Arginine deprivation, growth inhibition and tumour cell death: 2. Enzymatic degradation of arginine in normal and malignant cell cultures

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A major aim of our work has been to lower arginine levels in vitro and in vivo to arrest the growth of cells, and selectively cause the death of cells of malignant phenotype (Scott et al., 2000; Wheatley, 1998). Normal cells with stringent checkpoints respond to nutrient deprivation by arresting cells in G0, from which the majority can be recovered several weeks later. Lowering arginine levels presents no difficulty in culture, and most previous studies have concentrated on arginine-deficient media containing dialysed serum. A closer comparison with in vivo work would be to degrade arginine in situ with enzymes, the three main contenders being arginase, arginine decarboxylase and arginine deiminase. The ultimate objective is to sustain free plasma or medium arginine at <5 μM (preferably ~1 μM) long enough to procure cell death, which is clearly going to be much more difficult to accomplish in vivo than in culture. Our present experiments were designed to give further insight into the comparative efficacy of two of the arginine-catabolising enzymes in vitro, viz arginase and arginine decarboxylase, which could lead to improvements in their deployment in the in vivo treatment of cancer. Arginine deiminase will only be discussed here, because it generates ammonia and its toxic action on cells presents a problem that makes it less useful as a potential therapeutic agent (Wheatley and Campbell, 2002).

The specific objectives in the experiments reported here were to demonstrate that (i) arginase will quickly and effectively mop up all free arginine in culture medium for a considerable time measured in days after a single treatment, (ii) citrulline in the absence of arginine will generate arginine, which is not accessible to degrading enzymes in the medium (i.e. the ‘free’ arginine does not equilibrate with the medium), and (iii) different cell types clearly have different citrulline requirements, which correlates with growth rate. Thus, the rate of conversion of citrulline to arginine becomes the rate-limiting step, and this becomes most obvious the faster the cells grow.

Removal of arginine from the body under normal physiological conditions results in a powerful boost to the homeostatic mechanisms by which free amino-acid levels in the blood are restored. In addition to the release of considerable amounts of free amino acids liberated by skeletal muscle breakdown, the gut/kidney axis (Wu and Morris, 1998) is involved in arginine metabolism, since citrulline biosynthesis occurs in the former organ and is converted to arginine in the urea cycle by the actions of argininosuccinate synthetase (AS; EC 6.3.4.5) and argininosuccinate lyase (AL; EC 4.3.2.1) in the kidney (Schimke, 1964).

Some cells are clearly more adept than others at circumventing arginine deficiency by recycling enzyme products. The conversion of ornithine to citrulline requires ornithine carbamoyl transferase...
Arginine degradation in culture

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Table I Properties of some of the more relevant arginine-metabolising enzymes in this study

| Enzyme                           | EC number     | Substrate(s)              | Cofactors/ions/etc | Products                          | $K_m$ (mM) |
|----------------------------------|---------------|---------------------------|--------------------|-----------------------------------|------------|
| Arginase                         | 3.5.3.1       | Arginine                  | Mn$^{2+}$          | Ornithine and urea                 | 10.5       |
| Arginine decarboxylase           | 4.1.1.19      | Arginine                  | Pyridoxal phosphate| Arginine and CO$_2$               | ~1         |
| Arginine deiminase               | 3.5.3.6       | Arginine                  | Mg$^{2+}$          | Citrulline and NH$_3$              | ~0.1–0.2   |
| Nitric oxide synthase            | 1.14.13.39    | Arginine (made from citrulline) and O$_2$ | NADPH→NADP$^+$     | NO and citrulline                 | 0.03       |

Arginine has arguably the most complex metabolic pathways of any amino acid within the cell and the body. It can be removed by dietary restriction, dialysis or enzymatic destruction. The first method has always presented problems in the preparation of suitable diets, and of compliance (Wheatley, 1998). The second is experimental, and the technology needed to achieve low blood arginine probably will avert the need for dietary or dialytic efforts until the third can be adopted (Sugimura et al., 1992), and therefore ornithine rarely substitutes for arginine in culture (Eagle, 1955). On the other hand, Eagle (1959) found that citrulline can substitute for arginine, and this is so in all malignant and normal cells cultured in arginine-free medium (AFM) that we have so far investigated (Wheatley et al., 2000). The question now is whether citrulline metabolism interferes with or circumvents enzymatic efforts to reduce free arginine to negligible levels in cultures.

