Noncolorimetric Measurement of Cell Activity in Three-Dimensional Histoculture Using the Tetrazolium Dye 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide: The Pixel Image Analysis of Formazan Crystals

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We describe a novel system for measuring the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction in three-dimensional histoculture which is no longer dependent on colorimetric determination of extracted formazan, but rather is based on a pixel image analysis of formazan crystals, and which allows intratumor heterogeneity to be taken into account. The MTT test is based on the enzymatic reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan crystals by living, metabolically active cells, but not in dead cells. The reaction was carried out in situ in six-well plates on gel-supported histocultured human tumors. After a 24-h incubation with different drugs the tumors were incubated with a solution of MTT. Frozen sections of the tumor pieces were made and the slides were then stained with a propidium iodide solution, whose fluorescence is proportional to the number of cells present. We demonstrate here that the formazan crystals, formed by MTT reduction, reflect polarized light and that this can be quantified by using an image analysis system based on bright-pixel quantitation directly on a frozen section of the original tissue. Combined with the use of the fluorescent dye propidium iodide, also measured by pixel analysis, we can express a ratio between the total amount of MTT reduction and the total number of cells present in the specimen that expresses the effect of drugs on the histocultured tumors. Since histology is well maintained in histoculture it is possible to take into account the heterogeneity present in the tumor with regard to drug response. This assay system should be of potential clinical use for predicting the drug response of cancer patients. © 1992

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architecture (9,10). Drug response in the histoculture assay is thought to be potentially more similar to in vivo response because of the preservation of the original tissue architecture and function (11). In addition the histoculture system allows the growth of essentially all types of solid tumors with an 80% or better evaluability rate for drug response, multiple agents can be tested sequentially, and a relatively low amount of tissue is required. Using histoculture with [3H]thymidine incorporation measured by histological autoradiography, it was shown, in an in vitro—in vivo correlation study, that in vivo drug resistance was accurately predicted but in vivo drug response was accurately predicted for some drugs but not for others.

In this study we describe a novel system for measuring drug response in three-dimensional histoculture. This endpoint is based upon the capability of the tumor to reduce a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which has been used in a number of other assays (12–16) including histoculture, where good in vitro—in vivo drug response correlations have been obtained (17). The MTT endpoint as developed in our report, however, is no longer dependent on colorimetric determination of extracted formazan as it was previously, but is measured by a pixel image analysis of formazan crystals (PIAFC). We demonstrate here that the formazan crystals, formed by MTT reduction by mitochondrial succinate dehydrogenase, reflect polarized light and that this can be quantified by using an image analysis system based on bright-pixel quantitation directly in a section of the original tissue. With the combined use of the fluorescent dye propidium iodide, the intensity of which is proportional to the number of cells present and also determined by pixel analysis (PIAPI), we can now express a ratio between the amount of MTT reduction and the total number of cells present in the specimen (PIAFC/PIAPI) as an endpoint for measuring the in vitro effect of a drug on a tumor. Since histology is well maintained in histoculture it is also possible to take into account the heterogeneity of drug response present in a tumor specimen. Clinical usefulness is expected from such a safe, simple, and convenient in vitro chemosensitivity test.

MATERIALS AND METHODS
Histoculture

Human tumor specimens derived from surgery were explanted on sponge gels, following the technique described by Freeman and Hoffman (9) and previously developed by Leighton (7,8). Briefly, human tumor specimens were minced aseptically into 1-mm³ pieces and placed on collagen gels manufactured from piggleskin (Health Design Inc., Rochester, NY). The sponge-gel-supported tumors were cultured in six-well plates with 2 ml of Eagle’s minimum essential medium containing 10% fetal bovine serum and 1% nonessential amino acid, for 24 h before antitumor drugs were added. Gentamicin sulfate (Gemini Bioproducts, Inc.) was added to the media at a concentration of 0.1 mg/ml, plus 100 units/ml of penicillin G (Sigma) if colon tissue was cultured.

Drugs

Drugs used were mitomycin-C (100 ng/ml), doxorubicin (29 ng/ml), 5-fluorouracil (4.0 μg/ml), cisplatin (1.5 μg/ml), melphalan (1 μg/ml), carmustine (0.2 μg/ml), vincristine (7.3 ng/ml), vinblastine (23 ng/ml), and bleomycin (210 ng/ml). The concentrations of these drugs represent the levels achievable clinically and are termed “1×” concentrations (6). The drugs were obtained from Sigma Chemical Co. and dissolved in physiological saline, except for melphalan, which was dissolved in ethanol.

MTT Assay

3-(4,5-Dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. It was dissolved in a stock solution of 8 mg/ml in phosphate-buffered saline, pH 7.3, and filtered through a 0.2-μm membrane filter (Millipore, Bedford, MA). The phosphate-buffered saline was composed of 138.7 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄.

