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Vinblastine, Vincristine and Vindesine: Anti-invasive Effect on MO_4 Mouse Fibrosarcoma Cells *In Vitro**†

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Abstract—*Inhibition of the invasiveness of* MO_4 *mouse fibrosarcoma cells by the vinca alkaloids, vinblastine (VLB), vincristine (VCR) and vindesine (VDS), has been examined* in vitro. *At doses between* 0.006 μg/ml *(minimal effect) and* 0.1 μg/ml *(complete inhibition) these drugs interfered with the invasion of* MO_4 *cells from an aggregate confronting a fragment of embryonic chick heart in three-dimensional culture. We have also examined the effect of these drugs on the following activities of* MO_4 *cells: growth, directional migration and assembly of the cytoplasmic microtubule complex. Growth and directional migration were affected by the same doses of vinca alkaloids as invasion. In contrast with the vinca alkaloids, 5-fluorouracil at* 1 μg/ml *inhibited growth but allowed directional migration and invasion. At a dose of* 0.3 μg/ml *VLB, VCR and VDS interfered with the assembly of cytoplasmic microtubules, as visible after immunocytochemical staining with tubulin antiserum. Ultrastructural analysis demonstrated that inhibition of invasion in three-dimensional culture corresponds with abolishment of the cytoplasmic microtubule complex. Anti-invasive concentrations of VLB, VCR and VDS represent clinically achievable plasma concentrations. We concluded that the anti-invasive effect of the vinca alkaloids may contribute to their antitumor activity.*

INTRODUCTION

THE ANTITUMOR activity of the naturally occurring vinca alkaloids, vinblastine (VLB) and vincristine (VCR) and the semi-synthetic VLB-derivative vindesine (VDS), has been demonstrated in experimental and human cancer [1, 2]. How the vinca alkaloids exert their antitumor activity has been a matter of discussion [3, 4]. Since most attention has been paid to the effect of the vinca alkaloids on mitosis, clinical trials have been established on the basis of the antiproliferative activity of these drugs [5]. We have reported that microtubule inhibitors (including VLB and VCR) interfere with the capacity of MO_4 mouse fibrosarcoma cells to invade normal tissues in three-dimensional culture, and that abolishment of the cytoplasmic microtubule complex is responsible for this anti-invasive effect [6–8]. Invasion is a characteristic of malignant cells and, next to unbalanced growth, responsible for the fatal outcome of many tumors [9]. The relevance of invasion in three-dimensional culture has been shown in as much as all cell lines that were invasive *in vitro* produced invasive tumors in syngeneic animals [10]. Furthermore, the histopathology of invasion *in vitro* resembled that of invasive tumors in the animals [11]. The wide use of VLB, VCR and VDS in the clinic prompted us to investigate whether these drugs are anti-invasive *in vitro* at clinically achievable plasma concentrations. Furthermore, we examined the effect of these compounds on activities of MO_4 cells which might be involved in invasion: growth, directional migration and

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assembly of the cytoplasmic microtubule complex.

MATERIALS AND METHODS

MO_4 *cells*

MO_4 cells are virally transformed fibroblastic C3H mouse cells [12] which produce invasive [13, 14] and metastasizing [15] tumors after transplantation into syngeneic mice. MO_4 cells were maintained in monolayer culture using Eagle's Minimum Essential Medium (MEM, Flow Laboratories Ltd., Irvine, Scotland) plus 10% fetal bovine serum (Flow Laboratories Ltd., Irvine, Scotland) and 0.05% L-glutamine (hereafter called culture medium). The same culture medium was used in all the present experiments. Aggregates of MO_4 cells were prepared by incubating a suspension of trypsinized cells on a gyratory shaker at 70 rpm as described previously [16]. Unless stated otherwise, spheroidal aggregates with a diameter of 0.2 mm were used in the present experiments.

Drugs

The following vinca alkaloids (Eli Lilly and Company, Indianapolis, IN) were added to the culture medium at concentrations between 0.001 and 10 μg/ml: VLB, VCR and VDS. The structure and function of these molecules have been recently reviewed by Gerzon [1]. Stock solutions were prepared in balanced salt solution at 100 μg/ml and stored at 4°C for no longer than one week. The drugs were further diluted in culture medium before use. Unless indicated otherwise, cultures were run in the presence of the drug from the beginning of the experiment and the medium was kept unchanged. To study the reversibility of the effect of the drugs, cultures were washed four times with 10 ml fresh warm culture medium and reincubated with daily refreshment of the medium. In some of the experiments the effect of the vinca alkaloids was compared with that of 5-fluorouracil (Roche Laboratories, Nutley, NJ) at 1 μg/ml. We have reported previously that 5-fluorouracil inhibits the proliferation of MO_4 cells, but allows their invasion into the chick cardiac muscle *in vitro* [6, 8, 17]. In all experiments control cultures were made without drugs.

Assay for growth

Growth was evaluated by daily measurement of the diameters of aggregates incubated individually at 37°C in a 5 ml Erlenmeyer flask containing 1.5 ml of culture medium on a gyratory shaker at 120 rpm as described previously [17]. In spheroidal aggregates of cells *in vitro* and in solid tumors *in vivo*, growth results from both cell proliferation and cell loss; the volume of individual cells and the intercellular space are additional factors, as discussed previously [17].

