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Comparative Growth of Human Tumors in Pharmacologically Immunosuppressed, Immune-deprived, Cyclosporin A-treated and Nude Mice*

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Abstract—*The growth of three human tumor xenografts, namely an Ewing sarcoma, a colon carcinoma and an osteosarcoma, was compared in nude and conditioned mice. Conditioning protocols included* (1) *immune deprivation (thymectomy, lethal irradiation and cytosine arabinoside pretreatment);* (2) *immunosuppression with procarbazine, cyclophosphamide and antithymocyte serum; and* (3) *continuous administration of cyclosporin A. Similar tumor growth was seen in nude mice, immune-deprived mice and mice treated with the medium and high-dose immunosuppressive protocol. Cyclosporin A allowed only very modest tumor growth. In the main comparative experiment with the Ewing sarcoma and the colon carcinoma, overall survival was lowest with nude mice* (17%), *higher with immune-deprived mice* (61%) *and best with immunosuppressed mice* (81 *and* 87%). *For the screening of anticancer agents the immunosuppressive protocol consisting of synergistic procarbazine, cyclophosphamide and antithymocyte serum may be added to the already available models. It allows adequate tumor growth with good animal survival and does not require operative procedures and irradiation.*

INTRODUCTION

HUMAN tumor xenograft models may be useful in studying metabolism and biological characteristics of tumor cells and in testing the therapeutic susceptibility of human cancers. In this paper we do not propose to discuss whether human tumor xenografts are more promising models for screening anticancer drugs[1–4] than experimental rodent tumors or *in vitro* test systems, but we want to *compare* the diverse possibilities of growing human tumors *in vivo*. Congenitally athymic 'nude' mice are used successfully for this purpose [5–7]. However, they are expensive, not always readily available and susceptible to infection by pathogenic microorganisms and thus early death. With conventional mice the xenograft barrier has to be overcome. If they are made immunodeficient by thymectomy and total body irradiation (TBI) combined with syngeneic bone marrow transplantation or cytosine arabinoside (ARA-C) pretreatment[5], tumor xenografts are accepted and antitumor agents can be assessed[6–12], but the technical requirements of this 'immune deprivation' conditioning regimen (hereafter designed as ID) may compromise its use for large-scale screening procedures. Recently, short-term pregraft immunosuppression with a synergistic protocol consisting of procarbazine hydrochloride (PCH), cyclophosphamide (CY) and antithymocyte serum (ATS) has been introduced (hereafter designed as IS) and shown to permit the take of human tumor xenografts in conventional mice[13]. Finally, the newly discovered agent cyclosporin A, displaying potent immunosuppressive effects against the allograft response in animals[14] and man[15], could be expected to be useful for tumor xenografting.

In the present study we have compared nude mice and the three conditioning regimens with regard to their capacity to support the growth of human tumors and to allow survival for tumor evaluation.

MATERIALS AND METHODS

Mice

The experiments were carried out with male

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C3H mice (Gl. Bomholtgaard, 8680 Ry, Denmark), weighing 18–23 g and fed a commercial pellet diet and tap water *ad libitum*. For the thymectomies, 3 to 4-week-old male C3H mice were used.

Nude mice of similar weight and of Swiss mice genetic background were obtained from Iffa Credo (Lyon, France). They were kept under SPF precautions in conventional boxes and received sterilized water to which 10 mg polymyxin/l and 100 mg neomycin/l had been added.

Drugs

Cytosine arabinoside (ARA-C; Alexan®) was dissolved in distilled water and injected by the intraperitoneal route in 0.1 ml/10 g body weight. Procarbazine hydrochloride (PCH; Natulan®; courtesy Dr. W. Bollag, F. Hoffman-La Roche & Co. AG, Basel) and cyclophosphamide (CY; Endoxan®; courtesy Max Ritter AG, Zurich) were dissolved and applied similarly. Cyclosporin A (CyA) was a gift from Dr. J. F. Borel (Sandoz AG, Basel) and was injected subcutaneously in 0.05 ml/10 g. Its solvent, Miglyol 812 (Dynamit Nobel), was also injected subcutaneously at the dose 0.05 ml/10 g in the controls. Rabbit anti-mouse thymocyte serum (ATS, Lot 40295; Microbiological Associates, Biggs Fort Road, Walkersville, MD 21793, U.S.A.) was purchased from Dynatech AG (Zurich) and administered by the subcutaneous route. Its activity was ascertained by grafting BALB/c skin to C3H hosts. With two doses of 0.5 ml, applied subcutaneously on days 0 and 5, the median survival time of the skin grafts amounted to 26.5 ± 6.1 days as compared to 9.3 ± 2.1 days in the controls.

