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REVIEW

Arginine Catabolism, Liver Extracts and Cancer

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Although it is self evident that cells will not grow in amino acid deficient medium, an observation less well appreciated is that malignant cells are particularly vulnerable to such deprivation, which can lead to their rapid demise. Indeed, the more flagrantly malignant the phenotype (anaplastic the tumor), the more susceptible the cells seem to be to deprivation. While some attempts to employ this strategy in cancer treatment have been made, the difference between normal and malignant cells should be more fully exploited as a means of *selectively* eliminating tumor cell populations. To be successful, information on differences between the normal and the deranged cell cycle engine and checkpoints, especially how these are affected by deprivation, is of crucial importance. Since it is only recently that the controls at restriction points have been elucidated, it is little surprise that earlier attempts to control tumor cell growth by limiting the availability of an essential amino acid have met with limited success. Studies havebeen sporadic and isolated, often with little more than anecdotal descriptions as far as clinical work was concerned. This review concentrates on what has been accomplished primarily *in vitro* and since about 1950 with regard to *arginine* catabolism, while recognising that other essential amino acids have also been the focus of attention by some investigators. Treatments have included medium and plasma manipulation, dietary control, enzymatic degradation, and the use of liver extracts. On some occasions, substitution of amino acid analogues has been explored. It is argued that current knowledge, combined with past experience, calls for a much closer examination of the full potential of amino acid (and specifically arginine) deprivation as a means of controlling tumor growth, with greater attention to protocols that might be used to treat human cancers. (Pathology Oncology Research Vol 8, No 1, 18–25, 2002)

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Introduction

A relationship has been recognised for many years between arginine and cancer, while latterly, another relationship between arginase and cancer has emerged. Sufficient information exists for us to pay closer attention to both these correlations, and furthermore to explore how these correlations might themselves be related, as would seem obvious at face value. However, in the first review, we will consider the former relationship. Before presenting some of the data, it is worth keeping in mind several questions.

(i) Will reducing the availability of arginine selectively affect tumor growth?
(ii) Furthermore, can catabolic destruction of arginine not only arrest growth, but actually kill cancer cells?
(iii) Can tumors that are most likely to respond strongly to deprivation be quickly and easily identified?

Accepting that there may be reason to believe that some of these can be answered in the affirmative, one further question is whether these correlations in particular, as opposed to many hundreds of others that can be made in the field of cancer, merit deeper examination in the interests of the detection, diagnosis, treatment and subsequent prognosis of cancer.

We are deliberately focusing on arginine because this is the amino acid – for reasons explained in full elsewhere[1] – that might prove most suitable to manipulate in future cancer treatments. Studies from quite different groups support

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this contention (e.g. Currie and Basham,[2] and see below), and this review sets out the main reasons for pursuing this strategy based on an appraisal of what has been achieved to date. And finally by way of introduction, mention must be made of the diametrically opposite stance to arginine deprivation in cancer treatment, i.e. arginine supplementation. Since this has been discussed and reviewed in as many words as those reporting the hard data behind them,[3,4] suffice it to say that the two rationalisations for arginine supplementation of patients with cancer have been that it stimulates the immune surveillance in tumors,[5] and can be a precursor of nitric oxide generation, which in free radical form assists macrophages or tumor cells themselves in expressing their full cytocidal potential. In the absence of adequate proof from clinical trials that an extra 30 grammes of arginine daily has any clearly beneficial effect,[4] even when combined with other treatment modalities, we might now consider the opposite strategy of arginine deprivation might be worthy of evaluation clinical.

Deprivation: a different rationale

Amino acid deprivation offers a refreshingly different approach in that it moves away from traditional chemotherapy[6] and does not *per se* introduce toxins into the system, although with the proviso that deprivation can also cause physiological and metabolic imbalances,[7] and these complications have to be overcome (controlled). To provide a rational protocol, application needs to be based on an intimate knowledge of cell cycle perturbations in malignant cells, which has fortunately been emerging in recent years.