Assay for directional migration

The mean diameter of the circular area covered by MO_4 cells that had migrated radially from an aggregate explanted on glass was used as an index of directional migration as described previously [17]. We have reported [18] that MO_4 cells reach the border of this area by active migration and that the direction of migration of individual MO_4 cells is conditioned by collision with other MO_4 cells. All cultures were examined daily under the stereomicroscope. For histological examination, cultures were fixed after 2, 4 and 7 days in a solution of alcohol: formalin: acetic acid (75:20:5) and stained with hematoxylin and eosin.

Immunocytochemistry with tubulin antiserum

Immunocytochemical staining of microtubules by the unlabelled antibody enzyme method (including controls) was done as described by De Mey *et al.* [19]. Therefore, MO_4 cells were cultured from a trypsinized suspension on coverslips in Leighton tubes and fixed after 2–3 days in glutaraldehyde (1% in cacodylate buffer, pH = 7.4). The tubulin antiserum (kindly provided by J. De Mey and M. De Brabander, Janssen Pharmaceutica, Beerse, Belgium) was diluted 1/25–1/200 in phosphate-buffered saline. This method specifically revealed both assembled microtubules and unassembled tubulin [19–21]. In each experiment duplicated cultures were used.

Analysis of invasion into chick cardiac muscle

Invasion *in vitro* was studied using the method described earlier [16].

Briefly, MO_4 cell aggregates with a diameter of 0.2 mm or 0.4 mm were confronted with pre-cultured fragments of 9-day-old embryonic chick heart (diameter, 0.4 mm) on semisolid medium. After 2 hr, individual confronting pairs were transferred to 5 ml Erlenmeyer flasks and incubated with 1.5 ml of culture medium on a gyratory shaker (New Brunswick Scientific Company, Inc., New Brunswick, NJ) at 120 rpm. Triplicate cultures were fixed after 2 hr–7 days either in Bouin Hollande's solution for embedding in paraffin and complete sectioning into 8 μm thick sections, or in glutaraldehyde and osmiumtetroxide for embedding in Araldit (Serva, Heidelberg, FGR) and

sectioning into 1 μm thick sections. All sections were stained with hematoxylin and eosin. Selected areas from cultures embedded in Araldit were processed for transmission electron microscopy at 80kV (JEM 100B, Jeol, Tokyo, Japan). Therefore, 600–800 Å thick sections were counterstained with uranyl acetate and lead citrate. MO_4 cells inside the heart tissue could be easily identified because they contained intracisternal A-type particles [12].

RESULTS

Growth

Typical growth curves from individual cultures of MO_4 cell aggregates incubated with VLB, 5-fluorouracil or without drug are shown in Fig. 1. It is obvious that both drugs had the same effect on the growth of the aggregates. Figure 2 shows the effects of VLB, VCR and VDS on the growth of the aggregates. Large variations between individual cultures were observed at minimally effective doses (0.006–0.01 μg/ml). At these doses VLB appeared to be slightly more effective than VDS or VCR.

Directional migration

Comparison of the effects of VLB and of 5-fluorouracil on the directional migration of MO_4 cells from an aggregate explanted on glass is shown in Fig. 3. Whereas both VLB and 5-fluorouracil similarly inhibited growth (Fig. 1), VLB affected directional migration more rapidly and to a larger extent than 5-fluorouracil. We have shown previously that the increase of the diameter of the area

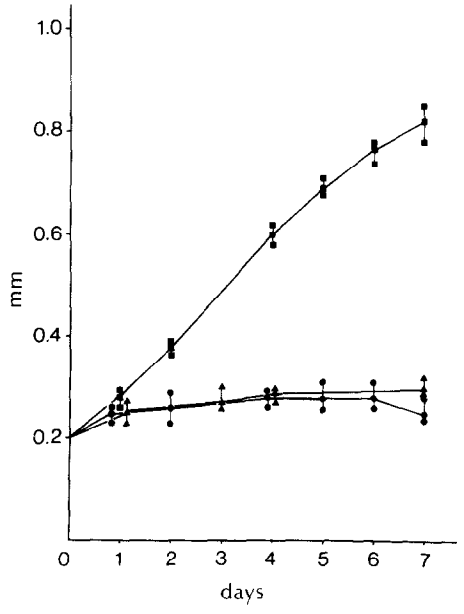

Fig. 1. Growth of individual aggregates of MO_4 cells: untreated (■); with 1 μg/ml 5-fluorouracil (▲); with 0.1 μg/ml VLB (●). Ordinate: diameter of aggregates; median and extreme values from 5 cultures. Abscissa: time of incubation.

