Rhabdomyosarcoma is the most common soft tissue sarcoma in childhood and represents between 5% and 15% of all malignant solid tumours in children under 15 years of age. Rhabdomyosarcomas are subdivided into alveolar, embryonal and pleomorphic types (Enzinger & Weiss, 1983).

Only a few human rhabdomyosarcoma cell lines have been recently studied (McAllister et al., 1969; Giard et al., 1973; Chapman et al., 1974; McAllister et al., 1975); whenever histologic type is specified, the original tumour was an embryonal rhabdomyosarcoma. Moreover, in vitro differentiation properties of human rhabdomyosarcoma cells have not yet been fully characterized. Therefore, a rhabdomyosarcoma cell line of alveolar origin with a residual ability to differentiate in vitro could be a useful tool for elucidating the differentiation process in neoplastic and normal muscle.

The cell line described here, RMZ, was derived from a bone marrow metastasis of a human alveolar rhabdomyosarcoma. In this paper we present data on its in vitro growth, unique differentiation properties and the expression of embryonic myosin in the parental cells and in clonal derivatives obtained from the second in vitro passage.

Materials and methods

Establishment of the RMZ cell line

The culture was established from a biopsy of a bone marrow metastasis. The primary tumour, diagnosed as an alveolar rhabdomyosarcoma (Istituto di Anatomia e Istologia Patologica, Università di Padova, Italy), developed in the left thigh of a 2-year-old caucasian male child and was removed one year prior to metastasis. The patient was treated with chemo- and radiotherapy.

Culture procedures

Cells were routinely cultured in Dulbecco's modified Eagle medium (GIBCO, Paisley, Scotland) supplemented with 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin (hereafter referred to as DMEM) and with 10% foetal calf serum (FCS, GIBCO). Horse serum (GIBCO) was used instead of FCS for the early in vitro passages and to study

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in vitro differentiation (Blau & Webster, 1981). Cell cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. RMZ cells were monitored for mycoplasma contamination by fluorescent staining with Hoechst 33258 (Chen, 1977) and found to be mycoplasma-free.

In vitro growth properties

The following parameters of in vitro growth were assessed as reported (Nanni et al., 1983): cell doubling time in the logarithmic growth phase; saturation density (maximum cell number cm⁻²); cloning efficiency (after seeding 2 × 10²–10⁵ cells in a 60 mm Petri dish) and mean cell diameter (determined on trypsinized cells with the aid of a micrometer).

Chromosome studies

Exponentially growing cells were harvested with trypsin-EDTA and treated for 3 h with 0.33 µg ml⁻¹ colcemid (GIBCO). Cells were then pelleted, swollen for 15 min at 37°C with 75 mM KCl, and repeatedly fixed in cold methanol:acetic acid 3:1 for 15 min. The cells were then dropped onto glass slides and stained with a 3% Giemsa solution. The cytogenetic analysis was kindly performed by Dr. N. Testoni and Dr. A. Zaccaria (Istituto di Ematologia L. & A. Seràgnoli, Università di Bologna).

Immunofluorescence and immunocytochemical staining

Desmin was identified with the monoclonal antibody DE-B-5 (Boehringer Mannheim, W. Germany) and with a polyclonal antiserum (DAKO, Santa Barbara, CA, USA) with identical results. BF-G6 is a monoclonal antibody which reacts specifically with embryonic-type myosin heavy chain present in foetal but not in neonatal or adult human skeletal muscle (Schiaffino et al., 1986).

Cell cultures and cytocentrifuged preparations were fixed with a 7:3 mixture of acetone:methanol and processed for the indirect immunofluorescence assay, or for immunoperoxidase staining, using the avidin-biotin complex (Hsu et al., 1981a, b).

Controls included non-immune sera and positive and negative tissues and the following cell lines: TG (Hernandez-Verduin et al., 1984), a human epithelioid tumour cell line (kindly given by Dr. A. Pession, Istituto di Patologia Generale, Università di Bologna, Italy); EUE (De Carlì & Larizza, 1978), a human epithelioid tumour line (kindly provided by Dr. P. Rocchi, Istituto di Cancrologia, Università di Bologna, Italy); Balb/3T3 clone A31, a cell line obtained from normal mouse embryonal fibroblasts (purchased from The American Type Culture Collection, Rockville, MD, USA).

Results

In vitro growth properties

The original tumour from which RMZ cells were derived was diagnosed as an alveolar rhabdomyosarcoma by current accepted criteria (Enzinger & Weiss, 1983). The tumour consisted of large sheets of cells separated by fibro-vascular septa. The tumour cells in the centre were frequently necrotic. The neoplastic population was formed by two different cell types: one constituted by small round elements having scanty cytoplasm; other cells, which were in the minority, displayed large eosinophilic cytoplasm and occasionally appeared multinucleated.