Arginine requirement and cancer

Although work on amino acids in cancer predates 1940, the first intimation that brought the idea on to the scene as far as arginine is concerned was a report by Bach and Lasnitski[8], which indicated that arginine stimulated mitotic activity in mouse tumors, evident from increased mitotic figures in histological sections. They surmised that rapidly growing tumors have a greater demand for arginine than the body can normally supply. Since body homeostasis tends to keep amino acid levels in the blood relatively constant, it is worth knowing whether their mice were unwittingly undernourished for this amino acid. Assuming an *ad libitum* diet was available and the body's own ability to generate arginine to a considerable extent through the urea cycle, as also in the kidney and also from gut bacteria, it is surprising that providing additional arginine should have this effect. However, it can also be argued that, since arginine is "semi-essential" (because growing animals require exogenous supplies of it), endogenous synthesis might not be able to keep up with demand. Similarly, the growth of a tumor in an adult mouse could put the same strain on the supply system as the growing neonate.[9] If this is indeed the case, then a key difference in supply exists that gives another excellent reason for targeting arginine.

Purified liver arginase

These findings set off a flurry of activity in Bach's group in Bristol, UK, during the 1950's and early 1960's that has received much less attention than it deserves and was quite astonishingly overlooked (ignored) by many approaching the problem from a different direction (see *below*). Vrat[9] and Wiswell[10] only partially confirmed Bach and Lasnitski's findings, but Irons and Boyd[11] found that crude arginase (EC 3.5.3.1) could reduce tumor growth. Bach and Maws[12] found that the Jensen sarcoma in rats utilised large amounts of arginine, and considered that arginase might therefore reduce tumor growth, and at much the same time Bach and Simon-Reuss[13] demonstrated that arginase inhibited or slowed the growth of these tumor cells in culture. Throughout this period and for some years afterwards, this group went diligently into the business of purifying bovine arginase (EC 3.5.3.1) for their experimental treatment of tumors. Bovine liver is relatively rich in this enzyme,[14,15] just as the livers of many other species, especially those of canivores, abound in it. They purified a 138 kD enzyme, and Bach and Swaine[16] subsequently treated CB rats with Walker 256 carcinoma i.p. daily or every other day for 4 days with it. Within that short space of time, 31-71% reduction in tumor growth was recorded, but paradoxically high dose levels of enzyme seemed less effective. More surprising is that, having obtained this level of response so quickly, these findings were not followed up with more prolonged treatments at the most effective dose level. Furthermore, the work failed to attract interest elsewhere during a period when new approaches to cancer therapy were being actively sought.

One possible explanation is that a different strategy was emerging, since during the preceding decade the structure and importance of DNA had been elucidated. It followed that, if protein synthesis can be curtailed by amino acid deprivation through their catabolic destruction, the semi-conservative replication of DNA during S-phase might be a point of attack, one method being to destroy the supply of nucleotide bases. Enzymes such as xanthine oxidase became the focus of attention of the anti-cancer drug scene.[17] While this might be just speculation, whatever the true explanation might be, a parallel story was unfolding.

Liver extracts: a separate story

Lieberman and Ove[18] were among the first credited with the observation that an extract of liver inhibited tumor cell growth in culture. Their paper has been extensively quoted and yet it is a brief note with far too little methodology

to repeat their experiment, as well as a great deal of unwarranted (unsubstantiated) speculation. But knowledge of the work of the Bach group should have led to the obvious connection, and the literature should have quickly provided the clues, even in those days. Since arginase breaks down an essential amino acid, and it was already common knowledge that the liver is a rich source of it, the extract story is a classic case of "reinventing the wheel". It subsequently became clear through the observations of others, notably investigators such as Freed and Schatz,[19] who confirmed that arginine deprivation resulted in chromosomal abnormalities in dividing cells in culture, that liver contained some agent that inhibited cell growth. At much the same time, Holley[20] confirmed these original observations, and conclusively showed that arginine rescued cells treated with liver extract, which must therefore have been rich in arginase, since no other amino acid was effective. Further corroboration came from others, including Umeda et al,[21] Freed and Sorof,[22] and Sasaki and Terayama,[23] the last group suggesting that two extractable arginases might be present, a membrane bound version of 40 kD (protein) and a cytosolic one of 100-120 kD (a proteoglycan). By now the notion that arginase was not solely a requirement of the urea cycle had been accepted. Miyamoto and Terayama[24] inhibited DNA synthesis in Ehrlich ascites and AH-414 hepatoma withdrawn from mice and treated with liver extracts, but argued that arginase alone might not necessarily be or was not entirely responsible for the action because thymidine hydrolase might also be present. Caution has always been needed in interpreting such data because "extracts" are complex mixtures that invariably contain many active enzymes and factors,[25,26] which is why Bach and his co-workers had struggled for years to isolate pure arginase.

