THE INCREDIBLE SHRINKING CHALONE

Leonard M. PATT and John C. HOUCK

Virginia Mason Research Center, 1000 Seneca Street, Seattle, WA 98101, USA

1 Introduction

Over thirty years ago it was proposed that the mitosis of cells may be controlled by a negative feed-back mechanism [1,2], an idea which was substantially applied to the regulation of epidermal cell growth [3,4] These workers suggested that endogenous mitotic inhibitors were produced by cells which would inhibit only that specific cell type Historically, these inhibitors have been referred to as 'chalones', from the Greek word meaning to brake or slow down. The history of the chalone concept has been reviewed [5] and is too vast to be treated here. The primary characteristics of a classic chalone have been defined as: (1) total cell specificity, (2) species non-specific, (3) reversible; and (4) non-cytotoxic. Houck [6] covered the literature up to 1976 in detail and summarized the major biological evidence for the existence of chalones, a point which has not been unequivocally established It is a general criticism of chalone research that the theories have always been far in advance of the data. This has come about possibly because of the inherent difficulties in measuring cell inhibition in a specific manner, as opposed to measurement of cell growth stimulation However, it may not be justified to discard an attractive theory simply due to technical difficulties and weak supporting data

Here we are not concerned with the phenomenology of chalones and consider only recent developments in several areas which show progress toward purification and understanding the chemistry of various chalones Obviously, much of the biology and theory will not be relevant until the purified inhibitor is at hand Thus we do not present a comprehensive review of the literature and cite only general or representative papers in any area We consider two specific chalones in detail, lymphocyte and granulocyte, and several others briefly Considerable progress has been made with these two and they may be representative of chalones in general We also mention some problems recognized in research on growth inhibitors

2 Chalone systems

2 1 *Lymphocyte chalone*

Inhibitors of lymphocyte transformation have been shown in vitro in extracts of lymphoid tissue [7–15] This work (reviewed [16–18]) showed that extracts of spleen, thymus, or lymph nodes, contained a factor (or factors) with an app. M_r of 10 000–50 000 as determined by ultrafiltration, which would specifically inhibit the lectin-stimulated transformation of lymphocytes as well as graft-versus-host response and allograft rejection [19–21] Subsequently it was found [18,22–24] that part or all of the activity could be present at a much lower M_r Pre-treatment of the extracts with ribonuclease, acid, or streptomycin sulfate could cause the inhibitory activity to pass through a 10 000 M_r ultrafilter and be retained on a column of Sephadex G-25 The recognition of the low M_r nature of lymphocyte chalone has accelerated progress in its purification and (as always has been the case in chalone research) presented several new problems We present first the recent steps toward the purification of this factor

Most progress in the purification of an immunosuppressive factor has been reported by Lenfant et al [25,26] working with an extract of spleen This isolation scheme also gives some insight into the possible chemical nature of lymphocyte chalone The activity is extracted from spleen and a low M_r fraction obtained by ultrafiltration This is absorbed to and eluted from neutral alumina by organic solvent mixtures, fractionated on a Sephadex LH-20 column in an ethanol water mixture, and subjected to thin-layer

chromatography on cellulose plates. At this stage of purity, the factor was active at a dose of 2×10^{-7} mg in reducing the haemolysin plaque-forming response of mouse lymphocytes. They subjected their most purified fraction to high-pressure liquid chromatography (HPLC) where the activity was one of the last components eluted from the C_{18}-reversed phase column. No data has been forthcoming concerning the structure of their factor, nor on its specificity. It should be noted here that the authors reported that the activity was lost upon prolonged storage at $-20°C$, or by repeated freeze–thawing, which may hamper efforts to accumulate quantities of the active factor!

