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Evaluation of response to chemotherapy in retinoblastoma heterotransplanted to the eyes of nude mice

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Summary. The role of chemotherapy in the treatment of retinoblastoma (RB) is unsatisfactory and clinical research is severely limited. A xenograft model for testing chemotherapeutic and other agents has been developed by the heterotransplantation of human RB cells into the anterior chamber of the nude mouse eye. A grading system for visually monitored tumor growth was designed to allow serial observations and documentation of the response to therapy in the model. This method of monitoring compared favorably with histopathologic, photographic, or other criteria in the reproducible, sequential evaluation of tumor status. Six chemotherapeutic agents [vincristine (VCR), doxorubicin (DOX), actinomycin D (ACT-D), dimethyltriazeno-imidazole carboxamide (DTIC), cyclosphosphamide (CPM), and diaziquone (AZQ)] were then tested in the model against a patient-derived xenograft line. Results were expressed as the delay in tumor progression judged by serial grading. CPM produced a consistent response in all treated tumors, as did DTIC to a lesser, more variable extent. In 3 of 10 tumors treated with CPM and in 1 of 18 treated with DTIC, complete responses were maintained for at least 20 weeks. VCR, DOX, and ACT-D were ineffective, producing patterns of tumor progression no different from those in the control group. AZQ was most effective, producing responses far exceeding those of conventional agents. The model allows quantitative documentation of the response to therapy in heterotransplanted human RB. Further testing of new agents and combinations is warranted. AZQ may be active against RB.

Introduction

The contribution of chemotherapy in the treatment of retinoblastoma (RB) has been difficult to evaluate in the clinical setting, and the modality remains of doubtful benefit [12]. Although the mortality and, in particular, morbidity of current treatment would be improved by the availability of effective chemotherapy, clinical research has been unable to design reliable and reproducible regimens [12]. The ethical and biologic obstacles to clinical research in the settings of (a) local, (b) micrometastatic (adjuvant), and (c) relapsed disease have previously been reviewed [12]. Therefore, experimental in vitro and in vivo systems have been sought to enable appropriate evaluations of chemotherapy in RB.

Gallie et al. [6] reported a successful model for the heterotransplantation of RB into the anterior chamber of the nude mouse eye, the culmination of 10 years of research into potential in vivo systems. The methodology was reproducible in a majority of fresh tumor explants as well as established RB cell lines [6]. In subsequent reports, the requirements for and characteristics of successful tumor growth were delineated and a response to therapeutic agents was suggested [7]. In particular, the effectiveness of radiation therapy and radiosensitizing agents was evaluated by the in vitro colony-forming ability of cells harvested after treatment [8].

The model was reproduced by Benedict et al. [1] at the Children's Hospital of Los Angeles, where a large number of in vivo cell lines have been established by this method. The histologic and karyotypic consistency of the heterotransplanted and serially passaged tumors to the original patient material was emphasized. A response to photoradiation was demonstrated by the evaluation of histologic criteria for tumor necrosis in treated and control eyes [2].

The experiments reported herein aim to test the reproducibility of the model, ascertain the most appropriate method for evaluation and monitoring of response, document the relationship between histologic and visually observed criteria, and apply the model to the testing of a number of potentially useful chemotherapeutic agents.

Materials and methods

Experimental animals. Swiss background nude mice were bred and maintained in a protected and controlled environment [1]. Laminar air-flow shelving units and work hoods minimized the risk of infection. Sterile feed and water were provided ad libidum and the cages were changed and sterilized weekly. Anesthesia for potentially painful procedures and ethical aspects of animal care were carried out in accordance with institutional regulations. Mice were killed by anesthetic overdose when they showed signs of poor health or persisted in losing weight, when tumors showed evidence of perforation, or when the experimental endpoints had been reached.

