DOSE-RELATED IMMUNOLOGICAL AND MORPHOLOGICAL CHANGES OBSERVED IN RATS WITH WALKER-256 CARCINOSARCOMA AFTER PHOTODYNAMIC THERAPY: A CONTROLLED STUDY

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Experiments were performed on six batches of Wistar infrared rats with Walker-256 carcinosarcoma 7 days post-transplantation. Animals from batches I and II were exposed to photofrin II (21 mg/kg body mass) or HeNe laser (10 mW, 632.8 nm), respectively; the animals in the batches III-V were given photofrin II, intraperitoneally, 24 h before 60-min laser treatment; one, three and six photofrin/laser treatments, respectively, were applied at an interval of 3 days. The control batch (batch VI) consisted of animals presenting with untreated Walker-256 tumours. The results were as follows: photofrin II or HeNe laser alone (photoexposure to low doses of 15 J/cm²) had no significant effects on tumoural volume and the survival of the rats. Photoexposure to multiple doses of PDT led to complete regression of tumoural volume (65.8%); the cure rate was 31.5% and concomitant survival rates increased. Cell-mediated immunity tests (performed at 1 and 26 days post-treatment) underlined superior values in batch IV and V animals photoexposed to multiple PDT doses, in comparison with immunosuppression noticed in batches I-III and the control batch VI. Data presented in this work demonstrate that photodynamic treatment exposure using multiple doses stimulates cell-mediated antitumoural activity, induces modifications in tumoural histological structure, increases survival rates and reduces tumoural incidence in Walker-256 carcinosarcoma in the rat model.

KEY WORDS: Photodynamic therapy, Walker-256 carcinosarcoma, Photofrin II, HeNe laser, Immune response, Concanavalin A

Introduction

Destruction of malignant tumours, previously sensitized with haematoporphyrin derivative, by photodynamic treatment, represents a comparatively new method of tumour therapy, especially in Romania. Haematoporphyrin derivative and photofrin II purified product are complex mixtures of dissolved monomeric porphyrins.¹,² Both preparations, haematoporphyrin and photofrin II, have antitumoural activities³ when activated by visible light. Although haematoporphyrin derivative (HpD) was the first to be used in clinics, photofrin II is more active, both against murine tumours⁴ and human ones.⁵ The high efficacy of photofrin II is determined by the release of biologically inactivated monomeric porphyrins by normal tissues and also by their localization in high amounts within tumoural tissues.¹

Photodynamic therapy (PDT) of malignant tumours includes biological, photochemical and photophysical processes.⁶ These processes involve: (a) absorption of photosensitizing agent; (b) selective retention of the photosensitizer in tumours; (c) irradiation of sensitized tumour by laser radiation.⁶ Although many factors determining the response to PDT have been investigated, there are still areas in PDT biology that need further study. Thus, photoinmuno-logic effects of photodynamic therapy have been studied to some degree, but in other studies cell-mediated anti-tumoural immunity has been systematically investigated after local X-ray irradiation.⁷-¹⁰ In the present work we study histopa-
thological modifications which appeared in Walker-256 carcinosarcoma and immunological reactivity of animals after local exposure to photodynamic therapy.

Materials and Methods

Animals, Tumour and Phototherapy

Animals and Tumour Implantation. Walker-256 carcinosarcoma was received from Oncology Institute, Bucharest, Romania and maintained by serial transplantation in inbred Wistar rats. Large tumours were dissected under sterile conditions, macroscopically viable tumour tissue was minced with scissors and forced through sterile needles of decreasing dimensions. Tumoural suspension (0.2 ml) was subcutaneously injected in the dorsal region of syngeneic animals. Tumours were visible in 7 days after transplant and had a volume between 80–120 mm³. At the start of the study, the animals were 8–10 weeks old, weighing 150–180 g. The animals were from Cantacuzino Institute farms and were kept in subdiet and water ad libitum.

Photosensitizer. Photofrin II (Photofrin Medical Inc. Cheektowage, N.Y.). Rats were intraperitoneally injected with a drug dose of 20 mg/kg body mass. Twenty-four hours post-injection, rats were photoirradiated.

Laser Phototherapy. Our light source was a divergent beam from an LG HeNe Laser (632.8 nm; 10 mW) whose output was checked periodically with a Spectra Physics C power meter.