Several other research groups, including ourselves, have reported on the purification of low M_r inhibitors of lymphocytes. Maschler and Maurer [27] have begun the isolation of an M_r 1000–2000 chalone from bovine thymus which will inhibit colony growth of lymphocytes under serum free conditions. Two activities were observed by ultrafiltration, an unstable activity at M_r 10 000–30 000 and a stable activity at $M_r < 10 000$. The low M_r fraction eluted after bacitracin on Bio-Gel P-6 columns, indicating an $M_r < 1400$, and could be partially purified by DEAE-cellulose columns. This fraction was carefully tested and found to be non-cytotoxic and reversible, both properties necessary for a chalone. The activity, in contrast to that reported by Lenfant, was stable to repeated freeze–thaw cycles, and to storage at $4°C$ up to 120 days or $37°C$ for 24 h. The authors suggested, as have others, that the higher M_r inhibitory activity, which was unstable and variable in yield, was due to large carrier molecules which associated with the smaller factor.

Grundboeck-Jusko [24] has reported very similar results to the above in antimitotic fractions isolated from bovine spleen and assayed in vivo by examining the number of mitotic cells in lymphoid organs of treated animals. Two activities were observed at M_r 38 000 and 2100 from smooth microsomes and one at M_r 2500 extracted from rough microsomal fractions. From biological studies, it was concluded that the M_r 38 000 inhibitor was specific for B-cells while the smaller species demonstrated specificity for the T-cells.

We have found both high and low M_r inhibitory activity in our studies of a thymus derived factor which inhibits the alloantigen stimulation of lymphocytes in mixed leukocyte culture (MLC). In the original studies [17,18], ultrafiltration was used to prepare

a fraction of M_r 10 000–50 000 which exhibited the properties of a lymphocyte chalone. Similar material could be prepared from media conditioned by cultured lymphoid cell lines [17]. Treatment of the ultrafiltrate from thymus with ribonuclease would enable the activity to pass through a 10 000 M_r filter and be retained above a 500 M_r filter [22]. This suggested to us that the chalone may become associated with RNase-sensitive molecules during tissue extraction, possibly transfer RNA of this molecular size. Gel filtration on columns of Sephadex G-25 confirmed the low M_r nature of the activity [18,28] and indicated that it was in fact $<2000 \, M_r$.

The low M_r fraction is isolated from thymus by 60% ethanol extraction followed by acetone precipitation. The crude fraction is then separated on a column of Sephadex G-25 which demonstrates only one active area when aliquots are added to MLC in the presence of human serum (fig.1A). The activity is detected
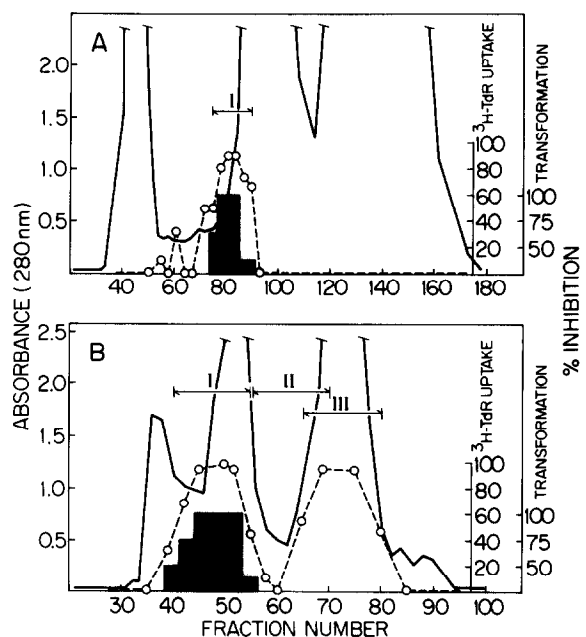

Fig.1. Column profile of 60% ethanol soluble fraction from calf thymus resolved on Sephadex G-25 (A) and then Sephadex G-10 (B). Column fractions are screened for A_{280} (——) and inhibition of the mixed lymphocyte culture in human serum determined by [³H]thymidine incorporation (o– – –o) and by visual estimation (■). (A) The pool indicated I was lyophilized and applied to the Sephadex G-10 column. (B) Pool I indicates area of activity determined by both methods, pool II, the area of elution of NaCl, and pool III, fraction containing inhibitors of label incorporation only.