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diluted 1:10 and dialysed against 0.9% saline along with the cofactors pyridoxal-5-phosphate (1.2 μM) and MgCl₂ (4 mM).

**Enzyme activity**

Enzyme activities were analysed *in vitro* over time at 37°C in a humidified atmosphere of 5% CO₂. Arginase was added to basic RPMI medium (without foetal calf serum) with L-arginine at 1 mM. The reaction was stopped at selected time points by the addition of 10% sulphosalicyclic acid (Sigma), which precipitated the protein at 4°C for 30 min. The resulting samples were centrifuged at 13 000 rpm for 10 min in a microcentrifuge and the supernatant was filtered through a Millex R-GV 0.22 μm filter (Millipore, Bedford, MA, USA). Arginase and ornithine were measured using a Biochrome 20 Amino Acid Analyser (Amersham Pharmacia Biotech, Cambridge, UK). Arginase activity was calculated in units, 1 unit being the amount of enzyme catabolising 1 μmol of arginine to ornithine (and urea) per minute per milligram of protein. Similarly, a unit of arginine decarboxylase was the amount of enzyme catabolising 1 μmol of arginine to ornithine and urea at pH 9.5 and 37°C. Specific activity of arginase can therefore be defined as the amount of enzyme required to convert 1 μmol arginine to ornithine and urea per minute per milligram of protein produced, calibrated against known standards within the range 10–100 U/C.

**Ornithine assay**

Arginase activity was also assayed by the method of Ochoa et al. (2000), which uses ninhydrin to detect the ornithine concentration, within each sample by a colorimetric procedure measured on the spectrophotometer at ~515 nm wavelength (Chinard, 1952). The specific activity of arginase can therefore be defined as the amount of ornithine (μmol) per minute per milligram of protein produced, calibrated against known standards within the range 10–100 μmol.

**Protein analysis**

An adaptation of the Lowry method (Oyama and Eagle, 1952) was used in conjunction with the above ornithine assay to determine accurately the protein concentrations of the various samples. The specific activities of the enzymes were required for accurate comparisons between the different preparations and batches.

**Statistics**

Comparisons have been analysed by the Student’s ‘t’-test only where appropriate, with significance being taken as P<0.05. Small deviations have not been subject to rigorous inspection at this stage because these will not provide the level of discrimination sought between malignant and normal cell responses.

**RESULTS**

**Human diploid fibroblasts**

Fibroblasts growing with a generation time of about 36 h were arrested in AFM. The cell number remained almost constant for >6 days (Figure 1A), although sometimes a small initial fall in number was seen. The cells could be kept at 37°C under these deprivation conditions for at least 3 weeks and probably considerably longer without serious loss of viability, although we show in Figure 1B rescue following a medium change into arginine-containing medium after 5 days of deprivation. There was little cell loss early in the rescue, but recovery required 5–6 days before growth kinetics approached those of the positive controls (which by this time had reached stationary phase).

Almost identical data were recorded for cultures in complete medium treated with either bovine arginase or *E. coli* arginine decarboxylase (hereafter ETM1 and ETM2, respectively) in situ (Figure 1A). Arrested cells entered G1/G0, as previously described in AFM by Scott et al. (2000). In terms of cell growth inhibition kinetics sampled after 3 days treatment, arginase was active at 0.25 – 0.5 units ml⁻¹ (IC₅₀ ~ 0.3 units ml⁻¹), the inhibition becoming increasingly felt with time. It was more immediately and completely inhibitory at 1 unit ml⁻¹ and above, but with higher enzyme concentrations of up to 5 units ml⁻¹, depression of cell growth occurred earlier. The same was true of arginine decarboxylase (Figure 1A), but dose -- response curves will be dealt with in regard to the malignant cell lines for L1210 cells below. However, these results refer here to slow-growing fibroblasts, and we already know (Wheatley et al, 2002) that 1 unit ml⁻¹ arginase can reduce arginine to negligible levels in a matter of a ~3 – 6 h *in vitro* (see below and Discussion).

Citrulline added to the ETM1-treated cultures without removal of the enzyme permitted growth at approximately control rates (P>0.05, and at the same rate in AFM cultures (only one curve shown in Figure 1B for the sake of clarity). It was noteworthy that arginase failed to restore cell growth in ETM1-treated cultures.

Data for ornithine ‘rescue’ have been included in Figure 1A, but will be omitted throughout the rest of the present results because it (a)
allowed no recovery under any of the deprivation conditions we have used (in agreement with earlier findings of Wheatley et al., 2000), and (b) lacked any observable inhibition of ETM1, on which it is supposed to act by feedback inhibition (see Discussion).