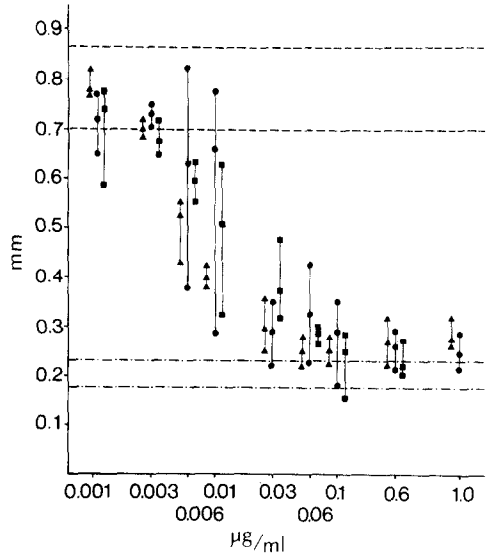

Fig. 2. Effect of VLB (▲), VCR (●) and VDS (■) on the growth of MO_4 cells in individual shaker culture. Ordinate: diameter of aggregates after 7 days culture; median and extreme values from 5 to 9 cultures.---: Extreme values from cultures without drug; -·-·-: extreme values at the onset of culture. Abscissa: doses of vinca alkaloids in μg/ml (log scale).

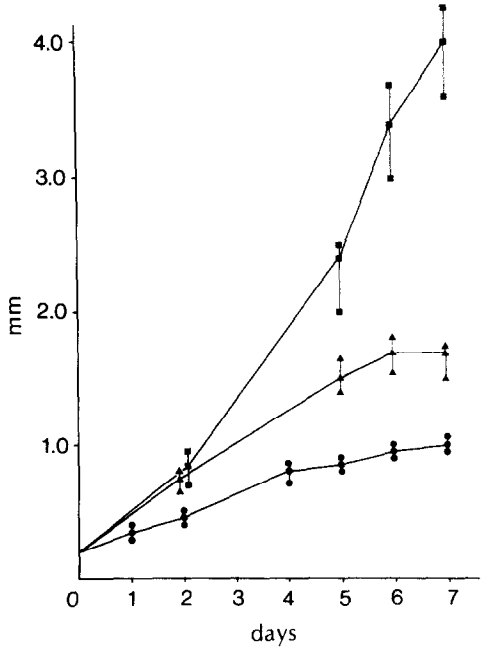

Fig. 3. Directional migration of MO_4 cells from individual aggregates explanted on glass: untreated (■); with 1 μg/ml 5-fluorouracil (▲); with 0.1 μg/ml VLB (●). Ordinate: mean diameter of the circlar area covered by the cells that have migrated from the aggregate; median and extreme values from 5 cultures. Abscissa: time in days.

covered by MO_4 cells in presence of 5-fluorouracil is lower than in controls because the number of cells is lower [18]. The data about the effect of various doses of vinca alkaloids on directional migration are summarized in Fig. 4. Addition of 1 μg/ml of VDS 1, 2, 3 or 4 days after the onset of the culture promptly inhibited directional migration (Fig. 5). Washing of these cultures 5 days after addition of the drug and further incubation in fresh medium restored directional migration (curves not shown in Fig. 5). Light microscopy of untreated cultures showed a majority of radially oriented, spindle-shaped MO_4 cells. In cultures treated with the vinca alkaloids at doses of 0.03 μg/ml or higher, polygonal cells formed a small corona around the original aggregate. At the beginning these cultures contained C-mitoses, as defined by Levan [22]. Later, most cells became multimicronucleated, as defined in [23]. With suboptimal doses of the drugs both affected and non-affected types of cells were found.

Reversibility of inhibition of directional migration after treatment with the three vinca alkaloids at 1 and 10 μg/ml for 2, 4 and 6 days is shown in Fig. 6. Treated cultures were only slightly retarded as compared to controls. This indicates a high degree of reversibility at doses of the drugs much higher than those needed for complete inhibition of directional migration. Histology of cultures fixed 6 days after

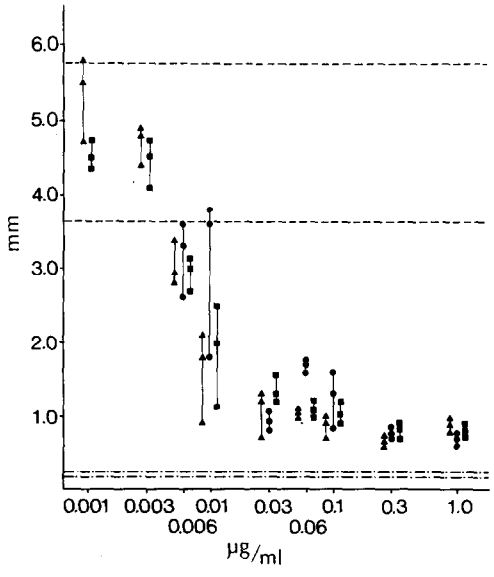

Fig. 4. Effect of VLB (▲), VCR (●) and VDS (■) on migration of MO_4 cells from an aggregate explanted on glass. Ordinate: mean diameter of the circular area covered by cells after 7 days; median and extreme values from 5 to 9 cultures. ---: Extreme values from cultures without drugs; -·-·-: extreme values at the onset of culture. Abscissa: doses of vinca alkaloids in μg/ml (log scale).

