secondary acute lymphocytic leukemia (AML). This patient did not

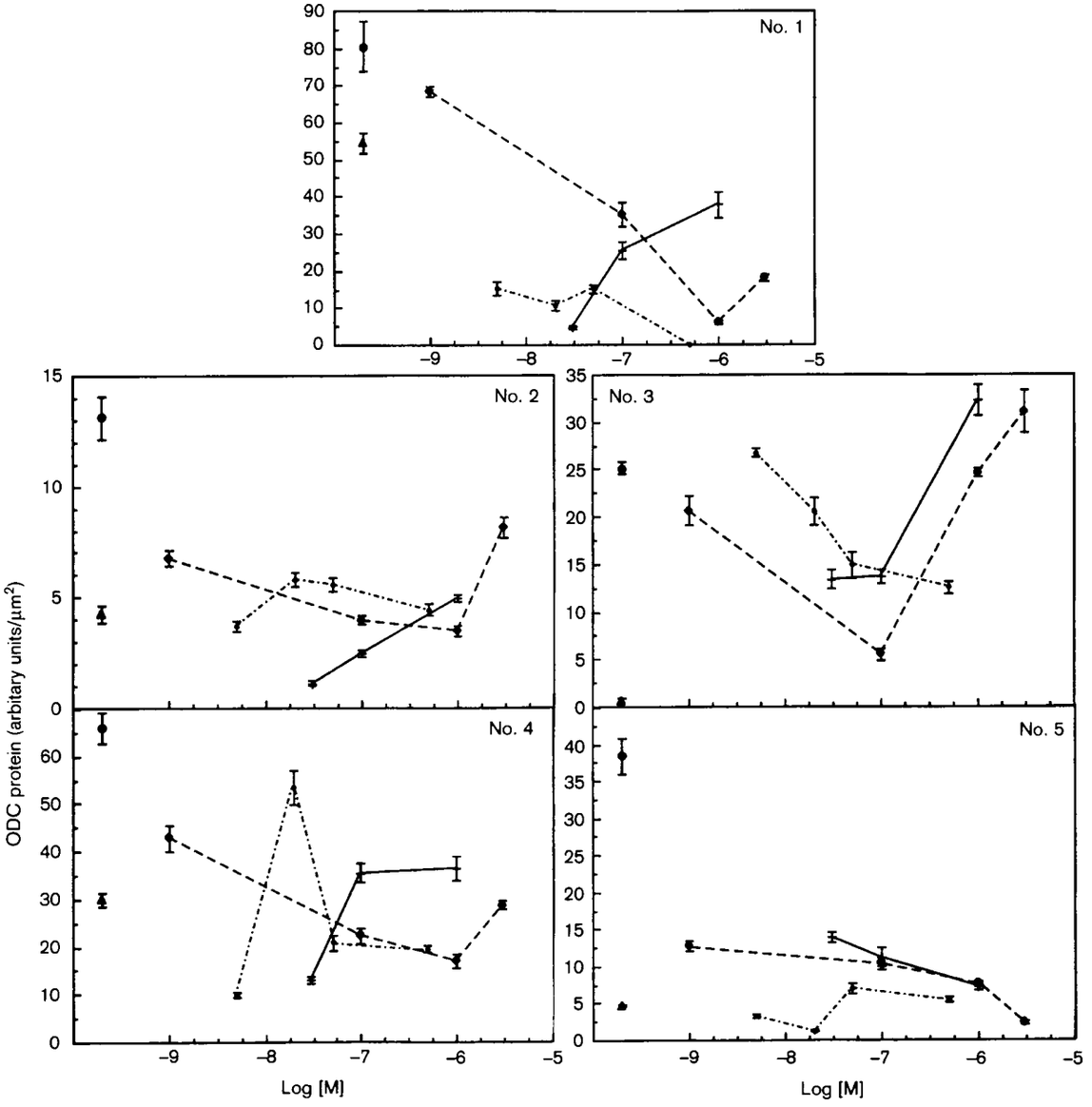

Figure 5. Detection of ODC in lymphocytes of CML patients. Treatment: ●, 2 μg/ml PHA; ▲, non-stimulated controls; +—+, stimulated lymphocytes in the presence of adriamycin; ◆- - - -◆, stimulated lymphocytes in the presence of methotrexate; ■·····■, stimulated lymphocytes in the presence of vincristine. All of them responded to clinical treatment.

respond to the treatment with anti-cancer drugs, was apparently multidrug resistant and died. Patient no. 2 suffered from chronic lymphocytic leukemia (CLL) and did not respond to chemotherapy. He, too, died. Patient no. 3 did not respond to the treatment with drugs at 10^{-9} to 10^{-} M concentration (Figure 3). In all of the three patients, multidrug resistance could be detected, and a good correlation was found between the predicted chemosensitivity and the failure of the therapy.