The sponge-gel-supported tumor pieces, after 24 h incubation with drugs, were transferred to drug-free media and were incubated for 1–3 h at 37°C in a humidified sterile atmosphere, containing 95% air/5% CO₂, with 2 ml of a new solution composed of MTT at a final concentration of 0.4 mg/ml in phosphate-buffered saline.

At the end of this reaction, the gels were removed from the incubation media containing MTT and placed in 2 ml of cold phosphate-buffered saline.

The specimens were kept at 4°C until 4-μm frozen sections were made. Water-soluble embedding media (Tissue Tek OCT Compound (Baxter) and a Leitz Cryostat 1720) were used to make frozen sections.

The slides were then dipped in a 1.25 μg/ml propidium iodide solution (Sigma) prepared in distilled water, for 30 s. After being dried, they were ready for the PIAFC and PIAPI measurement.

The image analysis system consisted of a Nikon Optiphot microscope connected to an RCA TC-1501 video camera, a Hitachi monitor, and an IBM personal computer.

The slides were analyzed microscopically under a mer-
FIG. 1. Composite illustrating frozen sections under polarized and fluorescent light. See text for details. (A) Human colon tumor after 24 h culture in the presence of doxorubicin and after 1 h incubation with MTT showing how the formazan crystals appear if visualized under polarized light. (B) Same field as (A) visualized under fluorescent light, showing by propidium iodide fluorescence the total number of nuclei present in this field. (C) Frozen section under polarized light of the same tumor incubated with cis-platinum showing the absence of formazan crystals. (D) Same field as (C) under fluorescent light. (E) Untreated piece of tumor, the control, under polarized light. (F) Control under fluorescent light.
FIG. 2. Composite illustrating the heterogeneous behavior of histocultured human colon tumor to mitomycin-C. (A) Picture of gel-supported tumor in six-well plates after 24 h incubation with mitomycin-C and 2 h with MTT. The arrow indicates the field that is shown magnified 85x in (B). (C and E) Frozen sections of this tumor under polarized light. (D and F) Corresponding fields visualized under fluorescent light.
corresponds to the absence of formazan crystals on the other hand, thus demonstrating that MTT is being reduced only by cells. By measuring the PIAFC and the PIAPI only in the areas where crystals were present, it was possible, using the formula

\[ \text{Inhibition rate} = 100 - \left( \frac{(\text{PIAFC}_{\text{Treated}}/\text{PIAPI}_{\text{Treated}})}{(\text{PIAFC}_{\text{Control}}/\text{PIAPI}_{\text{Control}})} \times 100 \right), \]

to demonstrate that the tumor is not sensitive in vitro to the drug doxorubicin (29 ng/ml) after a 24-h exposure.

Figure 1C shows, under polarized light, a different frozen section of the same tumor incubated with a different drug, 1.5 μg/ml cis-platinum, for 24 h.

In Fig. 1D the same field is visualized under fluorescent light. In this case the PIAFC is very low with respect to PIAPI, so that their ratio divided by the control value is only 0.25%. In this case we considered the tumor to be 99.75% sensitive in vitro to this drug using the above equation.

Figure 1E represents an untreated piece of the colon tumor, the control, visualized under polarized light. Figure 1F shows the same field under fluorescent light. It is important to note that in the control the presence of large amounts of formazan crystals also corresponds to a large number of nuclei.

In Fig. 2A one can observe macroscopically the heterogeneous behavior in the human colon tumor, after an incubation of 2 h with MTT. This tumor fragment was treated for 24 h with 100 ng/ml mitomycin-C. Figure 2B is a particular field of Fig. 2A that shows an 85× magnification of one of the areas where the MTT reduction is present. Even though the total amount of formazan crystal formation in the tumor fragment is very low, the colon tumor after PIAFC/PIAPI measurement was considered quite in vitro resistant to mitomycin-C because of the presence of some areas of very active MTT reduction. Figures 2C and 2E represent frozen sections of the same tumor as that shown in Fig. 2A, under polarized light and with a 170× magnification. Figure 2C shows one area where MTT reduction is absent and Fig. 2E shows one area where MTT reduction is very active. It can be observed in Figs. 2D and 2F that the total number of nuclei is similar for both areas, which is confirmed by measurement of PIAPI. The PIAFC/PIAPI measured in the areas where formazan crystals were observed, was very similar to that obtained in the control. Thus, comparing this measurement with the control value using the above formula, the tumor showed only an 18% sensitivity in vitro, thus indicating resistance of the tumor to the drug mitomycin-C.

Figure 3 summarizes these results graphically, demonstrating the high inhibition rate with cis-platinum and the low inhibition rate with doxorubicin.

Thus our assay demonstrates that the heterogeneous histology of the tissue has a relevant importance in the
evaluation of chemosensitivity. With this new PIAFC technique it is possible to observe the tissue histology while observing the MTT endpoint, thus incorporating the advantages obtained with three-dimensional histoculture and the rapidity, low cost, and ease of the MTT endpoint. The assay described here should make it possible to obtain in a very short time more accurate data on the efficacy of antineoplastic drugs.

REFERENCES