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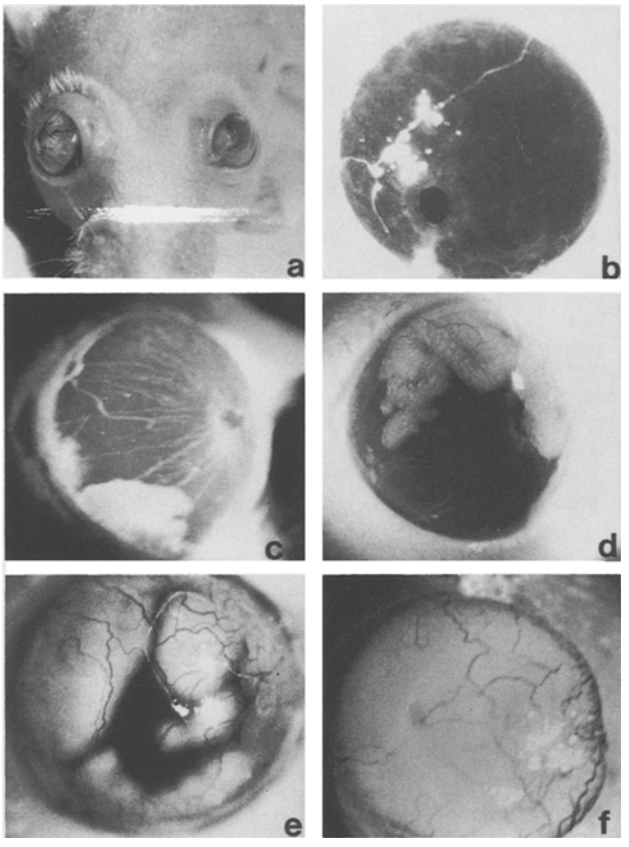

Fig. 1. Heterotransplanted human retinoblastoma in the anterior chamber of the nude mouse eye: (a) bilateral tumour growth, (b) normal eye (with light reflection artefact), (c) grade 1 tumour, (d) grade 2 tumour, (e) grade 3 tumour, (f) grade 4 tumour.

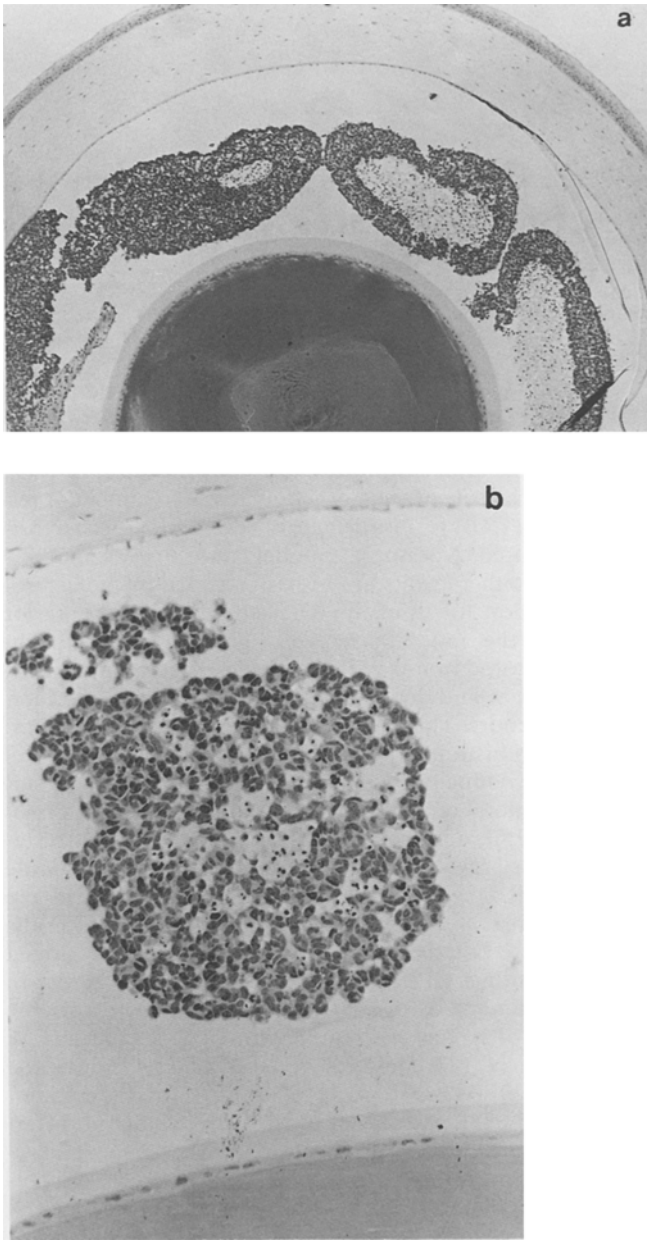

Fig. 2. Cross-sectional photomicrographs demonstrating tumour growth in the anterior chamber of the nude mouse eye (lens *below*; cornea *above*): (a) multilobular pattern filling a majority of the chamber (grade 3–4 tumour), (b) single lobule showing cellular morphology (H&E stain)

Tumor source and heterotransplantation. Tumors were originally obtained from the enucleated eyes of children with RB and maintained by serial passaging in the nude mouse intraocular model [1]. For the passage of tumors, the mouse eyes were enucleated, washed, and then opened in a sterile dish using the tips of 19-gauge needles. A suspension of tumor cells was obtained by teasing with scalpel blades or large (19-gauge) needles and agitation in RPMI-1640 culture medium. Cells often remained in clumps of two to five, and the estimated count per milliliter was between 1×10^6 and 5×10^7 cells. These cells were washed and resuspended in culture medium with 100 IU/ml penicillin and 100 μg/ml streptomycin.