ETM2 had a similar effect at 1 unit ml\(^{-1}\) to arginase (Figure 1A), and also responded to citrulline in an almost identical manner (Figure 1B, curve not shown for the sake of clarity). Arginine once again failed to allow recovery of ETM2-treated cultures in the continued presence of the enzyme, although the recovery of AFM-treated cells was identical to the positive control. With regard to the latter, recovery after a 5-day treatment with AFM required \(3 \times 10^3\) days to re-establish normal growth kinetics in fibroblasts (Figure 1C).

HeLa cells

The behaviour of HeLa cells (generation time 23 – 24 h) to the presence of ETM1 and ETM2 was also very similar to our previous findings using only AFM, and are included in the current experiments for comparative purposes (Figure 2A). After the second day, cell death became progressively more obvious and by 5 days, few, if any, cells remained intact for some large multinucleated cells (Figure 3A and B). The effective dose – response curve was similar to that of fibroblasts, and citrulline circumvented the effects of AFM and ETM1, but here a significantly reduced growth rate of HeLa cells given 0.4 mM citrulline was observed, that is a level corresponding to the

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**Figure 1** (A) Growth kinetics of human diploid fibroblasts in (■) arginine-containing medium (positive controls), (□) AFM, and (○) arginine-containing medium plus 1 unit ml\(^{-1}\) arginase, or (▲) the same except with arginine decarboxylase at 1 unit ml\(^{-1}\), starting at day 1 (the latter was controlled with its own vehicle without arginine present in the curve with the Δ symbols). The total number of cells in each of three wells was averaged at each time-point, and the bars given are 1 s.d. around the mean. The significance of the differences between (□) and (○) or (▲) is very highly significant (\(P < 0.001\)), but the differences between (□), (Δ) and (○) are not significant. (B) Growth kinetics as in (A), but for cells with attempted rescue in the continued presence of the enzyme of the arginase-treated fibroblast cultures with 0.4 mM arginine (○), 0.4 mM citrulline (▲) or 0.4 mM ornithine (△). The difference between the positive control and the citrulline rescued cells was not significant (\(P > 0.05\)). (C) Recovery of fibroblast from 5 days of arginine deprivation prior to the addition of 0.4 mM arginine to the culture medium without a medium change. (■) Positive controls that have had AFM but arginine restored immediately after a medium change at day 1, compared with a second group (○) in which arginine was restored only on day 6, that is, after 5 days of complete arginine deprivation. Cell loss during this whole experiment was minimal.
0.4 mM arginine used throughout this work (Figure 2A, \( P < 0.05 > 0.01 \)). Once again, adding arginine to cultures treated with enzymes failed to rescue growth because, unlike citrulline, it was quickly degraded.

The response of HeLa to arginine decarboxylase was very similar to that seen in fibroblast cultures, with good inhibition seen at 1 unit ml\(^{-1}\), but more immediate and obvious cell death occurred with 5 units ml\(^{-1}\) (Figure 2B), although in terms of cell growth arrest measured after 3 days, the dose-response curve for ETM2 looks particularly sharp (see data below for L1210 cells). It was noteworthy, however, that 0.1 units ml\(^{-1}\) brought about cell arrest and the start of cell death after 2 days (\( P < 0.1 \)) and a highly significant fall by day 4 (\( P < 0.01 \)), indicating persistence of the enzyme as it gradually got to work with time (this enzyme being of...
higher affinity but lower capacity than arginase). Recovery with citrulline was effective, whereas arginine was again ineffective (the data mimic that of arginase recovery and are therefore not shown here).

To determine whether agmatine, the product of arginine decarboxylase, might per se have some effect on cell growth, we analysed it by adding agmatine as an exogenous preparation in the presence and absence of arginine. In the presence of 0.4 mM arginine, agmatine had no obvious effect, except some cytotoxicity at 5 mM. In the absence of arginine, agmatine was decidedly cytotoxic, progressively reducing HeLa cell number to well below that of the negative controls ($P<0.01$) as the dose increased and most notably at and above millimolar concentrations (Figure 2C; see Discussion).