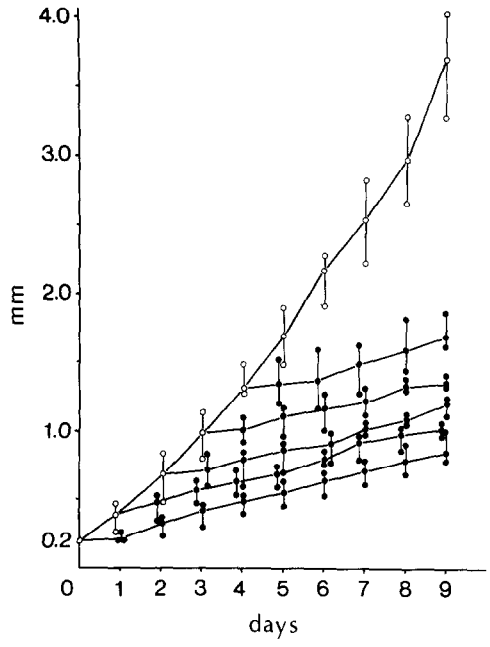

Fig. 5. Effect of 1 μg/ml VDS on directional migration of MO_4 cells on glass. Ordinate: mean diameter of area covered by cells that have migrated from the aggregate; median and extreme values from 5 to 9 cultures. Abscissa: time in days. VDS was added (●–●) at the onset, or after 1, 2, 3 and 4 days. ○–○: In absence of drug.

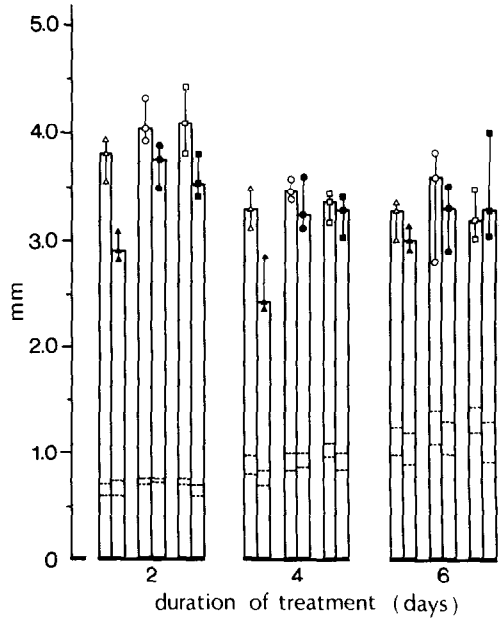

Fig. 6. Reversibility of inhibition of directional migration after 2, 4 and 6 days of incubation with VLB (triangles), VCR (circles) or VDS (squares) at 1 μg/ml (open symbols) or 10 μg/ml (filled symbols). Columns indicate the mean diameter of the circular area covered by the cells on the 6th day after removal of the drug (median and extreme values from 5 to 11 cultures). Treatments were started one day after explantation of the aggregates on the coverslips. ---: Extreme values at the moment of removal of drug.