The nude mice were anesthetized with 40 mg/kg i.p. pentobarbital. Under a dissecting microscope, the RB cell suspension was injected directly into the anterior chamber of the mouse eye with a 30-gauge needle on a tuberculin syringe, using digital pressure. Some of the cell suspension would reflux through the perforation created by the needle and an estimated 4–8 ml fluid were retained in the anterior chamber. Therefore, the retained cell number was estimated to be between 4,000 and 400,000, depending on the variations in the eye anatomy, technique, and cell concentrations.

Experiment 1. A total of 32 mice were injected bilaterally with patient-derived RB cells (LARB-72) in their 4th passage. The mice were monitored weekly and their eyes examined under a dissecting microscope, using light ether anesthesia. In all, 27 mice produced either unilateral or bilateral tumor growth, resulting in a total of 40 evaluable tumors; 3 mice (5 tumors) were put under observation as control animals and the remaining 24 (35 tumors) were assigned to one of three treatment groups. Cyclophosphamide (CPM) was given to each of the three groups as a single i.p. injection at intervals of 3 weeks, repeated up to 4 times, depending on the degree of weight loss and other toxicity in the animals. Treatment was ceased when a weight loss in excess of 10% lasting for 2 consecutive weeks was noted.

Treatment group 1, consisting of 10 tumors in 7 mice, was given 250 mg/kg CPM; group 2 (12 tumors in 9 mice)

Table 1. Treatment groups and results in experiment 2

Chemotherapy						Time (weeks) to grade 4	
Group	Agent	Dose	Regimen		Tumours treated (*n*)	Mean	SD
1	Saline control	0.1 ml	weekly	5 doses	11	2.3	0.9
2	Vincristine	1 mg/kg	every 2 weeks	3 doses	10	4.3	2.2
3	Doxorubicin	3 mg/kg	every 2 weeks	3 doses	9	2.7	0.7
4	Actinomycin D	0.3 mg/kg	every 2 weeks	3 doses	12	3.3	1.1
5	D.T.I.C.	200 mg/kg	weekly	5 doses	18	6.7[a]	3.1
6	Cyclophosphamide	100 mg/kg	weekly	5 doses	10	13.9[b]	3.0
7	Diaziquone	2 mg/kg	daily	5 doses	9	8.4	1.5
8	Diaziquone	10 mg/kg	weekly	5 doses	7	19.0[c]	–
9	Diaziquone	10 mg/kg	every 2 weeks	3 doses	8	15.3	1.4

[a] One tumour, no regrowth; mean calculated from $n = 17$
[b] Three tumours, no regrowth; mean calculated from $n = 7$
[c] Six tumours, no regrowth; one tumour regrowth to grade 4 in 19 weeks

was given 200 mg/kg; and group 3 (13 tumors in 8 mice) was given 150 mg/kg. The degree of tumor growth in the anterior chamber at the start of the therapy was varied in each of the three groups to allow a spectrum of observations and monitoring data. The weekly observation and recording of tumor status was complemented by serial macrophotography according to methods described elsewhere [11] (Fig. 1). A total of 14 mice (19 tumors) were sacrificed at intervals, either before or after a dose of chemotherapy, to ascertain their histopathologic status at different stages of growth and in relationship to the treatment (Fig. 2). Thereby, four mice (six tumors) from group 1, six mice (seven tumors) from group 2, and four mice (six tumors) from group 3 were histopathologically evaluated and the findings were correlated with visual and photographic observations. The remaining three mice (four tumors) in group 1, three mice (five tumors) in group 2, and four mice (seven tumors) in group 3 were followed undisturbed after treatment was completed to monitor long-term tumor outcome and sequelae of therapy.