both by visual examination of the cultures for lymphoblast formation and by labeled thymidine incorporation. The active area is concentrated and applied to a column of Sephadex G-10, the elution pattern of which is shown in fig.1B. This column separates the low M_r inhibitor (pool I) from the salt (pool II) and unlabeled nucleotides (pool III). The pool III material only inhibits labeled thymidine incorporation into stimulated lymphocytes without any effect upon morphological transformation. This will be discussed in more detail in section 3.1. Although considerably cleaner than the crude starting material, the inhibitory fraction from the Sephadex G-10 column is still extremely complex, containing at least 40 distinct components by analytical HPLC analysis (D. Barrantes, unpublished).

It is difficult to ascertain true M_r values using exclusion chromatography since chemical adsorption effects must be considered. The solubility properties of this active fraction indicate that it has considerable hydrophobic nature.

It can be generally noted now that lymphocyte inhibitory activity extracted from lymphoid tissue residues in a low M_r molecule which is either specifically carried by a much larger molecule, or becomes associated with one during extraction procedures. It is this association which sometimes allows the inhibitory activity to masquerade as a much larger molecule. The low M_r of this inhibitor, while making the isolation somewhat more difficult, raises the possibilities of eventual synthesis of active molecules which could be of extreme importance in immunosuppression.

The relationship of lymphocyte chalones to other inhibitors of lymphocytes such as inhibitory lymphokines, lymphotoxin, inhibitor of DNA synthesis (IDS), and soluble immune suppressor substance (SIRS) [16,29] or inhibitory activity from thymus-derived thymosin peptide fractions [30–32] or FTS (factor thymique serique) [31,33] is too complex a topic to be fairly treated in this limited space. On the surface it seems that nomenclature may be the major source of differences as the word 'chalone' is not treated with universal respect among most investigators.

2.2. Granulocyte chalone

Another example of a low M_r inhibitor is that of the granulocyte chalone. Early work on the granulocyte chalone, its biology and role in control of granulopoiesis has been reviewed [34,35] and the methods for its assay have been examined [36]. Briefly, it has

been demonstrated by numerous groups that extracts of granulocytes, or media conditioned by contact with granulocytes, reversibly inhibited granulocyte precursor cells in cultures of bone marrow. This activity has been shown to exhibit the classic chalone properties of reversibility, species non-specificity, cell-specificity, and non-cytotoxicity.

In a series of papers from the laboratory of W. R. Paukovits, the long, often complicated, steps in the purification of this chalone activity are detailed. Paukovits [37] and others [38] found that the inhibitory activity present in granulocyte conditioned media could pass through a 10 000 M_r ultrafilter and be retained above a 500 M_r filter. This was in agreement with the elution from Sephadex G-75 which indicated an M_r of 4000 [39]. The activity was found to migrate on several other types of Sephadex and Bio-Gel P gels in a manner to suggest considerably lower M_r, the activity usually eluting between the leucine and thymidine markers. Finally [40,41] it was demonstrated that Sephadex G-10 could provide the needed resolution and the chalone would elute from this gel matrix ahead of the salt and thymidine peaks. This indicated an M_r of ~600. The activity did not stick to Dowex resins but could be further purified by thin-layer chromatography on cellulose.

Maschler and Maurer [42] have found similar active fractions in either culture media or ascites fluid. Sephadex G-25 columns indicating an M_r <10 000 and in some cases ~500. Another group [43] have not presented their preparation in detail but indicate activity at an M_r of 500–1000 for a chalone extracted from leukocyte conditioned media.

The observation that the granulocyte chalone activity was dependent upon thiol groups [44], has resulted in considerable progress in the purification. The activity from leukocyte conditioned medium, referred to as granulopoiesis inhibiting factor (GIF), was absorbed to and eluted from thiol propyl Sepharose followed by a reversed phase C_{18}-column and ion-exchange chromatography [45]. After these techniques a single spot was demonstrated in several thin-layer chromatography (TLC) systems. Other analyses have indicated that the activity resides in an M_r 500–600 acidic peptide with an N-terminal pyroglutamyl residue, a thiol group, three carbonyl groups and one lysine amino group. Presumably, further analysis must await the purification of greater amounts of material, but, with the data indicated already, model peptides can be synthesized and tested.