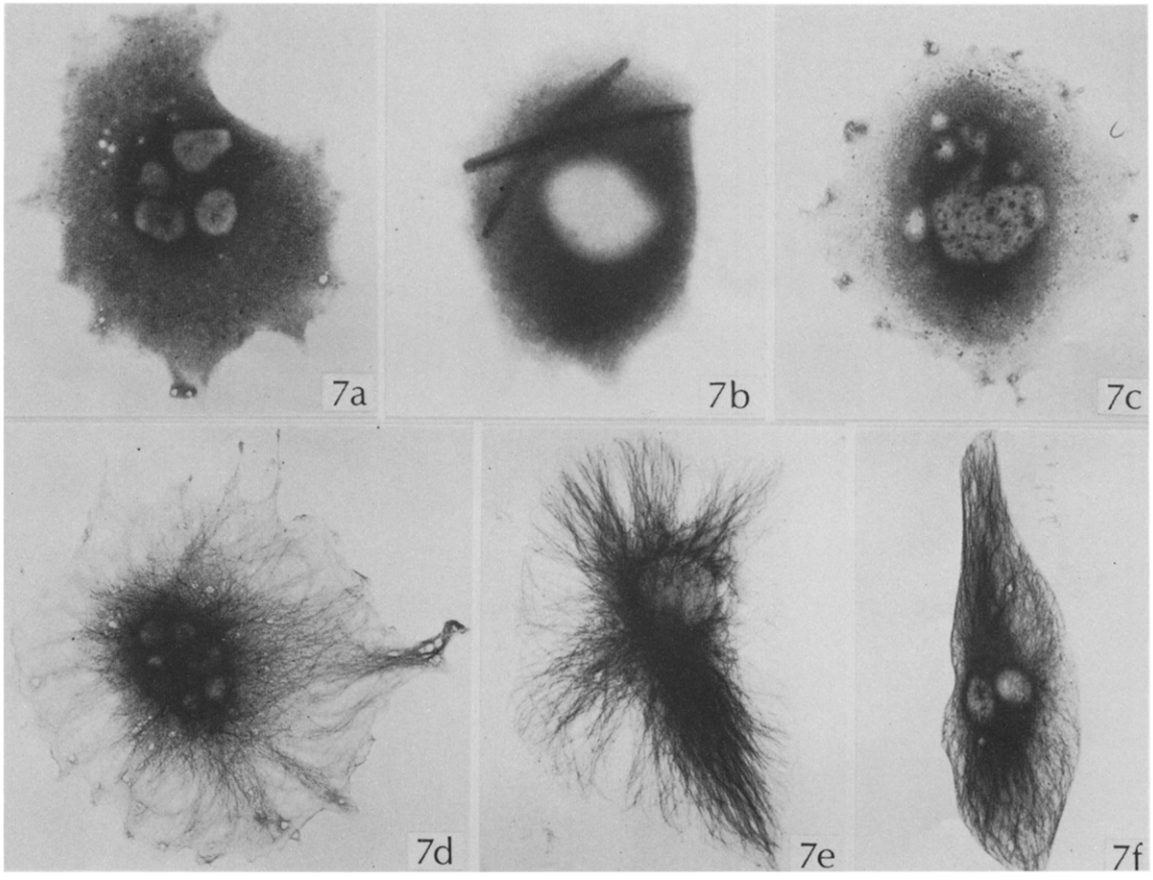

Fig. 7. Light micrographs of MO_4 *cells stained with tubulin antiserum following the unlabelled antibody enzyme method (see Materials and Methods) from cultures treated with*: (a) *VDS at* 1 μg/ml; (b) *VLB at* 1 μg/ml; (c) *VCR at* 1 μg/ml; (d) *VDS at* 0.01 μg/ml; (f) *5-fluorouracil at* 1 μg/ml; (e) *control culture* × 300 (*e, f*); × 430 (*a, c, d*); × 730 (*b*).

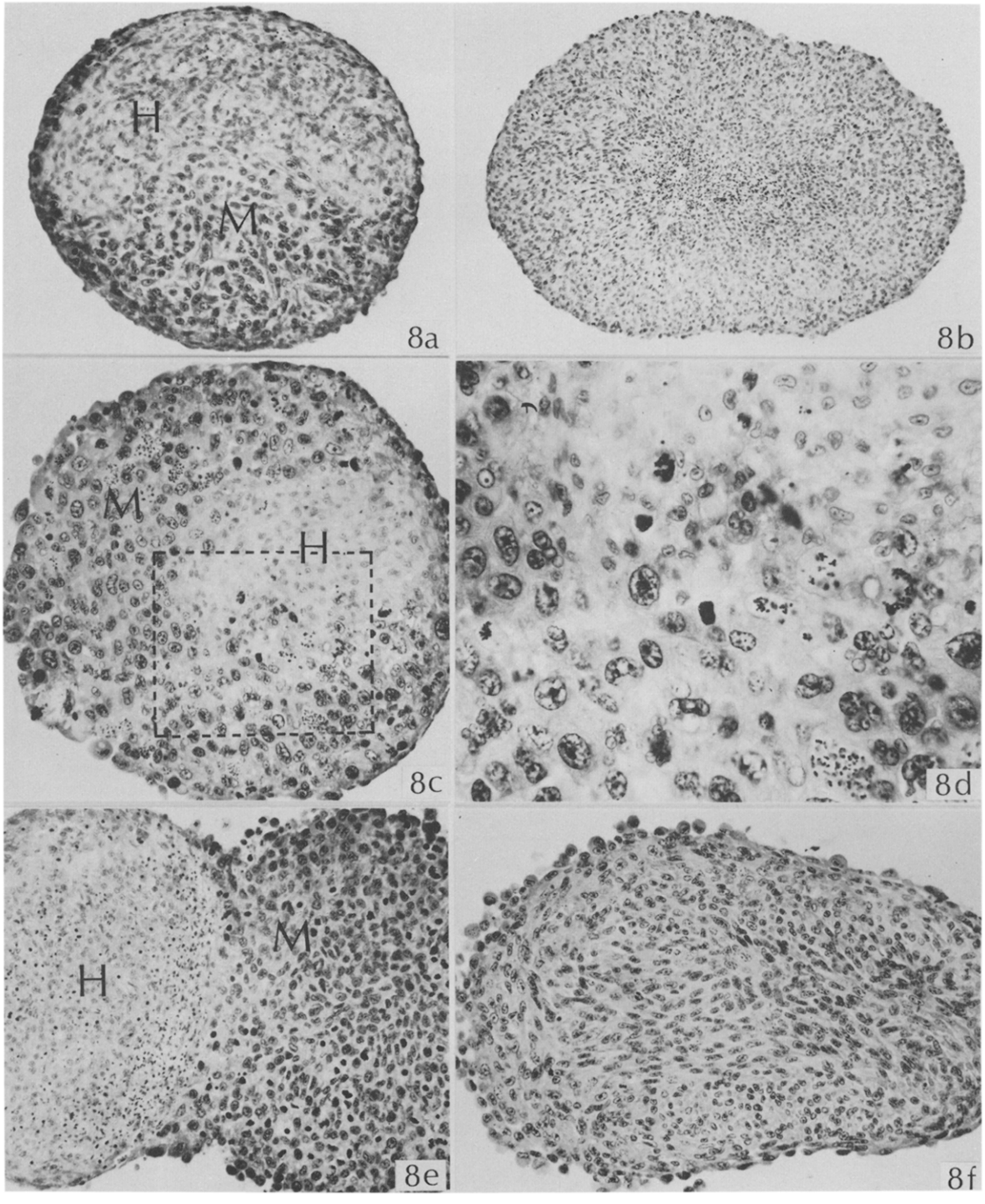

Fig. 8. Light micrographs of 8 μm-thick sections from confrontations of a MO_4 cell (M) aggregate with a precultured fragment of chick heart (H). Staining with hematoxylin and eosin. a: Fixation after 2 days (×175); b: after 6 days (×87); c: after 2 days without drug plus 4 days with 1 μg/ml VCR (×175); d: detail from 2c shows MO_4 cells inside the cardiac muscle (×435); e: after 4 days with 1 μg/ml VCR (×175); f: after 4 days with 1 μg/ml 5-fluorouracil (×175).