Conditioning protocols

Immune deprivation (ID) with ARA-C protection was performed according to the technique of Steel *et al.*[5]. In short, 3 to 4-week-old C3H mice were thymectomized under anesthesia with nembutal by sucking out the two thymic lobes after a suprasternal incision through a glass tube connected to a vacuum pump. The skin was closed with a Michel clip. The postoperative mortality was 33% in our hands. Four weeks later the thymectomized mice were given 900 rad total body irradiation (TBI) from a Linac 8 X-ray machine at a dose rate of 220 rad/min. Two days before the irradiation the mice had been treated with a intraperitoneal injection of 200 mg/kg of ARA-C. For untreated C3H male mice, 900 rad TBI were always lethal. The completeness of the thymectomy was always verified at autopsy.

Synergistic immunosuppression with PCH, CY and ATS (IS) was carried out as reported earlier[13, 16]. The mice were injected by the subcutaneous route on days -3 and -1 with PCH and CY at different sites. On days -2 and 0 (day 0 = day of tumor transplantation) the mice received 0.15 ml/10 g ATS, also subcutaneously. PCH and CY were applied at three dose levels: low (90 mg/kg PCH, 30 mg/kg CY), medium (135 mg/kg PCH, 45 mg/kg CY) or high (180 mg/kg PCH, 60 mg/kg CY).

No postgraft treatment was used in either the IS or ID protocols. It is therefore unlikely that any test drugs used for the screening of anticancer activity are affected by pharmacological interactions.

CyA was injected daily from day -1 to day 30 in 0.05 ml/10 g at two dosages, 50 and 100 mg/kg.

The solvent of CyA, Miglyol 812, was given similarly at a dose of 0.05 ml/10 g.

Tumor specimens

The three human tumors used, namely an Ewing sarcoma, a colonic carcinoma and an osteosarcoma, have been characterized before[13]. They had been obtained by surgery and were maintained by serial passage in nude mice without evidence of morphological change as ascertained by microscopy[13]. For our experiments, tumors were excised from the nude donors, placed in tissue culture medium TC 199, cut into small (1–2 mm) fragments and grafted subcutaneously by trocar over the posterior rib cage on one side of the animal. The implantations were performed six hours after the completion of the irradiation or the administration of the last dose of ATS, CyA or Miglyol.

Tumor assessment

The tumor size was followed in all mice first twice, then after one month, once, weekly by measuring the three largest perpendicular diameters and calculating the product (in mm^3) of these three diameters.

The presence of viable tumor cells in growing tumors at 14 days after grafting and subsequently has been confirmed histologically[13,16]. With regressing tumors or with tumors which failed to grow satisfactorily *ab initio*, it cannot be ruled out that small ($< 100\ mm^3$) nodes contained no viable tumor cells but only necrotic remnants within scar or granulation tissue. However, the mean values for the tumor volumes per group as presented in the text-figures undoubtedly reflect the overall tumor growth realistically.

Survival

The survival of all xenografted mice was scored daily up to conclusion of the experiment on day 60.

RESULTS

Figure 1 depicts the growth of a human osteosarcoma grafted either to nude mice or to IS mice (low dose protocol). The osteosarcoma reached only a smaller size in the IS mice. This may be due to the use of the lower dose immunosuppressive protocol and also to the fact that this tumor grew in the IS mice in a more differentiated fashion, forming extensive osteoid and ground substance as contrasted to the highly cellular anaplastic tumor in the nudes[13]. The more abundant growth in the nudes was, however, compromised by the death of all animals by day 62. In contrast, no fatalities occurred with the IS mice. Six out of the 10 nude mice succumbed with tumors whose volumes were below 1000 m^3.

Similar results as above were seen when the growth of the Ewing sarcoma in nude and in low dose immunosuppressed mice was compared (Fig. 2). With a less conspicuous difference in tumor size, again all nude hosts were dead by day 55, when the average tumor volume in the IS mice had just reached its maximum. The fatalities in the nude mice occurred again without a clear relationship to the tumor volume. Three out of ten nude mice succumbed with tumors $< 1000\ mm^3$, four with tumors between 1000 and 2000 m^3, and three with tumors $> 2000\ mm^3$. No fatalities occurred in the IS panel.

In the experiments illustrated in Figs 3 and 4 the growth of the colon carcinoma and the Ewing sarcoma was compared not only in nude and IS mice, but also in ID mice. With the use of the medium and high dose IS protocol, both tumors reached approximately similar sizes as in nude or ID mice. Successfully growing tumors displayed a phase of rapid enlargement up to approximately day 30. No significant growth was provided by treatment with CyA.