Renewed interest in arginase in cancer treatment

By now the relevance of these findings to cancer had taken on a new significance. Osunkoya, Adler and Smith[27] noted that Burkitt lymphoma responded to arginase. Storr and Burton[28] followed this by clearly demonstrating that mouse lymphoma cells newly taken into culture in Fisher's medium (containing a low level of 80 μM arginine) responded to arginase treatment by dying within 6-24 h. They attempted unsuccessfully to treat mice with L1210 and L5178Y ascites, but noted that arginase must have been actively working because of the generation of high levels of ornithine in the ascitic fluid. Since ornithine can be reconverted to arginine in the body, the effect on tumor cells was not surprisingly poor and minimal, especially in mice.

Subsequently and rather anecdotally, a number of reports have shown reasonable anti-tumor activity with arginase,[29-32] but again these bursts of research activities occurred without much co-ordination and follow up. But one valuable development was that the arginase attack on L5178Y cells could be sustained for considerably longer by "pegylating" the enzyme, i.e. extending the protein by the covalent attachment of polyethyleneglycol chains, which reduced the half-life of the enzyme both *in vivo* and *in vitro*[33]. This draws attention to a major problem *in vivo*, one of sustaining the action of a catabolic enzyme if tumor cells are to be suppressed for the lengths of time required for their arrest and/or demise, since the very rapid kill rate reported by Storr and Burton[28] has not been corroborated.

One conclusion to be drawn from these studies is that tumor cell seems to be susceptible to arginine withdrawal such that, at least *in vitro*, they die *rather than just arrest within the cell cycle*, contrasting strongly with the situation in normal cell populations.[2,34] Whether deprivation of cancer cells *in vivo* leads to the same sort of demise remains a key question, therefore, in the translation of the behaviour in culture to that which needs to be achieved in the body.

The difference in these two situations probably lies in the product of arginase activity and its fate. In culture, ornithine cannot be recycled to form arginine; the enzymes to do this are lacking, except in some minimal deviation hepatoma cell lines that retain a limited capacity to convert ornithine back arginine.[35] In the body, however, ornithine can be converted to citrulline, and this regenerates arginine through argininosuccinase synthetase (AS) and argininosuccinate lyase (ASL), along with the incorporation of aspartate. It would appear that an intact urea cycle *in vivo* should effectively subvert efforts to kill tumor cells, making it a puzzle as to why quite large suppressions in tumor growth have been reported in some of the above-mentioned experiments, unless it was simply that growth was slowed considerably in relative terms to the rapid growth of tumor in controls, and cell death was not significant. However, if we could establish why cultured tumor cells tend to die rather than arrest in cycle under arginine deprivation, an effective strategy that selectively eliminates tumor cells should emerge.

Importance of natural tissue (cellular) levels of arginase

The presence of arginase is important to all cells because they need to convert arginine to ornithine so that the precursor of putrescine in the polyamine pathway is supplied, from which spermine and spermidine can also be derived. Cells lacking or low in this enzyme may show curtailed growth, e.g. CHO A7 cells.[36,37] Unless such enzyme activity occurs by directed transfer (channelling), it is difficult to see how some of the extraordinarily low activity seen in many cell lines can cope with the requirement. *In vivo* the situation differs because release of arginase from the liver sustains a plasma level that seems to cope with demand. The leakage of excessive amounts of arginase from the

liver can be induced to send arginine down to very low levels. This follows injury to the liver, which under operative procedures can be achieved e.g. by controlled cryoablation.[38] This is a drastic measure that has had some limited success with widespread liver metastases, but can only be taken so far before a halt has to be called to repeated treatments. This topic is only briefly mentioned here, and will not be reviewed further, but continues to be a method whereby the natural arginase levels of the body are boosted by efflux from the liver and can quite drastically lower arginine availability.