**L1210 cells**

A series of similar experiments were carried out with L1210 cells, which have an exceedingly rapid growth rate (generation time 15 h) and often attain a final concentration of over 3 million cells per millilitre in suspension in normal medium. Clearly, this cell line has a considerably higher nutrient demand than HeLa cells, and a far greater one than fibroblasts. The basic findings confirm the above HeLa cell data, but the faster utilisation of arginine...
meant that cell death was more rapid in its absence, whether by AFM or ETM treatment (Figures 4A and B). Probability estimates are given in the figure legends.

The dose–response curve for arginine decarboxylase (Figure 4C) shows its considerable efficacy against L1210 cells after 3 days treatment. Most cells were irrecoverable after 4 days of AFM or ETM treatment. A few cells persisted, as shown by microscopic examination, these being predominantly large multinucleated cells, as seen with HeLa cell cultures (see Figure 3C and D, and Discussion).

Citrulline rescued the cells under AFM and ETM1 (arginase) or ETM2 (arginase decarboxylase) treatment, but arginine could only rescue cells from AFM (Figures 4A and 5A). Of interest is the finding that 0.4 mM arginine sustained normal growth when returned to AFM medium shortly after the start of incubation, but the kinetics of recovery were very significantly

*Figure 4* (A) Dose–response curve showing the inhibition of L1210 cell growth by Sigma (bovine liver) arginase used in the experiment shown in Figure 9. This preparation had about half its original activity after storage for some months, but shows that at a putative strength of 1 unit ml$^{-1}$ (approximately equal to 0.5 units ml$^{-1}$ of the original enzyme), it slowed cells after 3 days when cell counts were made, but not as immediately as a higher dose halted growth. The lowest values towards the higher enzyme concentrations correspond to the original inoculum of L1210 cells per well. (B) Effect of 0.5 units ml$^{-1}$ arginase (Sigma) on L1210 cells in suspension in arginine-containing medium given at day 1 ($\square$). The positive and negative controls in AFM are given as before by the symbols (■) and (□), respectively, but with dashed lines ($P < 0.01$). They show the expected responses. Enzyme-treated cultures (solid lines) and arginine (■) follow the same curve as the arginase alone (□), but the arginase-treated culture given 0.4 mM citrulline (Δ) shows recovery. However, its growth is $\sim 30\%$ the rate of the positive control, the difference being highly significantly slower ($P < 0.01$). The symbols of the last curve have been reduced to enable the 1 s.d. bars around the means of triplicate values to be seen. (C) Effect of arginine decarboxylase on L1210 cells in terms of the action of a Sigma preparation. The enzyme was freshly prepared and highly active at 1 unit ml$^{-1}$ within the 3 days at which assessment was made (cf. Figure 6A). The lowest value given here at the high concentrations corresponds approximately to the original inoculum density of the cells in each well (ie 100 000).
reduced with 0.4 mM citrulline (Figure 5A; cf HeLa cells in Figure 2A).

Recovery by removal of enzyme and arginine restoration

In the continuing presence of arginase, only large cells remained intact in L1210 cultures (Figure 3C and D), from which repopulation of the cultures on removal of the enzyme presumably occurred (see Discussion). Among L1210 cells that had been allowed to die for as long as 4 days in AFM, some began to show signs of recovery between 3 and 6 days later in arginine-containing medium, but cells left longer in AFM usually took 2 – 4 weeks to start growing exponentially again (Figure 5A). This experiment went for 27 days, by which time the cultures rescued after 8 and 9 days had not recovered. However, around 40 days later these cultures also rapidly grow. In subsequent experiments where cells were deprived for 14 days, a small percentage of wells (an average of ~2 – 3 per 12-well plate) showed that growth had resumed between 3 and 4 weeks after rescue (see Discussion).

Recombinant human arginase and ADC

The two preparations of recombinant human arginase expressed in E. coli had considerably higher specific activities than commercially available preparations of non-human enzymes (Table 2; Ikemoto et al, 1990). However, growth kinetics were affected in both HeLa and L1210 cell lines in a manner similar to any of the enzymes used in this study when they were given at equivalent units of activity (Figure 6A), all of which were fully effective for >3 days at 37°C. Pegylation of arginase reduces its activity.