RMZ cells were adapted to grow in DMEM supplemented with 10% FCS and were routinely subcultured approximately once a week at dilutions from 1:2 to 1:4. In vitro growth characteristics were studied around the 20th passage, in order to study a population as close as possible to the original tumour. Basic features remain unchanged during subsequent culture passages (RMZ cells are now around the 40th in vitro passage).

Adherent cell growth was found to be dependent on seeding density: when the seeding concentration was between 8 and 4,000 cells cm⁻², a cloning efficiency ≤0.01% was observed, whereas at higher seeding density mass growth was observed. RMZ cells, seeded at 20,000 cells cm⁻², had a doubling time of ~48 h and a saturation density of 1.6 × 10⁶ cells cm⁻²; mean cell diameter, measured in subconfluent cultures, was 15 ± 0.24 µm (range 9 to 21).

Chromosome analysis, performed on 44 metaphases, revealed a modal chromosome number of 84 (range 72 to 99). This hypotetraploid pattern is in agreement with that of another case of human alveolar rhabdomyosarcoma (studied on freshly-collected material) reported in the literature (Seidal et al., 1982). Forty metaphases carried structural aberrations: dicentrics, acentric fragments, double minutes, chromosome and chromatid breaks. Sixteen metaphases were photographed and karyotyped: 6 of them were characterized by abnormalities of chromosome #3 but with different breakpoints; 6 metaphases carried abnormalities of chromosome #1 (5 of 1q). Moreover, in 2 metaphases a double 12q+ was observed. Additional abnormalities involved chromosomes #5, #14 and #17. Several markers of different shape were observed in most metaphases. (De Giovanni et al., submitted for publication).
Attempts to induce tumours in nude mice with a subcutaneous injection of RMZ cells have insofar been unsuccessful.

In vitro morphology and differentiation properties

Different cell types were present in RMZ cultures: small mononucleated polygonal or spindle-shaped, actively proliferating, cells were observed along with a few multinucleated elongated cells, resembling myotube structures, and large giant, sometimes multinucleated, elements (Figure 1). Myotube-like structures were usually not present in freshly seeded culture and became more evident during the late logarithmic phase and in confluent cultures. They usually appeared to contain \( \geq 4 \) nuclei; however, a precise estimate of the average number of nuclei was hardly possible in cultures because of the extensive criss-cross growth pattern of these cells. The capacity of mononucleated elements to give rise to myotube-like structures was constantly observed during in vitro passages of RMZ cells; the expression of this characteristic was found to be dependent on seeding density and serum concentration. The lower the values these two variables had, the stronger was the tendency of cultured cells to become elongated and fuse. The growth pattern of RMZ cells seeded at a concentration of 6,000 cells cm\(^{-2}\) and cultured for 1 month is shown in Figure 2. Almost all the cells tended to a fusiform shape and to a mitotic arrest. RMZ cells, when cultured in the presence of 2% horse serum, revealed a pronounced increase in fusiform morphology.

We tried to isolate clones (Nanni et al., 1983) from RMZ colonies grown in semisolid medium (either agar or agarose 0.33%), but we did not obtain any appreciable cell growth on plastic. This could be due to the small dimension of the agar colonies (McAllister et al., 1975).

From a low density seeding (400 cells cm\(^{-2}\)) of the second in vitro subculture of RMZ cells, a few colonies were obtained which were separately collected with a rubber policeman and reseeded as above to obtain RMZ clonal derivatives.

An interesting feature of the set of clones derived from RMZ is that they showed, under identical culture conditions, different overall morphology, in vitro growth rate and degree of myotube formation. Two extreme clones are shown in Figure 3: clone RC2 had a low differentiative trend, being composed almost exclusively of small polygonal cells, and a relatively high proliferative rate (with a doubling time of \( \sim 38 \) h), whereas clone RC5 showed a high degree of myotube formation and a very low proliferative rate (doubling time \( > 100 \) h), which made its in vitro propagation very difficult.

Even though clones showed peculiar differentiative trends, they showed the occurrence of all the cell types described in the parental RMZ line (in quite different proportions). This suggests that the morphological heterogeneity described above is mainly due to the peculiar ability to differentiate in vitro and not to pre-existing, stable morphological variants, as observed in other experimental tumours (Dexter et al., 1978; Lollini et al., 1984).