Mild leukemia patients

Another group consisted of three mild cases of leukemia patients (Figure 4). These patients did not require chemotherapy. In two of the three patients, ODC protein was low in unstimulated lymphocytes and increased moderately after PHA stimulation (Figure 4). The lymphocytes of two of the patients were sensitive to the drugs tested, while the third one (which had relatively high ODC protein in non-

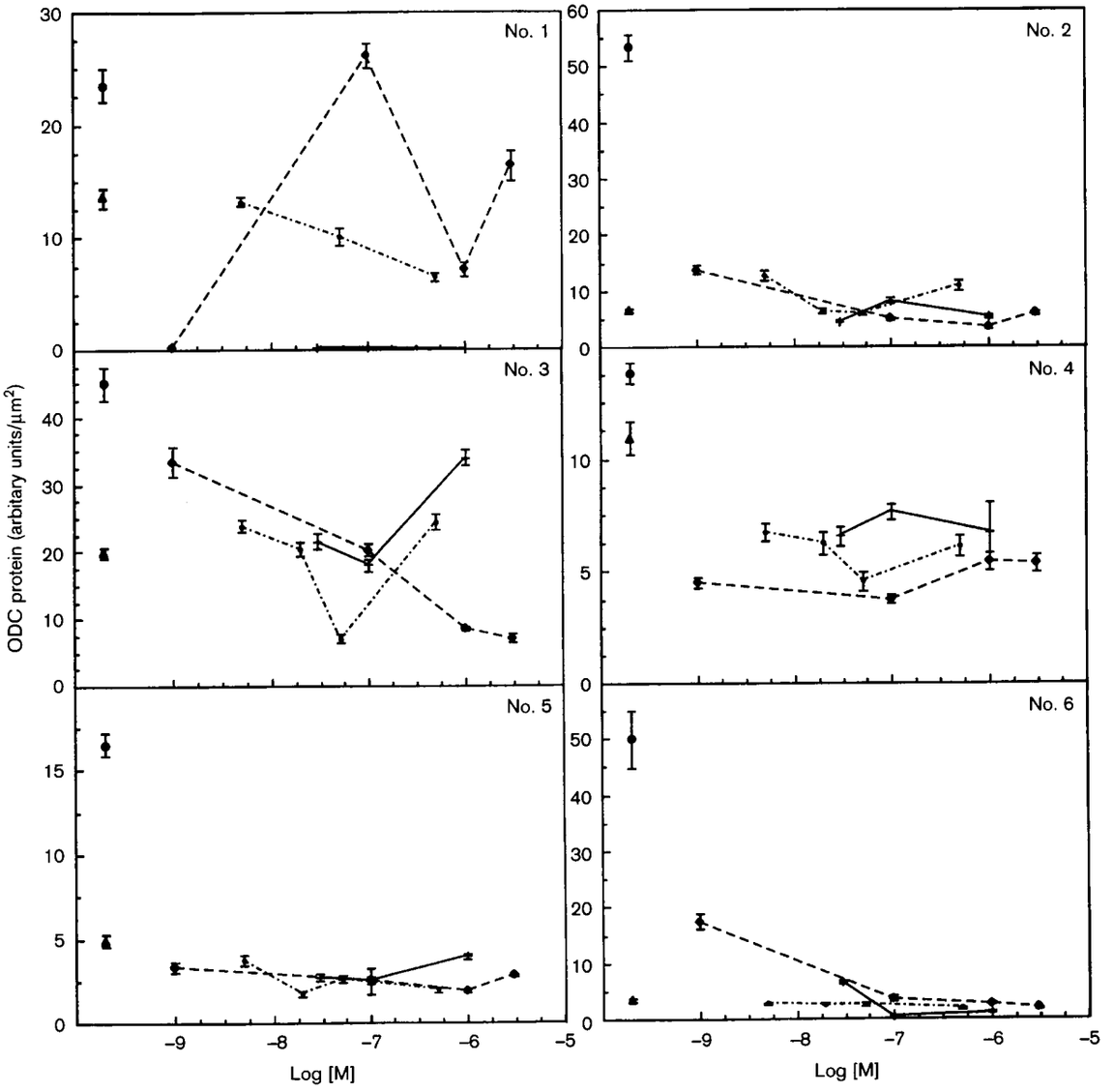

Figure 6. Detection of ODC in lymphocytes of non-Hodgkin's lymphoma patients. Treatment: ●, 2 μg/ml PHA; ▲, non-stimulated controls; +——+, stimulated lymphocytes in the presence of adriamycin; ◆- - - -◆, stimulated lymphocytes in the presence of methotrexate; ■-·-·-■, stimulated lymphocytes in the presence of vincristine. All of them responded to clinical treatment.

stimulated lymphocytes) was not affected by adriamycin (Figure 4, no. 3).