An interesting note here is that mouse lymphoma cell lines that demonstrate thiol dependence for culture may also secrete a thiol containing, small M_r, mitotic inhibitor [46].

2.3. Other chalone systems

We now briefly discuss several other chalones, or chalone-like activities, which have been suggested as being of low M_r and of hydrophobic nature.

Some interesting results have been presented concerning the properties of an inhibitor of the JB-1 ascites cells. These cells have been shown [47] to produce an inhibitor of M_r 10 000–50 000 with properties of a G_1-chalone. A preliminary report has appeared [48], which has suggested a considerably lower M_r. The inhibitor has been isolated from labeled ascites fluid by columns of DEAE-cellulose, Sephadex G-15, and AG1X-2 resin. This procedure yields a low M_r (400–700), acidic, and hydrophobic peptide. JB-1 ascites cells are ≥10–20-times more sensitive to the inhibitor than other cells. The properties of the JB-7 inhibitor are strikingly similar to those reported for the granulocyte chalone of Paukovits.

It has been suggested that the control of liver growth and regeneration is under the control of a chalone mechanism [49]. The traditional extraction procedure involves extraction of liver tissue and fractionation by cold ethanol solutions, the chalone activity was usually found in a 60–87% ethanol precipitated fraction. This material could be further fractionated by ultrafiltration or chromatography on Sephadex G-25 [50,51]. The M_r of this inhibitor has remained constant at ~2000, not changing as much as some of the others. Additional steps have been added to the isolation scheme for this chalone [52] which has involved chromatography on Sephadex LH-20 followed by silica-gel and thin-layer chromatography on polysilicic acid-impregnated sheets. All these methods suggest a considerable hydrophobic nature for the chalone. Amino acids were present in the active area on TLC but further purification is necessary for any firm conclusions.

A small M_r inhibitor of DNA synthesis which is specific for normal and malignant mammary cells has been described [53]. The inhibitor is purified from extracts of mammary tissue of Sephadex G-50, CM-Sephadex, and Sephadex G-25 columns, and had been suggested as being an M_r 2000–3000 peptide.

A fibroblast chalone which would inhibit the growth of human diploid fibroblasts was originally described as being of high M_r also by ultrafiltration and has been shown to be similar to lymphocyte chalone in its sensitivity to RNAse and elution from Sephadex G-25 [18,22].

The epidermal control mechanisms, history, and biology have been reviewed [54–56] and are too extensive to be mentioned here. It is commonly accepted that there exist two epidermal chalones, the G_1 and G_2. The G_2 chalone halts cell division at a point in the cell cycle after S phase prior to mitosis and is of high app. M_r, 20 000–45 000. Considerable heterogeniety has been noted in the material with activity noted as low as M_r 3000 [56–58].

The epidermal G_1 chalone owes much of its purification to the work reported by Marks [56]. Its properties indicate that it is a quite stable molecule resistant to heating (100°C, 1 h), phenol, urea, 1 M formic acid at 70°C, ionic detergents at 100°C, trypsin, pronase, and nucleases. The activity migrates at an app. M_r >300 000 but could be reduced to ~20 000 after rather brutal treatment with sodium dodecyl sulfate (SDS), dithiothreitol, blocking of –SH groups by iodoacetamide, and chromatography in 7 M urea and 0.5% SDS. The activity could be further purified by ion exchange and phenol extraction and could depress epidermal mitosis at a dose of 0.05–0.1 μg/mouse. Since the reported M_r ranges from >300 000 to <10 000, Marks [59] mentions that the lowest is probably the true value. This does not seem unreasonable considering the stability of the active species and the extreme efforts necessary to reduce the activity to lower M_r.