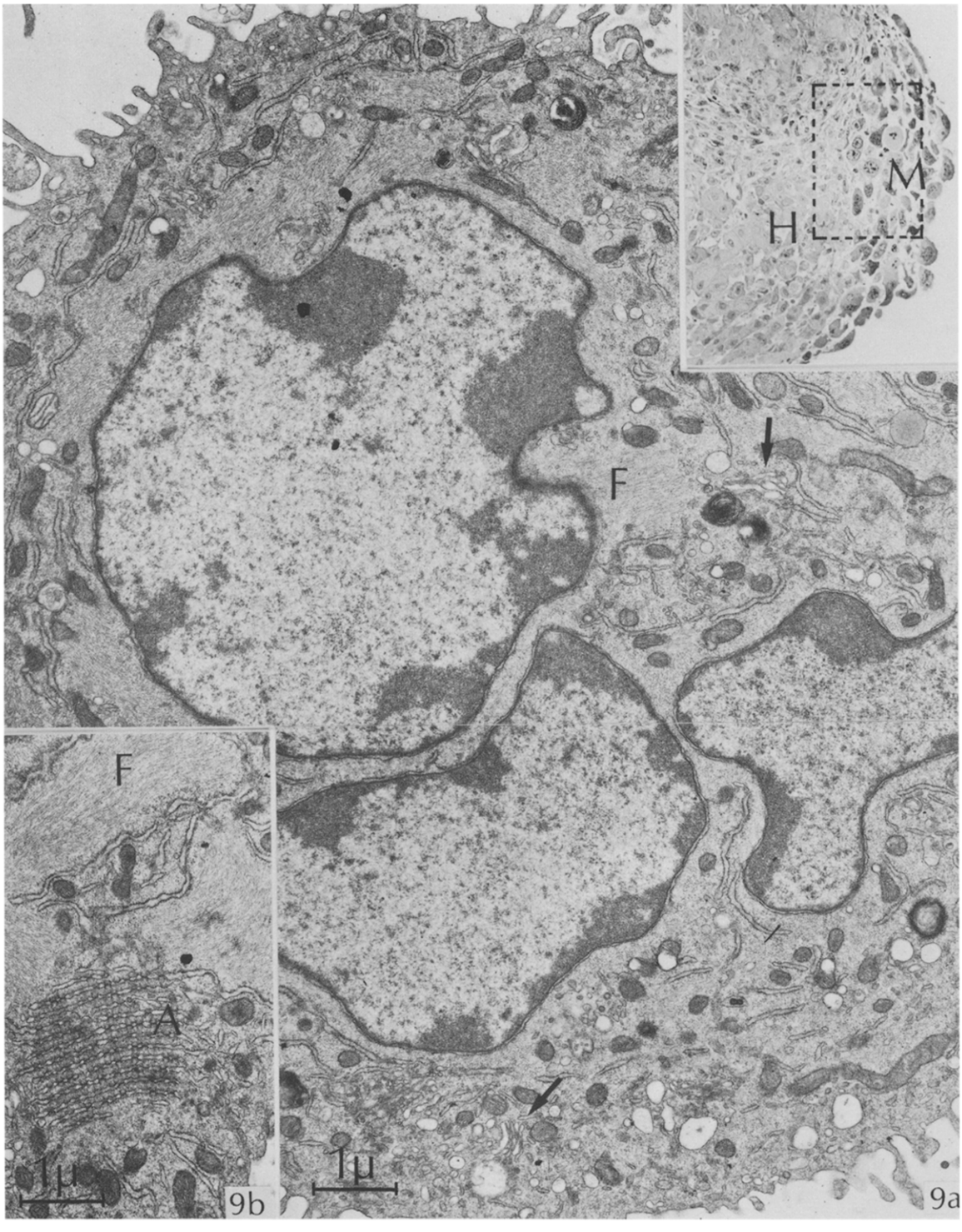

Fig. 9. Transmission electron micrographs of MO_4 *cells confronted with a precultured heart fragment in presence of* 1 μg/ml *VCR and fixed after* 4 *days. Inset: light micrograph of a* 1 μm*-thick section from which the area for ultrastructural analysis was selected.* $M = MO_4$ *cells;* H = *heart. Figure a shows perinuclear* 10 nm *filaments* (F) *and scattering of organelles over the cytoplasm. Arrows indicate Golgi cisternae. Figure b shows annulate lamellae* (A).

removal of the drug showed the following alterations as compared to controls. The number of cells per unit area was lower and mitoses were less abundant. Cells that had not completely recovered from mitotic arrest were found in most cultures. These cells were large, sometimes polygonal and contained one or several large nuclei; some were multimicronucleated. Remarkably, a number of multimicronucleated cells were spindle-shaped with a leading edge and a trailing end. Radial orientation of cells that had migrated from the aggregate was obvious in most cultures. The overall pattern of migration was interrupted by the large polygonal cells and by foci of cells without distinct orientation. The latter are interpreted as foci of incipient clonal growth.