Other leukemia patients

The next group included five chronic myelocytic leukemia (CML) patients, who responded to treatment. *In vitro* chemosensitivity tests demonstrated that the leukocytes of these patients were sensitive to the various drugs tested (Figure 5).

Non-Hodgkin's lymphoma patients were also tested. All the six patients responded to the therapy (Figure 6). Five patients suffered from CLL. All of these patients responded to chemotherapy. The sensitivity of the lymphocytes to the

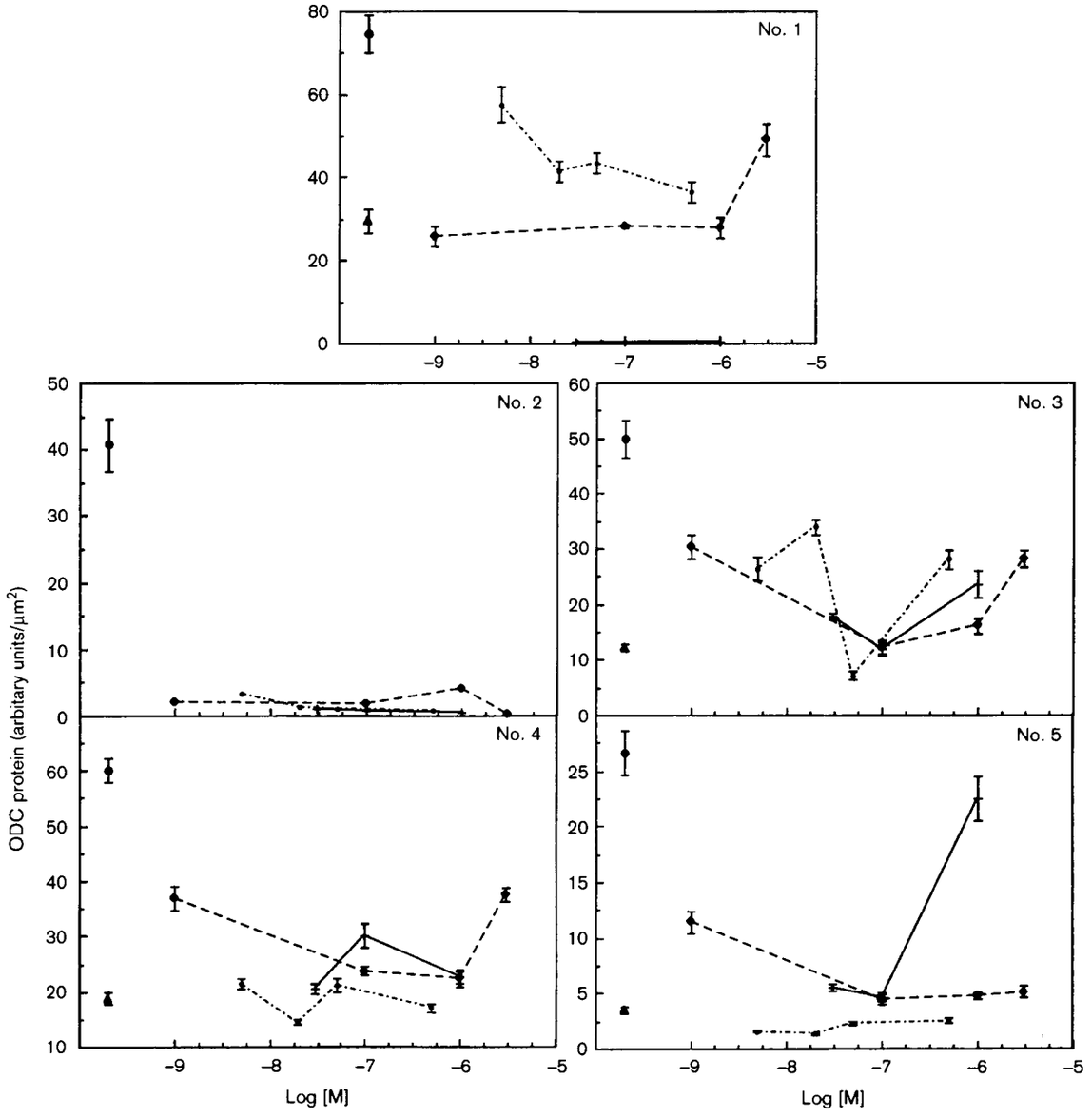

Figure 7. Detection of ODC in lymphocytes of CLL patients. Treatment: ●, 2 μg/ml PHA; ▲, non-stimulated controls; +——+, stimulated lymphocytes in the presence of adriamycin; ◆- - - -◆, stimulated lymphocytes in the presence of methotrexate; ■·····■ stimulated lymphocytes in the presence of vincristine. All of them responded to clinical treatment.