Thus, nude, ID and IS mice demonstrate all satisfactory and comparable tumor growth. However, if survival is followed (Table 1), nude mice display the highest lethality, with only 17% surviving at day 60 as compared to 61% of

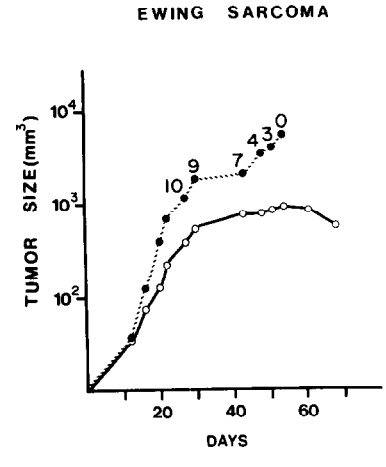

Fig. 2. Twelve C3H mice pretreated with the low dose immunosuppressive protocol and 10 nude mice were grafted on day 0 subcutaneously with the Ewing sarcoma. Ciphers indicate the number of surviving mice. O—O: *C3H*; ●\\\\\●: *nudes.*

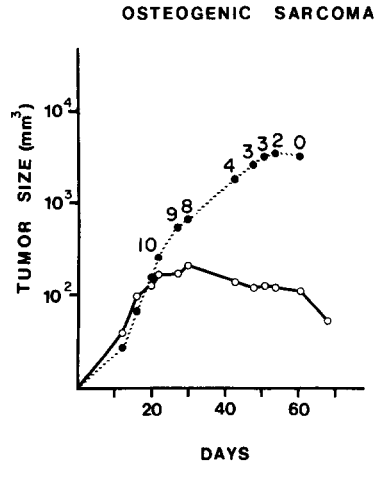

Fig. 1. Twelve C3H *mice pretreated with the low dose immunosuppressive protocol and 10 nude mice were grafted on day 0 subcutaneously with the osteosarcoma. Ciphers indicate the number of surviving mice.* O—O: *C3H*; ●\\\\\●: *nudes.*

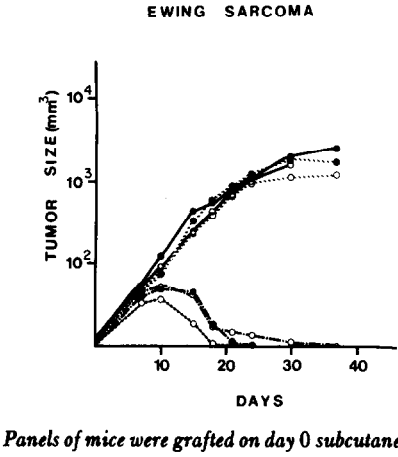

Fig. 3. Panels of mice were grafted on day 0 subcutaneously with the Ewing sarcoma. Ciphers in brackets indicate the number of mice in each panel. All nudes were dead by day 30. O—O: *Nudes* (10); ●—●: *immune deprived* (8); ●\\\\\●: *medium dose immunosuppressed* (8); O\\\\\O: *high dose immunosuppressed* (8); O——O: *CyA*, 100 mg/kg (8); ●——●: *CyA*, 50 mg/kg (8); O◆◆O: *Miglyol* 812 (6).

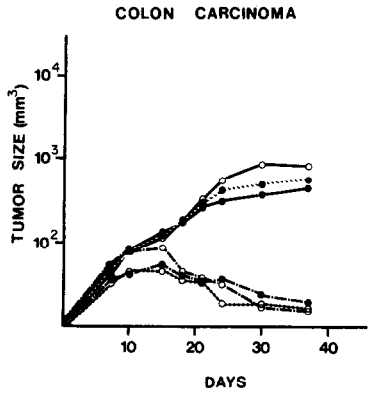

Fig. 4. Panels of mice were grafted on day 0 subcutaneously with the colon carcinoma. Ciphers in brackets indicate the numbers of mice comprised in each panel. O—O: *Nudes* (10); ●—●: *immune deprived* (8); ●\ \ \●: *medium dose immunosuppressed* (8); O—·—O: *CyA*, 100 mg/kg (8); ●——●: *CyA*, 50 mg/kg (8); O◆◆◆O: *Miglyol* 812 (6).

the ID and 81% (high dose) or 87% (medium dose) of the IS mice. Since the average tumor size was not significantly different in all three groups, the differences in survival cannot be related to the tumor volumes. The ratio of mice surviving by day 60 and still carrying tumors larger than 100 mm³ was highest in the ID mice (70%) and in the high dose IS mice (69%). With the medium dose IS protocol, 43% of the mice still supported tumor nodes at this time.

DISCUSSION

Our experiments show the growth of the three used human tumors in nude, ID and IS mice. In nude mice tumors grew well and progressively. The rate of only 50% of the nude mice carrying tumors at the completion of the experiment is due to the fact that three of the six nude survivors (from an initial 36 mice) had failed to display initially satisfactory tumor takes.