A grading system for the visual observation of tumor size was designed, in which the proportion of the anterior chamber occupied by tumor was expressed as grade 1, 2, 3, or 4 (Fig. 1). As the volume of the anterior chamber is estimated to be 4–8 µl, a grade 1 tumor occupying one-quarter of that space is expected to have a volume of 1–2 µl. Grade 2 (one-half) is 2–4 µl, grade 3 (three-quarters) is 3–6 µl and grade 4 is 4–8 µl or more due to expansion of the tumor. In practice, the monitoring system allows for eight grading categories, as the use of additional, intermediate grades in sequential observations was found to be optimal (i.e., grades ½, 1, 1½, 2, 2½, 3, 3½, and 4).

Experiment 2. In this study 68 nude mice (136 eyes) were injected intraocularly with human RB cells. The tumor cells (LARB-69, seventh passage) were derived from an enucleated tumor and maintained in the nude mouse model. The mice were examined weekly using light ether anesthesia, and tumor growth was monitored and graded by direct observation under a dissecting microscope.

Of 68 mice injected bilaterally, 65 produced a total of 94 evaluable tumors (in 29 mice, bilaterally simultaneous) and were randomized to one of nine treatment groups (Table 1). Treatment was begun at grade 1 or 2 tumor growth, and by subsequent weekly grading of tumors, the time interval required for progression to grade 4 was documented in each treated eye. The experiment was continued for a total of 20 weeks from the start of the therapy.

All agents were given in i.p. injections, as follows (Table 1): group 1 – control, 0.1 ml normal saline weekly, 5 doses ($n = 11$); group 2 – 1 mg/kg vincristine (VCR) every 2 weeks, 3 doses ($n = 10$); group 3 – 3 mg/kg doxorubicin (DOX) every 2 weeks, 3 doses ($n = 9$); group 4 – 0.3 mg/kg actinomycin D (ACT-D) every 2 weeks, 3 doses ($n = 12$); group 5 – 200 mg/kg dimethyltriazeno-imidazole carboxamide (DTIC) weekly, 5 doses ($n = 18$); group 6 – 100 mg/kg CPM, weekly, 5 doses ($n = 10$); group 7 – 2 mg/kg diaziquone (AZQ) daily, 5 doses ($n = 9$); group 8 – 10 mg/kg AZQ weekly, 5 doses ($n = 7$); group 9 – 10 mg/kg AZQ every 2 weeks, 3 doses ($n = 8$).

Results

Experiment 1

All mice treated with CPM showed varying degrees of tumor response. Both the size of individual dosages and the number of doses given were found to correlate positively with tumor response. Toxicity limited group 1 (250 mg/kg) doses to a maximum of three, at which time weight loss became irreversible in two of three mice followed to that point. The other groups could be treated with a maximum of four doses, and no toxic deaths were recorded. Furthermore, there were no other sequelae of therapy, and the unsacrificed mice lived at least 20 weeks from the start of the experiment, at which time the observations ceased.

Control mice showed tumor progression, filling the anterior chamber as well as progressing posteriorly, leading to perforation in four of the five evaluable tumors. Histological examinations confirmed extensive tumor invasion and necrosis at this stage. A majority of treated, responsive tumors regrew and subsequently proceeded to fill the eye in the same manner as those in control animals. In a total of three mice (four tumors), no tumor regrowth had been observed by the end of the experiment (20 weeks). These mice had received CPM as follows: (a) 250 mg/kg, three doses (group 1) in one mouse (two tumors); (b) 200 mg/kg, four doses (group 2) in one mouse (one tumor);

(c)150 mg/kg, four doses (group 3) in one mouse (one tumor).

The comparison of recorded weekly grades with photographic documentation revealed no advantage for the latter, and a high degree of concordance between individual observers was achieved. The histologic findings with respect to relative tumor grade were found to correlate strongly with visual observations (Fig. 2). Therefore, the proportion of the anterior chamber occupied by tumor on histologic analysis of multisectional cuts was consistent with the visually predicted grading of tumor size. However, attempts to evaluate the response to therapy by the degree of cell necrosis were both unreliable and unnecessary.

Experiment 2

Detailed results of experiment 2 are presented in Table 1. Control tumors as well as those treated with DOX, VCR, or ACT-D progressed rapidly to grade 4. The usual pattern in responsive tumors was one of regression beginning approximately 1 week after the first dose of drug, followed by a period of maintained remission and subsequent regrowth. In the case of DTIC, tumor growth was moderately retarded, with one tumor remaining in complete remission at the end of the observation period (20 weeks). CPM produced a most remarkable retardation of tumor growth, with three of ten tumors remaining in complete remission at the end of the observation period. However, the best results were obtained with AZQ: all three regimens (groups 7, 8, and 9; Table 1) were effective, and the mice that tolerated five weekly doses (group 8) showed complete, maintained remissions in six of seven tumors (20 weeks).