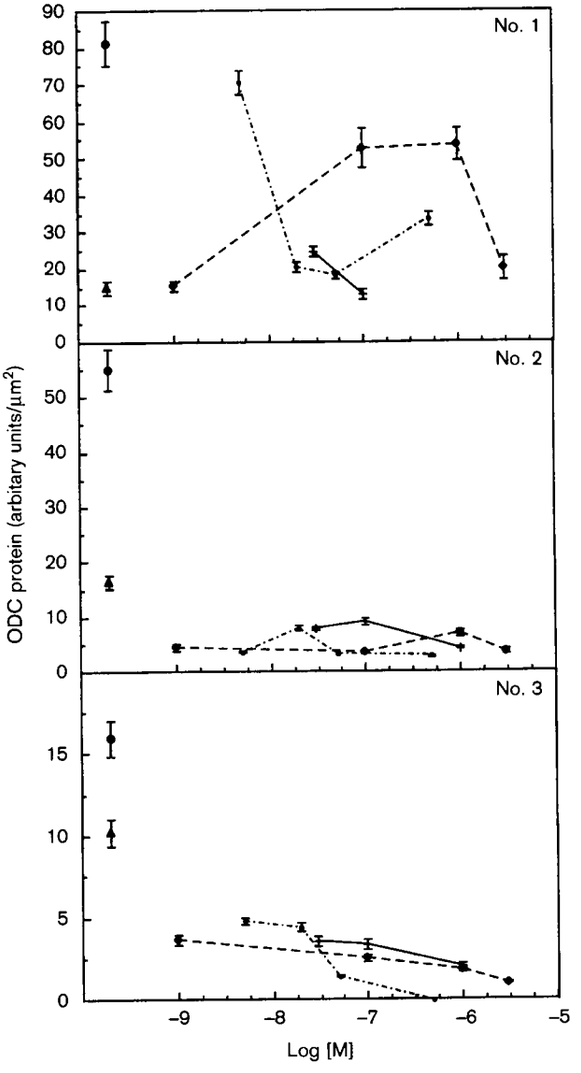

Figure 8. Detection of ODC in lymphocytes of other cancer patients. Treatment: ●, 2 μg/ml PHA; ▲, non-stimulated controls; +——+, stimulated lymphocytes in the presence of adriamycin; ◆- - - -◆, stimulated lymphocytes in the presence of methotrexate; ■·····■, stimulated lymphocytes in the presence of vincristine. Patient no. 1 suffered from hairy cell leukemia, no. 2 multiple myeloma and no. 3 essential thymocytosis. All of them responded to clinical treatment.

various drugs was confirmed by the *in vitro* chemosensitivity assay (Figure 7). In three other cancer patients suffering from hairy cell leukemia, multiple myeloma and essential thymocytosis, a good correlation between the outcome of therapy and the *in vitro* chemosensitivity test was observed (Figure 8).

Discussion

There is no doubt that a reliable *in vitro* chemosensitivity test will provide a better treatment for cancer patients. Thus, ineffective drugs may be identified and eliminated, so that the patient will be spared being subjected to 'standard' chemotherapy regimens and their associated toxicities. Unfortunately, the known procedures for the *in vitro* chemosensitivity assays are not satisfactory.[15-18] They are not accurate and in most cases the results can be obtained only after 7-14 days.[19,20]

In the present study we used a marker of proliferation rather than estimating cell growth or determining cellular metabolic activities. It is evident from Figure 1 that this approach, based on the immunohistochemical detection of ODC, can be used for *in vitro* chemosensitivity assays. Data presented in Figure 2 clearly show that results can be quantitated and that lymphocytes from normal individuals did not show drug resistance. On the other hand, three patients who did not respond to chemotherapy, showed multidrug resistance, based on the ODC assay (Figure 3). Lymphocytes from the three patients did not respond to the treatment with adriamycin, methotrexate, or vincristine and therefore could be defined as MDR. It is conceivable that these patients would not respond to the treatment with other drugs. On the other hand, *in vitro* chemosensitivity assays of mild cases (Figure 4), curable CML (Figure 5), non-Hodgkin's lymphoma (Figure 6) and CLL patients (Figure 7) suggested that those patients should respond to therapy. According to Von Hoff *et al.*[18] when therapy was selected according to the *in vitro* results, a clinical response was observed in 60% while the ability of the assay to correctly predict clinical resistance was 85%. It has been suggested that the real benefit of the *in vitro* assay systems may not be in predicting which drugs to administer, but rather which drugs not to administer.

In the proposed method we use ODC, which is not only a marker for proliferation, but also an oncogene.[21] It appears that the proposed immunohistochemical method could open new possibilities for the detection of multidrug resistance in hematological cancer patients. Obviously, more extensive studies should be carried out and the validity of the approach for patients with solid tumors should be tested.

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