Tumors grew equally well in ID and IS mice, with only a few tumors failing to take and grow progressively but with a moderate number of regressions with both protocols in the course of the experiment. After day 40 the regression was slightly more pronounced in the IS groups as compared to the ID panel. It had been found previously that the medium dose IS protocol allowed better tumor growth than the low dose regimen[13]. In the present experiment, the high dose IS did not secure a more abundant tumor growth than the medium dose protocol (Fig. 3).

The reason for the efficacy of the IS protocol has been related[13] to the fact that ATS acts predominantly on lymphocytes in the circulation, whereas PCH causes aplasia of lymphoid organs, particularly of the thymus. Being active before the antigenic challenge, both agents may synergistically deplete cells or their precursors that are involved in the initial phase of the reaction to antigen. CY may add an effect on rapidly proliferating B- and T-cells and contribute, with PCH, to the depression of the antibody formation against ATS.

Cyclosporin A, which was applied at up to 100 mg/kg continuously for 30 days [which is close to the highest tolerated dose (J. F. Borel, personal communication)], permitted only minimal tumor development. Further studies must determine whether this otherwise potent

Table 1. Survival of conventional mice made immunodeficient by various means and of nude mice grafted on day 0 with an Ewing sarcoma or a colonic carcinoma

Conditioning of recipient mice	No. of mice	Percentage of mice surviving on day 10	20	30	40	60	Percentage of the survivors on day 60 with tumors > 100 mm³
Thymectomy, ARA-C, TBI (ID)	38*	82	68	68	66	61	70
PCH, CY, ATA (IS)†	16	100	100	100	94	87	43
PCH, CY, ATS (IS)‡	16	100	94	87	87	81	69
CyA, 50 mg/kg/day§	31	100	100	100	100	100	0
CyA, 100 mg/kg/day§	31	100	100	100	100	100	0
Miglyol (solvent)§	17	100	94	94	94	94	0
Nude mice	36	97	97	81	36	17	50

*Survivors of 58 thymectomized mice.
†Medium dose IS protocol.
‡High dose IS protocol.
§Applied daily from day −1 to day 30.

immunosuppressant also fails, more generally, to affect the response to xenografts, or whether it displays an antitumor effect.

With regard to the practical usefulness of the described protocols for the *in vivo* screening of anticancer agents, an important question is at what cost of lethality the regimens can be applied. The table shows that the highest survival of 80–90% was achieved with the IS protocols. The reduced survival seen with the ID group was due to initial mortality of mice not recovering from the lethal TBI in spite of ARA-C protection.

The highest lethality, allowing only 17% of the tumor recipients to survive the end of the experiment, was observed with the nude mice. It is likely that some animals became cachectic by virtue of the large tumors which they were bearing. Since the IS and ID groups displayed tumors of similar mean size (Figs 3 and 4), one would expect that death by tumor cachexia contributes in a similar ratio to the lethality of all three protocols, unless metabolic or immunological differences between nude mice and the other mice are responsible for death of nude mice when their tumors are still relatively smaller than those in the other mice. The high lethality of the nude mice may also be due to an increased susceptibility to pathogenic microorganisms.

The relation between tumor growth and survival of the animals during the course of the experiment was satisfactory with the medium or high dose IS protocol. However, while the IS mice displayed progressive tumor growth up to day 30, regression could begin in about 50% of the tumors. For testing purposes it may thus be recommended to use this model for short-term screening procedures and to apply the anticancer agents to be investigated at about 10–14 days, when the tumors are well-established and continue to grow rapidly for at least two more weeks. Clear-cut differences between treated and control animals can be observed in this period[16]. Also, IS mice with persisting tumors from day 40 to day 60, when no more spontaneous regressions occur, can be used[16]. It is important to note that a second treatment with the IS protocol at any time after the tumor grafting did not improve tumor growth (experiments not shown). The antitumor effect of a test drug can thus be evaluated without being compromised by possible simultaneous immunosuppressive activity retarding tumor regression.

With the ID protocol some early lethality occurred, but the satisfactory tumor growth described by Steel *et al.*[5] was confirmed. An advantage of this system, namely the fact that the ID mice can be used as tumor recipients for extended periods after the irradiation[5], may be somewhat counterbalanced by the greater labour required for their preparation, including thymectomy, ARA-C administration and TBI.

The morphological characteristics of the tumors grown in the IS mice are, with the exception of the osteosarcoma, similar to the tumors grown in nude mice[13]. As with nude mice, metastatic involvement of other organs was not detected by gross macroscopic inspection of the deceased mice in the IS and ID groups.

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