There are two other situations in which levels of arginase *per se* become of interest. One concerns the level of the enzyme within cells themselves. Pohjanpelto and Hölltä[39] have shown high variability in three levels of arginase in established tumor and normal cell lines, from which the conclusion is clearly that formation of ornithine from arginine cannot usually be a rate-limiting step in cell proliferation. Nevertheless, if arginine becomes restricted for other reasons (i.e. than cells being in a depleted medium), then there could be consequences of an inadequate supply of the precursor of polyamines. Levels of arginase in cancers and in cancer-bearing host plasma have been examined,[40,41] but on the question of whether arginase will be a useful tumor marker, it is too early to comment, a great deal more data needing to be collected and correlated.

The second relates to Dvorak's suggestion[42] that tumors are like "wounds that do not heal". The one clear outcome of Barbul et al. findings[43] is that arginine plays a number of key roles at appropriate times in wound healing, and that the balance of local supplies of arginine and levels of arginase need to be finely tuned during the normal healing process. But when it is abnormal or behaves like a tumor, the balance may be lost, and we need to know what the changes are and if possible how to rectify them. We need to turn attention to two quite different enzymes that have been investigated in relation to the treatment of cancers.

Arginine deiminase

Action on non-leukemic murine tumors

This is an enzyme secreted by several species of mycoplasma, notably *Mycoplasma arginini*. Barile and Levinthan[44] noted that *Mycoplasma arginini* released an enzyme inhibiting lymphocyte proliferation, and Kraemer[45] made a similar observation on mycoplasma medium that inhibited the growth of lymphomas. Gill and Pan[46] identified the enzyme responsible as arginine deiminase (EC 3.5.3.6; or arginine dihydrolase). One notable feature of arginine deiminase is its much lower Km than arginase, Miyazaki et al[47] finding it about 1000 times more effective than the latter on a mass per ml basis (5 $\mu g\ ml^{-1}$) after purifying it from mycoplasma broth. It had a greater inhibitory effect on SV-40 transformed cell lines than control cells, but was also moderately inhibitory to 10 tumor cell lines. However, their data do not in general support the latter contention, except perhaps in the case of one heptoma cell line. While arginine deiminase isolation, purification and ability to degrade arginine have been reported by Sugimura et al[48] and Takaku et al,[49] its anti-proliferative activity results from the conversion of arginine to citrulline. However, citrulline is converted back to arginine by almost all mammalian cells in culture, and therefore it follows that a truly arginine-deficient state would be difficult to achieve. Nevertheless, diminution in the growth of a number of murine tumors has been noted.[50]

Takaku et al[49] tested the enzyme *in vitro* and found that their arginine deiminase actively suppressed growth in all 6 murine lines used, with low IC50 values of ~10 ng ml^{-1}. They also showed that as little as 0.2 mg $mouse^{-1}$ i.p prolonged the survival of mice well beyond those of the untreated controls. A bolus of 5 units i.v. catabolised free arginine to negligible level within 5 min and the level remained undetectable for no less than 8 days. Once again, these are quite remarkable results that should have provided the incentive for a more determined exploration of the anti-tumor activity of arginine deiminase.

Deiminase: a more potent antileukemic enzyme than asparaginase?

More recently attention has focused on its antileukemic action.[51,52] Gong et al[53] suggested that arginine deiminase is more effective against mouse leukemia than L-asparaginase, which has had a place in human antileukemic protocols for twenty years. However, L-asparaginase has some undesirable side effects, such as anaphylaxis and coagulopathy, that clinicians would prefer to avoid.[54] Komada et al[51] presented evidence that T cells of the lymphoblastoid series were inhibited by their deiminase enzyme whereas B cells and the myeloid line were unaffected. Latterly Gong et al[53] have shown that four malignant (leukemic) cells lines could be inhibited and undergo apoptosis as a result of arginine deiminase treatment. Their data are unclear as to whether inhibition of growth related specifically to arginine depletion rather than some "more direct" (unspecified) action they suspected was involved. Indeed, this might explain why a generally acting enzyme that depletes arginine availability might affect T lymphoblast cells but not touch the B series. Furthermore, they reported cycle arrest that in some cases seemed to be in G1 and in others S phase (or an as yet unrecognised S_0 state). This leaves the possibility that cell cycle control has been compromised by arginine deficiency (or some "direct action" of arginine deiminase) open to debate, with the possible consequences that cells might either recover (from arrested G1) or progress into apoptotic cell death (if prolonged S phase occurred). While these