Experimental Design. Five different modes of treatment were evaluated: Photofrin II only (batch I); HeNe laser only (batch II); PDT with an energy density of 15 J/cm² (batch III); PDT at a dose of 30 J/cm² (3 exposures × 60 min each, batch IV); PDT at a dose of 60 J/cm² (6 × 60 min each, batch V) and the control animals, saline injection, no laser irradiation (batch VI). Fractionated irradiation for animals in batches IV and V was performed at 3 days interval. Each batch included 25 animals. The rats were regularly assessed and tumour size was measured (in mm) along three orthogonal diameters (D₁; +D₂; +D₃), every third day, in the first 2 weeks and then weekly till treatment end (60 days). The volume (V) was calculated assuming spheroid geometry using the following formula:

\[
V = \frac{1}{6} \times D_1 \times D_2 \times D_3 \text{ mm}^3
\]

Immunological Tests

Mitogenic Response of Spleen Cells. Splenocytes from all six batches and also from a seventh control batch consisting of 27 healthy animals (not inoculated with saline or treated with HPD) were fractionated on a Nylon column according to the method of Julius et al., at 7 and 28 days post-treatment. The resultant enriched T-cells (Nylon wool-nonadherent) were cultured at a density of 2.5 × 10⁶ cells/well, in RPMI 1640 medium supplemented with 25 mM HEPES, 2 mM glutamine, 50 μM penicillin G, 50 μg/ml streptomycin and 10% foetal calf serum (Gibco, U.K., Europe). With or without the addition of Con A (Concanavalin A, Pharmacia Fine Chemical AB, Uppsala, Sweden), 5 μg/ml in triplicate wells, the cultures were incubated at 37°C in a CO₂ incubator (5% CO₂ + 95% air) for 48 h; 20 μl aliquots of [³H]-thymidine (spec. act. 20 Ci/mmol IFA, Bucharest, Romania) (5 μCi/ml), in RPMI medium were added to the well. After 48 h cultivation, the cells were harvested on glass filter papers, and washed with 5% trichloroacetic acid, methanol dried and then introduced into vials containing 10 ml of scintillation liquid using 0.4% PPO (2,5-diphenyl-oxazole) and 0.01% POPPOP (1,4-bis-[5-phenylloxazolyl]-benzene) solution in toluene. The radioactivity was read in a Beckman spectrometer.

Cytotoxicity Assay. Effector cells cytotoxicity was assayed by [³H]-uridine method described previously by Nishimura et al., at 7 and 28 days post-treatment. Briefly, aliquots (0.1 ml) of effector cells suspension were mixed with 0.1 ml [³H]-uridine (10 μCi/ml) (spec. act. 30 Ci/mmol, IFA, Bucharest, Romania) labelled target cells (2.5 × 10⁶/0.1 ml) on Coster 96-well round bottom plates at 200:1 effector to target ratio and cultured for 18 h at 37°C. The cells were harvested with the harvester and then the retained radioactivity determined by standard scintillation technique. Target cells lysis was calculated by the following equation:

\[
\text{% cytotoxicity} = \frac{1 - \left[ \text{cpm in culture of effector and target cells} \right]}{\text{cpm culture of target cells alone}} \times 100
\]

Target Cells. YAC-1a Maloney murine leukaemia virus T-cell lymphoma of A strain mice: the line was maintained as suspension culture in RPMI 1640 medium with glutamine (Gibco, Grand Island, N.Y.), supplemented with 10% foetal calf serum.

Cytostatic Activity of Splenic Cells. Cytostatic activity was assayed by the method described by Tsuchiya et al., at different intervals post-transplantation. Splenic cells (2 × 10⁶) were cultured in vitro with 2 × 10⁴ tumour cells (Walker-
256, obtained by trypsinization) in 0.2 ml RPMI 1640 medium, supplemented with 10% fetal calf serum in wells of flat-bottomed microtiter culture plates at 37°C for 72 h in 5% CO₂ atmosphere. Cells were harvested by a cell harvester. Twelve hours before radioactivity determination, in every well, 1.0 μCi tritiated thymidine was added, radioactive precursor incorporation (³H TdR) into tumoural cells being measured by a liquid scintillation counter. Cytostatic activity of splenica cells was expressed as percentage of the inhibition of ³H TdR incorporation into tumoural cells.

For calculation of the percentage inhibition of tumour growth we used the following formula:

\[
\% \text{ inhibition} = \left[ \frac{1\times(cpm \text{ of } tc + \text{ sc from irradi. rats} - cpm \text{ of sc only from irradi. rats})}{cpm \text{ of (tc + norm sc) - cpm norm sc}} \right] \times 100
\]

where tc = tumour cell, sc = splenic cell, irradi. = irradiated, and norm = normal.

**Histology.** For examination of lesions which appeared after PDT treatment, the tumours were removed from animals (from those groups of animals used for the immunological tests) after 10 days of each dose (15, 30 and 60 J/cm²) of PDT and also the control (saline) group; tissue was fixed in buffered formalin (10%), sectioned and stained with haematoxylin-eosin.