Figure 5  (A) Effect of human recombinant arginine decarboxylase on L1210 cells. The data show the pronounced inhibition of growth of L1210 cells in the presence of this enzyme at 1 unit ml$^{-1}$ ( ), which is the same as in the negative control cells in AFM ( ). Note that the 0.4 mM citrulline rescue (Δ) gives only ~40% of the growth rate of the positive control (■), the difference being highly significant from positive controls lacking the enzyme (P < 0.01), but also equally significant in extent against the unrescued control. (B) L1210 cells were treated with 1 unit ml$^{-1}$ human recombinant arginase for periods up to 27 days, but rescued by medium change into complete RPMI 1640 with 10% normal foetal calf serum at 1 (■), 2 (□), 3 ( ), 4 ( ), 6 (Δ), 7 (Δ), 8 ( ) and 9 ( ) days. Recovery in the last two cases finally occurred at around 40 days (not shown), but the kinetics were not followed by cell counts.
In contrast to arginase, pegylation of recombinant arginine decarboxylase completely abolished its catabolic activity.

**DISCUSSION**

*In vitro* work shows that arginine withdrawal can have a devastating effect on malignant cells relative to normal cells, irrespective of whether deprivation is achieved by straightforward manipulation of the medium or *in situ* enzymatic catabolism of arginine. This exercise is easy to carry out *in vitro*, but similar control over the availability of arginine *in vivo* will need to be gained. However, using enzymes as a treatment is going to be a more practical proposition than, for example, extensive dialysis of the blood (Takaku et al., 1993). Although the behaviour of a tumour in the body may not accord with their responses *in vitro* for many obvious reasons, we need to demonstrate that it does work as well in principle with enzymes as with deficient medium, already indicated in some previous reports (Storr and Burton, 1974; Terayama et al., 1982; Savoca et al., 1984; Miyazaki et al., 1990; Gong et al., 2000; Wheatley et al., 2002).

**Enzymes as potential therapeutic agents**

The action of arginase is relatively swift and its capacity high (*Figure 2B*); it therefore clears the medium of arginine relatively quickly. Theoretically, the rate of the reaction at 1 unit ml⁻¹ converts 1 μmol arginine to ~1 μmol ornithine per minute (*Figure 6A*). In a medium or solution containing 400 μM arginine, each millilitre contains 0.4 μmol. In the presence of 1 unit of arginase, just 20 s should suffice to complete the conversion to ornithine. However, this calculation is based on arginase reacting with excess (4 mM) arginine under the conditions of a biochemical assay. The rate of conversion at low concentrations is slower, mainly because arginase has a high Kᵣ, and ornithine in the next figure. (*B*) Alterations in the levels of arginine and ornithine in RPMI medium measured in the amino-acid analyser after incubation for 5 and 10 min, to show their relative rates of arginine loss in unpegylated (■) and (□) pegylated enzyme, and correspondingly the rise in ornithine in unpegylated (●) and pegylated (○) arginase treatment set at precisely 1 unit ml⁻¹ of the original preparation in both cases.

Savoca et al. (1984), and our pegylation produced a 60% fall in activity (*Figure 6B*); thus it took over twice as long to reduce arginine to below detectable levels in our assay (i.e. <0.5 nmol). Pegylated arginase was more stable than the unpegylated form, and persisted without diminished vigour in culture for at least 8 days and probably a lot longer. This is evidenced by the fact that adding arginine to pegylated arginase-treated L1210 cells and leaving them for periods of up to 2 weeks did not permit recovery when arginine was restored at 0.4 μM.

Recombinant ADC worked as effectively as the Sigma preparation, giving almost identical data at equivalent units per millilitre.
Although accurate assessments have yet to be made with time that clearly extends to weeks at 37 °C. It is noteworthy that arginine deiminase can similarly be pegylated (Galea et al., 1996), but unfortunately arginine decarboxylase cannot (Table 2). The purity of arginase is high, and since it is activated at 60 °C, most other proteins come out of solution and any contaminating enzymes are more likely to be inactivated. We have run many experiments with 5% dialysed serum in the medium, and have found that there is no need to give additional Mn²⁺ in order to improve catalytic activity, and most preparations have been lyophilised with manganous salts present (Roholt and Greenberg, 1956). The strong inhibition of tumour cells by arginase indicates that it should be a very effective antitumour agent for treating animals, provided this effect can be translated into an effective in vivo protocol, as would seem possible from the work of Storr and Burton (1974), who obtained considerable tumour reduction after a remarkably short treatment time (3 – 4 days).