Immunocytochemistry with tubulin antiserum

Immunocytochemical staining (Fig. 7) with tubulin antiserum of subconfluent cultures of MO_4 cells grown from cell suspensions on coverslips in absence of drugs demonstrated both cytoplasmic and spindle microtubules. In these cells, diffuse staining of the cytoplasm was also present. In cultures treated for 2 days with one of the vinca alkaloids at doses of 0.3 μg/ml or higher, spindle and cytoplasmic microtubules were absent. The cytoplasm of these cells was diffusely stained (Fig. 7a–c). Tubulin paracrystals were found at doses of 1 μg/ml, most frequently with VLB (Fig. 7b). At doses between 0.01 and 0.1 μg/ml, spindle microtubules were absent in contrast to cytoplasmic microtubules. These cultures were, however, different from controls, because varying numbers of cells lacked cytoplasmic microtubules and in most others the network of cytoplasmic microtubules was less abundant. Some multimicronucleated cells (defective mitotic spindle) had multiple cytoplasmic microtubules (Fig. 7d). The immunocytochemical aspect of the microtubule complexes in cultures treated with doses of 0.006 μg/ml or lower was the same as in controls. In MO_4 cells incubated for 2 days with 5-fluorouracil at 1 μg/ml, the cytoplasmic microtubule complex did not differ from that in controls (Fig. 7f).

Invasion into chick cardiac muscle in vitro

In all control cultures invasion of MO_4 cells into the chick heart was observed as described previously [16].

Briefly, MO_4 cells progressively occupied the cardiac muscle and almost completely replaced it after 4–6 days (Fig. 8a–b). MO_4 cells presumed to be invading were spindle-shaped with irregular extensions; they frequently contained phagosomes. Muscle cells in the close vicinity of MO_4 cells showed signs of degeneration. Cultures treated with one of the vinca alkaloids at doses of 0.001 and 0.003 μg/ml were not different from controls. At a dose of 0.006 μg/ml invasion was present in all cultures, but higher amounts of heart tissue were left after 4 days as compared to controls. At doses of 0.01–0.03 μg/ml complete inhibition of invasion was observed in some cultures. In most of them some spindle-shaped MO_4 cells were found between the muscle cells. At doses between 0.1 and 1 μg/ml invasion was completely inhibited in all cultures fixed after 4 days (Fig. 8e). The MO_4 cells formed a cap at the pole of attachment or partly surrounded the heart fragment. In control cultures the heart fragment was lined by a layer of flat fibroblastic cells, as described previously [24]. At the site of contact with MO_4 cells this layer was absent and the MO_4 cells faced the cardiac muscle. However, no MO_4 cells were found between the muscle cells. Most MO_4 cells were polygonal. MO_4 cells spread to some extent along the heart fragment were dome-shaped, with extensions pointing towards the heart tissue. C-mitoses were present both in the heart cells and in the MO_4 cells. Many MO_4 cells were multimicronucleated.

No consistent differences between the anti-invasive effects of the different vinca alkaloids were observed in these experiments. Treatment with vinca alkaloids at 1 μg/ml of larger MO_4 cell aggregates (diameter, 0.4 mm) confronting pre-cultured heart fragments (diameter, 0.4 mm) also resulted in complete inhibition of invasion (Fig. 8e). In contrast, invasion from larger aggregates (0.4 mm) in presence of 1 μg/ml 5-fluorouracil hardly differed from controls (0.2 mm) incubated without the drug (Fig. 8f). Addition of VLB, VCR or VDS at 1 μg/ml to confronting tissues two days after the onset of incubation demonstrated that the drugs not only prevent invasion but also are capable of inhibiting invasion during its course (Fig. 8c). Solitary MO_4 cells lying between the muscle cells were polygonal and eventually showed C-mitoses, or multiple micronuclei (Fig. 8d). In controls (Fig. 8a) such cells were predominantly spindle-shaped when in interphase. Transmission electron microscopic analysis of confrontations between MO_4 cell aggregates (diameter, 0.2 mm) and heart fragments concentrated on the ultrastructure of cells, the invasion of which was inhibited (Fig. 9a). These cells had Golgi complexes distributed all over the cytoplasm. Their perinuclear region was filled with 10 nm-thick

filaments and they contained annulate lamellae (Fig. 9b). Short microvillous extensions were present all around their periphery. In some of these cells short tracks of cytoplasmic microtubules could be found. Interestingly, both inhibited and invasive MO_4 cells could be observed in the same culture when doses of vinca alkaloids that partly inhibited invasion were used.

DISCUSSION

The present experiments confirm our previous observations on the anti-invasive effect of microtubule inhibitors [6–8]. They provide new evidence that inhibition of directional migration by abolishment of the cytoplasmic microtubule complex is the mechanism of anti-invasiveness.