**Statistics.** Data were expressed as the arithmetic mean ± the standard error (SE). The statistical significance of differences between groups was calculated by the Kruskal-Wallis test.

**Results**

**Inhibition of Tumour Growth after Local Photodynamic Therapy**

Growth curves for tumoural volumes of all six batches of animals are presented in Figure 1. In control animals (batch VI, saline injection, no PDT), tumoural volume values increased approximately 10 times, after 15 days. Growth was only slightly slower in batch (photofrin II only) and batch II (HeNe laser only). In batch III, animals exposed to photodynamic therapy at a dose of 15 J/cm², we noticed a relatively slower growth of tumoural volume, as compared to control batch VI. At exposure to the higher dosage of PDT (30 J/cm², batch IV), we noticed a slower rate of tumoural volume growth in the first 3 weeks, followed by a marked decrease in the following weeks, but without complete cure of the tumour by the 60-day mark. In contrast, in the batch V animals, treated with multiple PDT doses (6 × 60 min; 60 J/cm²²), we noticed a discrete growth of tumoural volume in the first two weeks only, followed by a complete cessation of growth.

Sixty days post-transplantation, examination of tumoural volume curves suggested two aspects: (a) local treatment with low PDT doses insignificantly influenced tumoural volume growth and (b) animals with photoexposure to multiple doses led to
tumoural volume diminution until a complete cure (cure rate = 31.5%) and also to an increase in the survival rates (Figures 1–3). Photofrin II alone and HeNe laser irradiation alone did not have a statistically significant influence on tumoural volume values and the animal survival rates (Figures 1 and 2).

In conclusion, in animals exposed to the optimal dose (60 J/cm²) of photodynamic therapy, the values of investigated experimental parameters were superior to the values noticed in the other batches. All these facts demonstrate the efficacy of that particular dosage rate of photodynamic therapy in Walker-256 carcinosarcoma.

Changes in the Mitogenic Response of the Host Splenic Cells by Single or Multiple PDT Doses

Cpm mean values, after lymphocyte stimulation with Con A (5 μg/ml), at 7 and 28 days post-PDT treatment, respectively, are presented in Table 1. Examining it we can notice that exposure to multiple doses produced a marked increase of radioactive precursor ³H-TdR incorporation into Con A-stimulated lymphocytes, when splenic cells were cultured at 2.5 × 10⁵ cells/well density; in contrast, after a single exposure to PDT (15 J/cm²) we noticed a significant reduction of mitogen response to Con A.

Twenty-eight days after starting PDT treatment, cpm values in batch V, after Con A stimulation, were superior to those after 7 days, especially in batches IV and V, as compared to control batches (batches I–III and VI).

From the percentage value [³H]-thymidine incorporation into splenic lymphocytes, obtained from photofrin II-injected animals or HeNe laser irradiated (60 J/cm²), was inferior to that noticed in batches IV and V and approximately equal to values in batches III and VI.

Augmentation of Cytotoxic Activity After Photodynamic Therapy. The experiments presented in Table 2 aimed to determine the capacity of photodynamic therapy to stimulate cytotoxic activity of splenic lymphocytes and to lyse target YAC-1 cells.

Results demonstrated that after a single exposure to PDT (15 J/cm²), cytotoxic activity was nonspecifically stimulated. Maximal cytolysis against target YAC-1 cells was noticed in splenic lymphocytes from multiple PDT doses treated animals (batches IV and V). In contrast, i.v. injection of
Table 1. Response of splenic lymphocytes to Con A in animals with Walker-256 carcinosarcoma locally treated with different doses of photodynamic treatment

<table>
<thead>
<tr>
<th>Batch</th>
<th>Treatment (J/cm²)</th>
<th>7 days after treatment</th>
<th>28 days after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Photofrin II</td>
<td>18 962 ± 1876</td>
<td>20 835 ± 1569</td>
</tr>
<tr>
<td>II</td>
<td>He-Ne laser</td>
<td>19 954 ± 1663</td>
<td>23 114 ± 2043</td>
</tr>
<tr>
<td>III</td>
<td>PDT—15</td>
<td>20 139 ± 1589</td>
<td>21 412 ± 2956</td>
</tr>
<tr>
<td>IV</td>
<td>PDT—30</td>
<td>23 746 ± 1983</td>
<td>28 458 ± 3097</td>
</tr>
<tr>
<td>V</td>
<td>PDT—60</td>
<td>27 994 ± 2663</td>
<td>34 724 ± 2688</td>
</tr>
<tr>
<td>VI</td>
<td>Control (saline)</td>
<td>19 365 ± 2365</td>
<td>22 673 ± 1724</td>
</tr>
</tbody>
</table>

*Healthy control animals: 32 965 ± 1146.