authors claimed that the earlier papers from the Japanese groups had confirmed their findings, their own evidence actually provides only weak confirmatory evidence in favour of these reports that *preceded* their own work. Indeed, careful analysis of the claim by Gong et al[53] that arginine deiminase is superior to asparaginase in the treatment of leukemia is readily challenged from their own data, because the number of units of arginine deiminase required is not that different from the units of asparaginase needed, assuming that the enzyme which they themselves purified had such a a remarkably high activity of 50-100, 000 units per mg (quoted as I unit per 10-20 ng). If there is any substance in their reports, then the experimental data needs to be substantially clearer and more convincing. The importance of such a supposition, however, cannot be ignored, and careful comparative work must be carried out before a new enzyme can be considered a potential replacement for the well tried and tested L-asparaginase used in the therapy of human leukemia. Furthermore, for reasons already mentioned above, we believe that arginase might be a better enzyme than arginine deiminase, because its product (ornithine) cannot be reconverted to the substrate by most cultured cells (other than by a few minimal- deviation hepatoma cell lines) and ammonia is not evolved (see below).

We also think it important to check cell recovery after treatment rather than rely on cell counts alone to suppose that cell death (by apoptosis) has occurred. In view of our more thorough coverage of the behaviour of cells in arginine-deprived medium, along with evidence that arginase achieves the same objective when added to arginine-containing medium, we have set out to make a strict comparison between the effects of arginine-free medium on L1210 cells (a very fast growing murine leukemia strain) and those of arginase added to complete medium. The latter was then compared with the effects of asparagine deprivation brought about by asparaginase treatment of complete medium or asparagine deprivation in the medium formulation. Our data indicates that arginase has a faster and more devastating effect on leukemic cells than asparaginase, but further works needs to be done to establish whether the treatment works on a wider panel of leukemic cell lines before making comparison between the two enzymes both *in vitro* and *in vivo* on appropriate murine model tumors.

Complications of arginine deiminase treatment

Although arginine deiminase still seems to show some promise, its main product other than citrulline is ammonia. Since citrulline can be efficiently reconverted to arginine by almost all cells, it is surprising that these reports and some subsequent ones (see below) were able to show effects on tumor cell growth *in vivo* and *in vitro*, and the answer to this problem remains to be resolved. It would be ironic if ammonia itself was primarily involved in causing tumor arrest, although the paradoxical result of Gong et al[53] that arginine deiminase worked better on leukemic cells given arginine than on cells treated in the same way but *without* arginine in the medium argues strongly that this is indeed the case.

The only way in which arginine deiminase can really be expected to work, therefore, is where tumor cells are deficient in the enzymes needed to reconvert citrulline to arginine, i.e. argininosuccinate synthetase and argininosuccinate lyase. Schimke[55] examined this pathway in detail in tumor cells and noted its suppression at high arginine levels, but a 2-15 fold elevation soon after arginine fell back to limiting (low) concentrations. In contrast, Snodgrass and Lin[36] found no such elevation of biosynthetic enzyme of the urea cycle in hepatoma $7800C_1$ cells in arginine deficiency. Unless cells are truly defective in this pathway, citrulline production as a result of enzymatic catabolism of arginine (by deiminase, or any other enzyme using it as a substrate) will always subvert attempts to achieve an adequately deficient arginine state. But Sugimara et al[48] claimed that human melanoma – and possibly some hepatoma – cell lines generally lack the first of the converting enzymes, argininosuccinate synthetase (AS), whereas most other cell types have an intact pathway from citrulline to arginine. This purported feature of melanoma cells is not shared by murine melanomas, since arginine-deprived B16F10 cells utilise citrulline (Campbell and Wheatley, unpublished). Nevertheless, the AS deficiency of some human melanomas and hepatomas could be the key to the specific application of arginine deprivation, especially as such tumors are currently given a high priority. The therapeutic use of arginine deiminase deserves to be followed up with alacrity.