Several authors have demonstrated that the vinca alkaloids bind to tubulin dimers and, therefore, interfere with the assembly of microtubules [1, 25, 26]. The ultrastructural alterations of MO_4 cells confronted with chick cardiac muscle in presence of anti-invasive doses of the drugs (Fig. 9) were characteristic of inhibition of the microtubule complex as described by others [27].

We have reported previously that a series of natural and synthetic microtubule inhibitors with unrelated chemical structures are anti-invasive *in vitro* [7]. In contrast, compounds without effect on the microtubule complex allowed invasion [6, 8]. Immunocytochemical staining with a tubulin antiserum of MO_4 cells cultured in presence of the vinca alkaloids at anti-invasive doses clearly demonstrated their effect on the microtubule complex (Fig. 7). Complete disappearance of all microtubules was found at doses of vinca alkaloids higher than those necessary for complete inhibition of invasion. Changes in the shape of the MO_4 cells cultured on glass [28] and ultrastructural alterations of MO_4 cells confronted with cardiac muscle in three-dimensional culture at anti-invasive doses, however, suggest that functional impairment of the microtubule complex occurred before complete disappearance of microtubules from immunocytochemical preparations.

At anti-invasive doses the vinca alkaloids inhibited the growth of MO_4 cell aggregates in individual shaker cultures (Fig. 2). Absence of normal mitotic figures, accumulation of C-mitoses [22] and formation of multimicronucleated cells [23] strongly suggest that interference of the drugs with the assembly of the mitotic spindle is responsible for inhibition of growth. Anti-invasive agents that do not interfere with growth have not been found. Therefore, we can present only indirect evidence that the anti-invasive effect of the vinca alkaloids is not due to inhibition of growth. Previous experiments with a number of growth inhibitors [6, 8] that do not affect microtubules make it unlikely that inhibition of growth is responsible for the anti-invasiveness of the vinca alkaloids. Increasing the number of cells in the confronting aggregate (diameter, 0.4 mm instead of 0.2 mm) did not alter the anti-invasive activity of the vinca alkaloids (Fig. 8e). In presence of 5-fluorouracil, invasion from a larger (0.4 mm) MO_4 cell aggregate occurred more rapidly than from a smaller (0.2 mm) aggregate. These data strengthen the idea that growth and invasion are basically unrelated activities of MO_4 cells. They indicate that growth might indirectly influence invasion by increasing the number of invasive cells. For one microtubule inhibitor (Nocodazole) we have presented direct evidence that the anti-invasive effect was not due to interference with spindle microtubules [6]. In this experiment MO_4 cells were treated with 5-fluorouracil, which arrested cells in S-phase but allowed invasion. The anti-invasive effect of Nocodazole on these cells could only be ascribed to abolishment of the cytoplasmic microtubule complex because spindle microtubules were not formed.

Abolishment of the cytoplasmic mictrotubule complex by vinca alkaloids might well explain their anti-invasive effect. Various authors have reported that directional migration depends on an intact cytoplasmic microtubule complex [17, 28–30]. It is highly probable, as reviewed in [9], that directional migration is a vital activity of invading cells.

In most of our experiments VLB, VCR and VDS produced similar effects at comparable doses. Most data about the tubulin binding of the vinca alkaloids, measured either as the concentration of the drug necessary to inhibit polymerization of tubulin in the test tube (1, 28, 31) or as the capacity of the drug to displace [^{3}H]–VLB from its binding with tubulin [1, 31], showed the following ranking: VCR > VDS > VLB. Ranking of vinca alkaloids following their anti-invasive effects did not reflect their tubulin binding capacity. This supports the opinion that it is difficult to correlate differential biological activities of the vinca alkaloids with differential binding to tubulin in the test tube [32].

It is a matter of discussion as to whether cells arrested during mitosis by the vinca alkaloids inevitably become necrotic, or are able to survive

and to produce a progeny of normalized cells after removal of the drug [3, 33]. Contradictory results have been explained by differential sensitivity to mitotic arrest of various cell types [28]. Vasiliev and co-workers [29] reported that inhibition of directional migration of embryonic fibroblasts by VLB was completely reversible. This agrees with our finding that the effect of VLB, VCR and VDS on directional migration at anti-invasive doses or higher is rapidly reversible (Fig. 6). Reversibility of inhibition of invasion after treatment during 2 days with 1 μg/ml VCR has been reported previously [34]. The histology of the cultures used in the reversibility experiments indicated heterogeneity within individual populations of MO_4 cells. Such heterogeneity was also obvious from cloning of cells treated with vinca alkaloids [5]. We have not further analysed this particular aspect of drug activity because our primary interest was in complete inhibition of invasion. Populations of tumor cells are usually also heterogeneous with regard to invasiveness [35, 36]. We consider that the tumor remains potentially malignant as long as a few cells are able to invade and to divide.

Caution is needed in extrapolating our *in vitro* results to the clinic. Anti-invasive concentrations *in vitro* represent, nevertheless, plasma levels which may be achieved with clinically employed doses [2, 37]. Therefore, it is legitimate to accept that the anti-invasive effect of the vinca alkaloids may contribute to their antitumoral activity.

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