Table 2. Dependence of cytotoxic activity of splenic effector cells from animals with Walker-256 carcinosarcoma and dose exposure to photodynamic therapy

<table>
<thead>
<tr>
<th>Batch</th>
<th>Treatment (J/cm²)</th>
<th>7 days after treatment</th>
<th>28 days after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Photofrin II</td>
<td>11.3 ± 0.3</td>
<td>10.4 ± 0.7</td>
</tr>
<tr>
<td>II</td>
<td>He-Ne laser</td>
<td>12.1 ± 0.5</td>
<td>14.7 ± 0.8</td>
</tr>
<tr>
<td>III</td>
<td>PDT—15</td>
<td>13.6 ± 0.4</td>
<td>15.9 ± 0.6</td>
</tr>
<tr>
<td>IV</td>
<td>PDT—30</td>
<td>19.2 ± 0.8</td>
<td>22.5 ± 1.4</td>
</tr>
<tr>
<td>V</td>
<td>PDT—60</td>
<td>24.3 ± 2.1</td>
<td>29.8 ± 2.6</td>
</tr>
<tr>
<td>VI</td>
<td>Control (saline)</td>
<td>11.8 ± 0.5</td>
<td>10.9 ± 0.6</td>
</tr>
</tbody>
</table>

*Splenic cells from Wistar inbred rats with Walker-256 carcinosarcoma were assayed at 7 and 28 days respectively for cytotoxic activity against YAC-1 target cells at a 200:1 effector:target cell ratio.

Healthy control animals: 27.9 ± 1.9.

The results suggest two aspects: (a) PDT local irradiation increases cytotoxic activity against target cells and (b) this activity takes place in a dose-dependent manner. Spontaneous release of the isotope from target cells was less than 12.5% during the test.

Cytostatic Activity of Splenic Cells from Tumour Photodynamic Therapy. In splenic cells from control animals (saline) and those treated with a single PDT dose (15 J/cm², batch III) we noticed an increase of cytostatic activity with a peak within 6–8 days post-transplantation, followed by a decrease nearly up to baseline.

In contrast, in splenic cells isolated from PDT multiple dose-tested animals (batches IV and V), we noticed a marked increase of cytostatic activity (inhibition of radioactive precursor [3H-TdR] incorporation into carcinomatous cells), starting in day 6 and maintaining high values during treatment, as can be seen in Figure 4.

Histopathology. The following morphological modifications were noticed in the different PDT (15–60 J/cm²) treated Walker-256 carcinosarcoma: in samples obtained from control batch VI (saline), we noticed tumoural proliferation with an average of small cells, oval or rounded, presenting nuclei with rich chromatin (hyperchromatic), comparatively reduced cytoplasm, weak basophilia; here and there, there were larger cells with budding or multiple nuclei, presenting characteristic atypia for tumour cells. The cells did not present adhesion, appearing individualized; between cells, spaces could be seen where red cells and proteinform type granular structures were noticed; also individualized blood-dilated or haemorrhaging capillaries in tumoural mass, could be seen. In the tumour mass, we noticed unequal zones of necrosis, leading to the conclusion the tumour was a weakly differentiated tumour of sarcomatoid type (Figure 5).
Figure 4. Change of cytostatic activity of spleen cells after tumour photodynamic therapy. O-O saline-control group; +++ PDT—15 J/cm²; ×-× PDT—30 J/cm²; Δ-Δ PDT—60 J/cm².

Figure 5. Control rat. Histological aspect of Walker-256 carcinosarcoma (not treated). Haematoxylin-eosin staining. × 572

Figure 6 presents a fragment of epidermis and dermis without significant modifications. The sample was obtained from animals with Walker tumours treated with a single PDT dose (15 J/cm²) and sacrificed after 10 days. In the prevalent layer, under the striated muscular tissue, an intricate arrangement of small-sized tumoural cells with numerous capillary neoforation and lympho-fibrocytic reactive elements may be noticed; in other ones the histological picture presents a mixomatous conjunctival tissue, with cells having star-like prolongations, near zones with less oedematous aspect, the cells acquiring a prolonged aspect, some of them being of fibrocytic type and others showing tumoural characteristics in volume, size, and nuclear hyperchromatism: these are typical aspects of fibrosarcoma.