Arginine decarboxylase

Arginine decarboxylase (EC 4.1.1.19) has been found in bacteria and many plants. It is inducible in bacteria,[56] and the human form of the enzyme can be synthesised by bacteria following transfection.[57-59] Its products are agmatine[60] (which can be damaging to cells) and carbon dioxide. Agmatine in the presence of arginine is not particularly toxic, but when its concentration reaches millimolar levels, especially with a falling concentration of free arginine, cell growth is inhibited (Wheatley et al, unpublished observation). The value of this enzyme is that it has a low Km and can rapidly degrade arginine both *in vivo* and *in vitro*, leaving a product that *does not* get converted back to arginine under normal physiological circumstances. This makes it a more desirable catabolic enzyme of arginine, but apart from our own experiences with it (to be published), no other group has seriously entertained the use of this expensive product. Recombinant human arginine decarboxylase produced by *E. coli*[59] is considerably more

active than Sigma enzymes prepared from natural straightforward enzyme extraction from other sources, both *in vivo* and *in vitro.* However, pegylated arginine decarboxylase loses almost all its activity, probably because it has far fewer lysine residues than arginase. While this will diminish its potential use *in vivo,* this enzyme still requires to be more fully evaluated.

General concluding remarks

Many of the issues discussed above have to be carefully considered if the full potential of these different catabolic enzymes of arginine as inhibitors of tumor cell proliferation *in vitro* and *in vivo* is to be realised. There is no question that the track record shows them all to have merits worthy of deeper investigation. Some of their disadvantages can almost certainly be circumvented, but preparation in quantities sufficient to treat human patients poses a problem that requires considerable investment in time, money and know-how for the future. Furthermore, an efficient means of selecting tumor-types and/or patients that would respond best to the treatment has to be operative. On this account, our *in vitro* studies of manipulation of arginine concentration are reatively easy, and quickly indicate which tumors are going to respond well. The 24 cell lines studied in our earlier work[34] now extends to about 36 malignant cell lines, with still over 80% being sensitive to deprivation, with none of the survivors of shorter term treatments developing resistance to repeated exposures.

These enzymes can probably be used in a rational way which is in keeping with our hypothesis that under deprivation conditions, normal cells enter quiescence from which they can later be recovered, whereas tumor cells generally remain in cycle and – for reasons yet to be fully elucidated – die within a relatively short space of time (days). An alternative strategy exist to using enzymes, namely the purging of arginine from the supply. This is a simple manoeuvre in cell culture, but a major problem for the clinican left with only currently available protocols. However, if the need or demand is great enough, such procedures will probably be attempted in the near future.

(Added note:

In this review, we have deliberately avoided dealing with the best controls for comparison with enzymatic degradation of arginine, that of studying cells in truly arginine-free conditions, which can be achieved in culture by careful medium formulation. This is because we have already dealt with this topic on an earlier occasion[61] as a lead, in to some of our own *in vitro* studies.[34] Nevertheless, it is worth mentioning that enzyme catabolism of arginine does achieve much the same end, although probably not quite as effectively as experimental manipulation of the medium content. The scavenging of arginine, particularly from existing proteins through turnover, has been generally overlooked, but since this does play an important role in achieving low arginine concentrations *in vivo*, it needs be examined more thoroughly *in vitro* (work in progress). As already indicated, the problems of lowering body arginine levels for therapeutic benefit are highly challenging, and require three elements: (a) strict control of diet, (b) the use of an arginine purging mechanism (enzyme or dialysis, or both), and (c) a means of preventing/reducing compensatory protein turnover, which normally subverts all efforts through the amino acid homeostasis mechanism. Progress towards this end is being made and will be reported elsewhere.

We have also ignored another enzyme that utilises arginine as its substrate, *viz*. nitric oxide synthase. The fact that arginine deprivation will affect nitric oxide generation, which in turn will influence angiogenesis in tumor development[62] and blood vessel tone in general, as well as altering macrophage function, indicates that there will be many complicating factors due to a lacko of a substrate for this constitutive and inducible enzyme, especially *in vivo.* However, this review is not the place to deal with this matter, which will require consideration in its own right.

Finally, there is now growing evidence of elevated levels of arginase in the blood or tissues of human patients with tumors. The relevance of this elevation is not understood, and we are still in the process of gathering sufficient data to get better insight into this finding. It will also have to be the subject of a later review.)

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