A summary of the progress toward the isolation of a chalone would be that many chalones, whether called that or not, are now recognized as being very stable, low M_r compounds, probably peptides. A number of these active species previously demonstrated much higher M_r values either due to specific or non-specific binding to carriers or due to very strong aggregation properties. Modern purification techniques such as HPLC show obvious promise in the resolution of complex tissue extracts containing chalone activities and should provide much information in the future. Obviously the controversy surrounding the chalone concept will not resolve itself until purified material is available in sufficient quantities.

3. Critique

3.1. Assay artifacts

No review of chalones could be considered com-

plete without first addressing itself to several problem areas which become apparent, and are constant criticisms, in the study of growth inhibiting substances. The most important are several artifacts which can commonly arise with the use of the popular in vitro proliferation assays. We will concern ourselves here with several high M_r components which can alter cell growth and with certain small M_r compounds which have received much attention in the literature, namely, the polyamines spermine and spermidine. Our mention of these effects, in reference to in vitro assays, must not be taken to imply that this type of assay is worthless, rather that careful attention must be paid to all possible influences on cell growth possible with tissue extracts. Whenever possible, reference to in vivo assay systems should be used to substantiate in vitro studies.

The organ extract, or conditioned media, which are used as chalone sources contain a wide variety of components which can specifically or non-specifically influence cell division in vitro. As the most common method to ascertain if a particular cell population has undergone division is a pulse of labeled thymidine, we will consider briefly various pitfalls to be avoided. A number of these which have direct effect on labeled thymidine have been mentioned [36] and can include breakdown or alteration of thymidine by kinases released in tissue homogenates or by phosphorylases resulting from low level mycoplasma infections [60]. The latter must be considered especially when using conditioned media sources.

Another macromolecular inhibitor which can arise in crude extracts as well as purified samples is bacterial infection and subsequent toxin production. A classic example of this problem is that of melanocyte chalone prepared from crude pig skin concentrate [61] which was subsequently shown to be associated with clostridium spores [62,63]. We have had similar experience with commercially prepared spleen powder extracts [64].

Various nucleotides, including thymidine, are always present in small M_r fractions from tissue [25,28] and can of course, inhibit labeled thymidine incorporation into cells by pool dilution and transport inhibition [36]. Interestingly, unlabeled nucleotides can be present in cell supernatants and conditioned media in sufficient amounts to cause inhibition of cellular uptake [65–68] and may result from breakdown of DNA from cell death present in the cultures [67,68]. All of the above influences on labeled thymidine can

usually be controlled, and eliminated, if the data obtained by label incorporation is compared with results obtained by some non-isotopic method. We have found that some low M_r fractions from thymus were about twice as active in an in vitro assay determined by isotope as compared to visual determinations. This difference could be eliminated by passage of the active fraction through a Sephadex G-10 column which (see fig.1B) separated a peak of activity with equal potency measured by isotope or visually from a peak of activity noted only by isotope incorporation. The last peak has been identified as a mixture of thymidine and other nucleotides.

3.2. Problems with polyamines

Perhaps the most interesting (at least the most controversial) small M_r inhibitors of cell proliferation to arise from chalone research recently has been the polyamines spermine and spermidine. Allen et al. [69] were among the first to suggest that a chalone (lymphocyte) was a complex of spermine and a higher M_r carrier molecule. They isolated a lymphocyte chalone by published procedures and found that the activity was dependent on the presence of bovine sera in the in vitro assays. Simultaneously, Byrd et al. [70] reported the potent inhibition of lymphocyte transformation by addition of synthetic spermine or spermidine to cultures containing bovine sera. Allen's group further showed that polyamines could easily survive various extraction techniques and purification steps such as dialysis by associating with high M_r carriers.

The inhibition of authentic polyamines was investigated by several groups [71–74] after the appearance of the above papers. Generally it was found that the inhibition of growth of lymphocytes and granulocytes by these polyamines was dependent on the presence of bovine or human pregnancy sera. Polyamines were not inhibitors in the presence of human AB serum. In all cases the inhibitory activity has been associated with the presence of the enzymes diamine oxidase (DAO) or polyamine oxidase (PAO) in the serum. The absence of the DAO and PAO in human serum is perhaps not absolute and high levels can cause inhibition in the presence of polyamines [72]. Addition of PAO to normal human serum containing polyamines causes inhibition of PHA-stimulated lymphocytes and lymphoblastic cell lines [73].