Discussion

In experiment 1, the optimal method for monitoring therapy in the intraocular RB xenograft model was established. We had anticipated that a potographic record would be necessary to show serial changes in tumor size reproducibly and accurately [11]. Using one of three treatment regimens with an effective chemotherapeutic agent, four methods of tumor-response monitoring were examined and compared:

1. Photographic documentation, while useful for presentations and publication, was not found to be necessary for reliable, reproducible tumor monitoring (Fig. 1).
2. Histopathologic analysis of treated and nontreated eyes revealed no information in addition to that obtained by visual observation, but it may be useful for occasional documentation (Fig. 2). Importantly, visually determined tumor grades were confirmed on histologic sections.
3. The time interval to perforation of the globe by tumor growth was found to be variable and was also considered to be an unacceptable endpoint on ethical grounds.
4. However, sequential visual monitoring under a dissecting microscope using a grading system of tumor growth was found to be reliable, reproducible, noninvasive, humane, and relatively easy. Furthermore, multiple and/or long-term chemotherapeutic trials are possible using this model and methodology.

In experiment 2, the comparison of currently available and often used chemotherapeutic agents provided useful information (Table 1). The chosen endpoint was visually documented progression to grade 4 tumor, in accordance with the findings of experiment 1. The model allows long-term monitoring of single or repeated chemotherapy, with reproducible results. As expected, CPM produced the most remarkable responses, and DTIC was moderately effective. The results with VCR, DOX, and ACT-D did not significantly differ from those of the control group. CPM is clearly the most effective single chemotherapeutic agent for retinoblastoma in clinical studies and reports [9, 12, 17]. Other experiments using our model have further confirmed that status [16]. VCR is the second most frequently used agent in the clinical setting; our data shows a trend suggesting tumor responsiveness, but no significantly delayed tumor regression was documented. Although VCR and DOX are often included in chemotherapeutic regimens for the treatment of RB [12, 14], a review of the clinical literature, particularly early phase-studies, suggest these to be relatively ineffective as single agents [9, 10, 12]. Preliminary data with s.c. xenografted RB cell lines (unpublished data) supports the clinical experience and findings of experiment 2.

AZQ is an experimental agent with expected CNS penetration [3–5], of potential clinical benefit in both local and disseminated RB. Our results would indicate an important role for this agent, worthy of further evaluation in both the model and clinical studies.

Each regimen of drug administration in experiment 2 was determined after pilot toxicity studies. The aim was to repeat a tolerable and effective single dose between three and five times over a period of 5 weeks to achieve the highest theoretical cytoxicity within the limits of animal tolerance. Weight loss exceeding 10% of the original weight or abnormal clinical signs in the mouse were considered to be the tolerable limits of toxicity. We anticipated that in these as well as future experiments the treatments would be given as one or more courses consisting of several doses of the respective drug. This strategy most closely resembles clinical therapy and also lowers the number of mice required.

The capacity to evaluate and monitor small tumor masses in this model may correlate with expected responses in clinical micrometastases. This method of heterotransplantation has been and will continue to be pursued for neoplasia other than RB [13]. The rate of engraftment for RB exceeds 80%; however, much lower rates have been observed in other malignancies. Several strategies for improving the heterotransplantability of all neoplasia are being developed.

The heterotransplantation of human RB into the anterior chamber of nude mice and the established therapeutic model produces responses that correlate with currently available clinical data [12]. Therefore, it will be possible to evaluate combinations of agents, or new, more effective drugs to improve their chemotherapeutic efficacy in this disease. Admittedly, the anterior chamber is not the natural place for such tumor growth in humans. Furthermore, the lack of vascularity of the model and dependence on the pharmacokinetics of the anterior chamber may impose certain limitations. A number of experiments are under way to ascertain the penetration and retention of various agents in this model. Current data indicate adequate drug access to the tumor cells [15, 16]. Subcutaneous (s.c.) xenografts from RB cell lines also respond to chemotherapy in a pattern similar to that of the intraocular model, but s.c. engraftment is unpredictable and particularly unrewarding from direct human RB explants.

As the results of the present experiments are so encouraging, further projects are under way to test other agents, modalities, combinations, and novel approaches in the nonsurgical management of RB. In particular, the agent AZQ is being tested in a variety of RB xenografts. Furthermore, correlations are being sought with data from recently improved clonogenic assays using established cell lines. We anticipate that the results will influence the treatment of local as well as regionally or systemically disseminated RB.

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