In contrast, arginine decarboxylase produces agmatine (Galea et al., 1996), which has the advantage both in vivo and in vitro of not being recycled to arginine. The bovine enzyme was as effective as the human recombinant form, and as arginase a unit for unit basis, but the former’s specific activity could be up to two, if not three, orders of magnitude less than that of human recombinant arginase (Table 2). Therefore, much more protein would have to be injected into animals to produce the same effect, which might create a problem if the preparation was significantly immunogenic. Our data indicate no obvious problem with agmatine toxicity in culture (Figure 2C), except when it exceeded millimolar levels in the absence of arginine, and therefore some doubt remains as to whether a build-up under such conditions is accompanied by toxic sequelae in the body. Agmatine had toxic effects similar to those of arginine both in vivo and in vitro, which further weakens this explanation, just as it does in other similar claims (Miyazaki et al., 1990; Sugimura et al., 1992; Takaku et al., 1992, 1993). Therefore, the only truly rational explanation remaining is that the reaction generates ammonia. In cultures, where ammonia cannot be detoxified, small traces of the free gas can be lethal to both normal and tumour cells. The irony is that arginase itself is required to assist in the detoxification of ammonia. We do note, however, that some cells are incapable of utilising citrulline instead of arginine (Campbell et al, in press), and these might prove to be more susceptible to arginine deiminase provided it can be effective when arginine concentration is not too high (so that ammonia generation is limited).

Complications with nitric oxide (NO) metabolism

One further enzyme might also be further mentioned, viz nitric oxide synthase (Wu and Morris, 1998). This produces citrulline and NO from arginine, but this arginase has to be channelled initially from citrulline in what amounts to a tight circular coupling (Chang et al, 2001). It is of little use, therefore, as a catabolic enzyme, especially with an extremely low Km (Tables 1 and 2; Tatoyan and Giulivi, 1998), and its ability to deal with only nanomolar quantities of arginine shows it to be both inadequate and inappropriate. One apparent problem with arginine-catabolising enzymes such as arginase is that removal of arginine from the body was originally thought to deprive NO synthase of its substrate, and lead to loss of vascular tone and signalling in many other vital functions. However, new evidence strongly suggests that a special arginine pool may be generated, which is closely linked or channelled with nitric oxide synthase in appropriate cells (Flam et al., 2001).

Implications for future applications

We still face problems with tumours and their hosts that are good converters of citrulline to arginine, although we have also clearly shown here (and the work in progress) that citrulline conversion cannot so often keep up with arginine demands of the faster-growing tumour cells. The most suitable subjects for treatment both in culture and in vivo will undoubtedly be those tumours that are poor converters, for example, certain hepatomas and melanomas (Sugimura et al., 1992).

A further problem that cannot be ignored in this complicated scenario is the control of the breakdown of nonessential proteins by the cells of the body (Grisolia and Wheatley, 1984), which can release amino acids in sufficient quantity to circumvent the best efforts to induce a true arginine-deficient state. We also do not know how much this, and scavenging from dead cells, plays a part in keeping at least some cells alive in arginine deprivation conditions after many days (Figure 5B). Some procedures can be introduced to help reduce protein breakdown, and these might be needed alongside the catabolic enzymes, although if the findings of Takaku et al. (1993) are correct, pegylated arginine deiminase can keep arginine below detectable levels for at least 8 days in mice injected i.v. with a single bolus of 5 units of enzyme. Thus, it is difficult to assess the extent to which enzyme-induced deprivation will be affected by protein turnover without many more careful studies. Nevertheless, some cells can and do survive enzyme treatment or AFM in culture, even if these are very exceptional cases, after a period of a week or more of exposure. We are convinced that the only cells that seem to persist for any length of time are the very large multinucleated and often highly polyploid cells, as seen in HeLa and L1210 cultures (Figure 3). If Erenpreisa and Cragg (2001) are correct, then these large cells, similar to those surviving apparently lethal doses of radiation, may later undergo ‘reduction divisions’, creating one or two small progenitor cells that repopulate the cultures. However, it will take time to
determine whether this is indeed the mechanism that also occurs in our cultures after arginine deprivation.

CONCLUSION

We conclude that enzymatic degradation is a reasonable procedure for removing arginine from culture media, and this has also been shown to be effective in vivo by others, especially in pegylated forms of arginine. Deiminase and arginase. However, each enzyme has its shortcomings, and additional strategies will be needed to circumvent them. Arginase remains a strong contender, however, because of its high specific activity, and production of ornithine rather than citrulline (Wheatley et al., 2002). The major obstacles are, (i) efficient recycling where an intact urea cycle operates, leaving only some of those in which the urea cycle has been compromised as susceptible, and (ii) in the large-scale protein turnover that can easily subvert efforts to maintain free arginine at micromolar levels.

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