In Figure 7, we present a cutaneous fragment with epidermis and hypodermis, harvested 10 days after last PDT exposure (30 J/cm²); it does not show significant histopathological modifications. At the dermal level, we detect granulation tissue, rich in cells and vascularized, with an intricate arrangement of tumour cells; distinguishing them from reactive elements within stromal reaction process is difficult in certain zones, except the deep peripheral zones near muscular tissue where these tumoural elements appear clear and individualized, having a more prolonged form, and mostly small in size; here and there larger cells appear with bigger and hyperchromatic nuclei. This enabled us to stress the richness of the vasculo-conjunctival reaction layer which determines changes into the cytological aspect of the tumour, but making difficult any identification, except at the peripheral limited zones lacking in reactive granulation tissue.

Samples obtained from batch V animals with 60 J/cm²-treated tumours (Figure 8) showed a segment without significant modifications and tumoural proliferation; under the striated muscular layer there are fusiform, round, oval infiltrates, separated by local oedema; cells with tumoural characteristics are isolated and rare (more varied volume, nuclei with hyperchromasia, atypic). Figure 9 presents a similar image where tumoural infiltrates with small, round cells appear under the striated muscular layer. At
various levels we notice vascular modifications of the obliterating endovascularitis type.

From the same batch (V), we obtained the section seen in Figure 10 showing a cutaneous fragment with normal-appearing epidermis and dermis. Pilose follicles present rate mononuclear, lymphocytic infiltrations. An inflammatory reaction with a lymphocytic component is seen together with fibrocytic elements having conjunctival fibres; also arterioles and venules with endovascularitis processes showing obliterating tendency are present. No tumoural proliferation is detected.

Discussion

In the present work and in previous ones\(^{13,14}\) we have shown that photodynamic treatment reduces mortality, volume and incidence of tumour. The maximal period of 2 weeks (7–21 days post-transplantation) was chosen because we had noticed, in previous studies, a significant response of cell-mediated antitumoural immune system\(^{15}\) within this time.

The values of the experimental parameters under study, during 53 days from the start of photodynamic treatment, amplified the variation of the immune
response between the different batches, due to each treatment type.

Experimental results showed that repeated exposure to photodynamic therapy led to stimulation and maintenance of high values of cell-mediated antitumoural activity for 28 days post-treatment (Tables 1 and 2 and Figure 4). In contrast, after a single photofrin II treatment or HeNe laser exposure, a significant increase of cell-mediated antitumoural immunity was not detected (Tables 1 and 2).

For the appearance of these beneficial effects, we could provide several explanations: (a) increase of cytotoxic lymphoid cells antitumoural activity; (b) increase in cytotoxic activity during a relatively long period (6–10 days), in such a manner that cytotoxic activity would contribute to elimination of tumoural cells, which aspect is relevant in our experimental design; (c) photodynamic treatment increased activity of lymphocytes and of other nonirradiated regions (liver, lung, blood; experiments in progress).

In support of our results, we can mention data provided by other researchers, showing that tumoural cell survival is strongly inhibited by host immune response after local irradiation.16 Host cell-mentioned antitumoural immunity was demonstrated to be induced or increased by local irradiation.
of the tumour and also splenic cells of such animals were proved to be capable of inhibition of the growth of tumoural cells. Recently, it been suggested that cell-mediated antitumoural immunity could depend on cooperation between non-killer T-cells and macrophages.

Our experimental observations are in agreement with works of some authors, sustaining the appearance of an increased immune response 7 or more days after local irradiation, but are contradicted by other authors who indicate such an increase in only a single day after irradiation. These discrepancies could be explained by different methods used for immunological activity evaluation.

At present, we do not know why cell-mediated antitumoural specific activity increases after tumour exposure to multiple PDT doses. It was suggested that it could be induced by modifications of surface tumoural antigens after irradiation or because of alterations in suppressor-effector T-cells ratio, as in the case of surgical excised tumours.

Histopathological modifications noticed 10 days after exposure to singular or multiple PDT doses are in full agreement with other authors' observations referring to possible mechanisms that could act in photodynamic therapy. Among the most important mechanisms we emphasize: (a) direct effects on malignant cells induced by oxygen presence as single oxygen, (b) superoxide production; (c) ischemia resulting from vascular lesions which could be the main cause of tumour destruction during photodynamic therapy; (d) potentiation of cell-mediated antitumoural immune response after photoexposure to multiple doses of PDT.

Data presented in this work demonstrate that exposure to local photodynamic treatment in multiple doses stimulates cell-mediated antitumoural activity, produces modifications in tumour histological structure, increases survival rate and reduces tumoural incidence in Walker-256 carcinosarcoma in the rat model.

References