Markers of myogenic differentiation

In order to better define the in vitro differentiative properties of rhabdomyosarcoma cells, we studied the expression of desmin and embryonic myosin in RMZ and cloned cells by means of immunofluorescence and immunoperoxidase techniques.
In the RMZ cell line the anti-desmin antibody strongly stained almost all the myotube-like structures (Figure 4); the brightest fluorescence labelling was seen at the ends of myotube-like structures. Small (polygonal and spindle-shaped) cells showed a gradation of staining from completely negative to strongly positive elements. Giant multinuclear elements were sometimes desmin-positive; fluorescent filaments were particularly evident in these cells. In order to obtain a precise estimate of the proportion of RMZ cells expressing desmin, cytocentrifuged preparations were stained by immunofluorescence. The proportion of desmin-positive cells in cytocentrifuged preparations was 81.3% ± 2.9 (mean ± s.e. of 4 independent experiments). Control experiments were performed with cell lines of human (TG and EUE) and murine origin (3T3), all of which were completely negative.

The RMZ cell line has been characterised for the expression of embryonic myosin, as shown by BF-G6 reactivity. All the myotube-like structures showed a strong positivity (Figure 5a) with fluorescent filaments and sometimes cross-striations (Figure 5b). Almost all the small cells were negative; giant cells were sometimes positive. In standard culture conditions the proportion of myosin-positive cells in cytocentrifuged preparations was 6.3% ± 0.9 (mean ± s.e. of 3 experiments). Control cell lines (TG, EUE, 3T3) were completely negative.

The expression of embryonic myosin heavy chain was investigated also in the two RMZ clonal derivatives previously described (see Figure 3), studied under standard culture conditions. Clone RC2 showed a BF-G6 positivity (Table I) not far from that of the uncloned parental cell line, whereas a strongly higher proportion of embryonic myosin-expressing cells was found in clone RC5. Therefore, as previously reported for cell morphology, the study of RMZ clones also showed that the expression of embryonic myosin was not clonally distributed, but was rather correlated to the proportions of myotube-like structures of the culture.
Both RMZ clones studied (RC2 and RC5) showed high and comparable proportions of desmin-expressing cells in cytocentrifuged preparations (Table I).

**Discussion**

The most interesting features of the new human cell line, RMZ, described here are the following: (1) it was derived from a rhabdomyosarcoma of the alveolar histological type; (2) it retained some ability to differentiate in vitro; and (3) in vitro morphological differentiation into myotube-like structures correlates with the expression of embryonic myosin heavy chain.

In the RMZ cell line some cells express a myosin heavy chain type antigenically similar to that present in human muscle during the early stage of development. RMZ cells are (at least partially) able to differentiate in vitro to long multinucleated myotube-like structures expressing both desmin and embryonic myosin. However, myosin- or desmin-negative cells were also represented in RMZ cell line. Further studies on the proliferative and differentiative ability and on the origin of the different cell types present in rhabdomyosarcoma might contribute to the understanding of the differentiation process in neoplastic and normal muscle. We are presently considering the distribution of desmin, embryonic myosin and other myosin isoforms and the use of modulating agents.

In RMZ cultures we observed a small fraction of large, flat, distended, sometimes multinucleated, cells which frequently exhibited immunoreactivity for desmin and embryonic myosin. It should be noted that cells morphologically similar to those described here have been observed in cell cultures

| Cells            | Embryonic myosin | Desmin     |
|------------------|------------------|------------|
| RMZ cell line    | 6.3 ± 0.9        | 81.3 ± 2.9 |
| Clone RC2        | 4.0              | 71.7       |
| Clone RC5        | 35.4             | 86.9       |

*a*By immunofluorescence.
obtained from Duchenne muscular dystrophy patients (Blau et al., 1983) and in two other rhabdomyosarcoma cell lines (McAllister et al., 1969; Chapman et al., 1974). It is possible that the presence of such cells could be related with developmental alterations.

We obtained a set of clonal derivatives from the second in vitro passage of RMZ cells. These clones share with the parental cells all the features outlined above, but differ quantitatively from one another in the capacity to give rise to differentiated myotube-like structures. We are presently trying to study the reciprocal effects on differentiation of culture media conditioned by the various clones and of cell co-cultures.

In the RMZ cell line, proliferative and differentiative abilities seem to be opposite. In this model the study of inducers of differentiation and of stimuli causing quiescent, differentiated cells to re-enter the cell cycle could have therapeutic implications.

It is considered that a comparison of the in vitro properties of RMZ with those of other rhabdomyosarcoma cell lines, derived from embryonal tumours, could be useful for the study of correlations between the behaviour of in vitro cultured cells and the histologic classification of myogenic tumours.

Note added in proof

Progressive tumour growth was obtained in Swiss nude mice after a s.c. or i.m. injection of $3 \times 10^7$ cells of clone RC2.

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