The amine oxidases catalyze the oxidation of the amino propyl moiety of polyamines to the corresponding amine aldehyde [75]. These amino aldehydes and

the breakdown of product acrolein ($H_2C=CH-CHO$) [76,77] are toxic to a variety of cultured cells. It is unknown if similar compounds are responsible for the inhibition observed in lymphocyte cultures, but it is not unlikely. However, the inhibition has been observed to be fully reversible (after even several days exposure to polyamines in the presence of bovine sera) and therefore does not appear to be associated with gross cellular toxicity. The degree of sensitivity to polyamine inhibition can vary considerably and this has been used [78] to eliminate fibroblasts from mixtures of fibroblasts and epithelial cells. Obviously more work must be done on identifying the active component resulting from the interaction of bovine sera and polyamines and capable of inhibiting lymphocyte proliferation.

Other products of the oxidation of polyamines to aldehydes by DAO and PAO are possible in addition to acrolein. Under certain conditions [79,80] amino aldehydes can form various condensation products with themselves to form larger polycations. Some of these as yet uncharacterized products are potent inhibitors of sodium transport [80].

The connections of polyamines, PAO, DAO, and certain immunosuppressed states is still of much interest. The two enzymes are increased in pregnancy [71], age [81], and polyamine levels increase in fetal and neoplastic growth [82]. It may be that such an association is mere coincidence and further research will resolve the difficulties.

A number of investigators, in response to the papers of Allen and Byrd et al., have subsequently published statements that their particular chalone preparation does not contain polyamines or that the activity is present in human as well as bovine sera. This has been done for lymphocyte chalone isolated from spleen [83,84], and thymus [28,72,85] and leukocytes [43] and for the granulocyte chalone [43]. It is noteworthy that the effects of polyamine are important to eliminate wherever possible as these molecules seem able to appear at various M_r fractions and can survive normal purification steps for large or small molecules. This is of special importance if in vitro assays are used in the presence of bovine serum.

4. Conclusions

In the seven years since this subject was last reviewed

here, relatively little has been added to our knowledge about the elusive cell-specific and endogenous inhibitors called 'chalones'. The epidermal, lymphocytic and granulocytic chalones have all been further purified, but until very recently (and only in the case of the granulocytic chalone) not to anything near homogeneity. The major increase in knowledge about these chalones is:

(1) That they are of much smaller size for the most part than we had previously perceived and believed; i.e., lymphocyte, granulocyte, and the JB-1 ascites chalone are now down to an M_r of 600–700, while fibroblast chalone is clearly $<M_r$ 10 000 and both the G-2 and G-1 epidermal inhibitors have been reduced in size (see table 1). This reduction in molecular size proceeds from the aggregative avidity of many of these chalones for various macromolecules.

(2) One of the major problems in the identification of endogenous inhibitors is that most tissues contain polyamines and that polyamines will inhibit the proliferation in vitro of most cell types to varying degrees, especially when cultured in fetal calf serum.

Thus, as the chalones themselves 'shrink' to smaller sizes, small cationic molecules (like spermine and spermidine) loom larger as major artifacts in the determination of specific endogenous mitotic inhibitor capacity of various fractions during purification attempts.

Table 1
Relative molecular mass estimates for representative chalones

Chalone	'Old' M_r	'New' M_r
Lymphocyte	30 000	< 2000
	30 000–50 000	500– 700
	38 000	2100
	10 000–30 000	< 1400
Granulocyte	2000– 3000	300– 700
		< 1000
JB-1 Ascites	10 000–50 000	500– 600
Mammary	2000– 3000	2000–3000
Liver	2000	2000
Epidermal	300 000	<10 000
Fibroblast	10 000–50 000	<10 000

M_r values for selected chalones can be found in references and reviews cited in the text

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