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FERRICYANIDE CAN REPLACE PYRUVATE TO STIMULATE GROWTH AND ATTACHMENT OF SERUM RESTRICTED HUMAN MELANOMA CELLS

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Summary: Addition of potassium ferricyanide to RPMI 1640 medium can stimulate cell attachment and replication, in a closely correlated fashion, of a human melanoma line when serum is a limiting growth factor. Ferricyanide is more effective than pyruvate on a molar basis but toxic effects at concentrations >0.03mM limit its full potential. Since ferricyanide cannot itself provide nutrients for the cell and is extracellular but may be involved in transmembrane electron flow, it is suggested that its mechanism of action may be to provide energy for cell surface processes concerned with attachment and thus secondarily for replication.

Since the seminal studies of Eagle (1), pyruvate has been recognized to be a required medium supplement for a variety of cell types growing at low density. It has been noted in at least 2 human cell culture systems that there is an inverse relationship between serum and pyruvate requirements (2,3,4). The effectiveness of a variety of other 2-oxocarboxylates was found to equal that of pyruvate as a supplement for growth of a human malignant melanoma cell line with little regard for the length or branching of the carbon skeleton - being limited only by solubility (4). The common 2-oxocarboxylate functions are paramount for activity (2,4). While it is possible that the 2-oxocarboxylates could all yield some single required intermediate, the diversity of structure suggests that they may fulfill a more general function. A global function which the 2-oxocarboxylates could modulate is the redox state of the cell (5). Millimolar concentrations of 2-oxocarboxylates could raise the $NAD^+/NADH$ by acting as an electron sink for appropriate NADH oxidoreductases .

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Cell proliferation and a more oxidizing state (higher NAD^+/NADH) in the cytosol have been associated in some mammalian cell types (6) although the correlation is not universal (7) and may depend on cell-type. While the increase of NAD^+ by this mechanism would possibly enhance glycolysis and increase ATP levels, recent evidence suggests that the electron flow *per se* is more important for cell transition from G_1 to S phase than is the production of ATP therefrom (8). Furthermore, our observation that cell adhesion and replication in this cell line are correlated tightly, places special emphasis on membrane activity in these cells. A wealth of literature attests the role of the plasma membrane as a transducer of mitogenic signals, and attachment-determined modulations of cell behavior, but the detail of the mechanisms of influence are scanty.

These considerations highlight possible roles for the recently described plasma membrane dehydrogenases (9). These membrane enzymes are differentiable from their counterparts in mitochondria and endoplasmic reticulum, and effect transfer of electrons from NADH to a variety of substrates which include, prominently, pyruvate and glyoxylate. The energy released by electron flow provides the driving force for ATP synthesis, classically via oxidative phosphorylation by the mitochondrial cytochrome system, but also through much simpler reactions (10). It has been suggested also that this energy, which can be stored in nonphosphorylated intermediates (10,11) for significant lengths of time (12), may be used directly to energize some membrane systems without the mediation of the high energy phosphate bond (11,13,14,15).

In an attempt to provide a fairly direct test to distinguish between a local surface membrane energizing function and a more classical role of the 2-oxocarboxylates via intermediary metabolism and glycolytic/mitochondrial energy production to modulate the melanoma cell attachment and growth, we sought a substrate for the membrane oxidoreductases which was unlikely to penetrate the membrane, and which was not available to intermediary metabolism.

The ferricyanide anion fulfils these requirements, has been shown not to penetrate erythrocytes (16,17) or mitochondria (18), and is a substrate for florid plasma membrane NADH oxidoreductase activity (9). Furthermore, Mishra and Passow (19) found that reduction of extracellular ferricyanide by human erythrocytes was accompanied by ATP synthesis, presumably accomplished as a result of transmembrane electron flow. It is thus of considerable interest that we have found that ferricyanide at concentrations of 0.003-0.1mM is capable of substituting for pyruvate or serum in promoting cell attachment and replication of a line of human melanoma cells.

Materials and Methods are described in detail elsewhere (3,4). Necessary basic information is included in the legends to figure 1 and table 1.

Results and Discussion. Cultures of MM96 wild type (wt) attach less avidly and grow more slowly when the serum concentration in their medium is reduced (3). This effect is magnified in sublines selected from the parental wt line (3,4). The effect of serum limitation is in the main reversed by supplementing the medium with pyruvate and a variety of 2-oxocarboxylates (4). Figure 1 illustrates a comparison between the effectiveness of pyruvate and ferricyanide on both wt and subline B cultures. At 0.0033 mM to 0.01mM ferricyanide reversed the inhibitory effect of serum deprivation on the growth of MM96 wt and produced a large increase in that of the B subline. Inhibition occurred with higher concentrations of ferricyanide. The maximum effect of pyruvate was obtained with a concentration of 1mM, as previously found (4). Between experiment variation in the magnitude of the ferricyanide and pyruvate reversal effects occurred in relation to the degree of attachment and growth of the cells in 10% fbs. However, in all cases, ferricyanide was much more effective than pyruvate at concentrations $\leq$ 0.03mM. Figure 1 also shows the large difference between the wt and B subline in their sensitivity to the effects of serum deprivation (1.5% fbs vs 10% fbs).

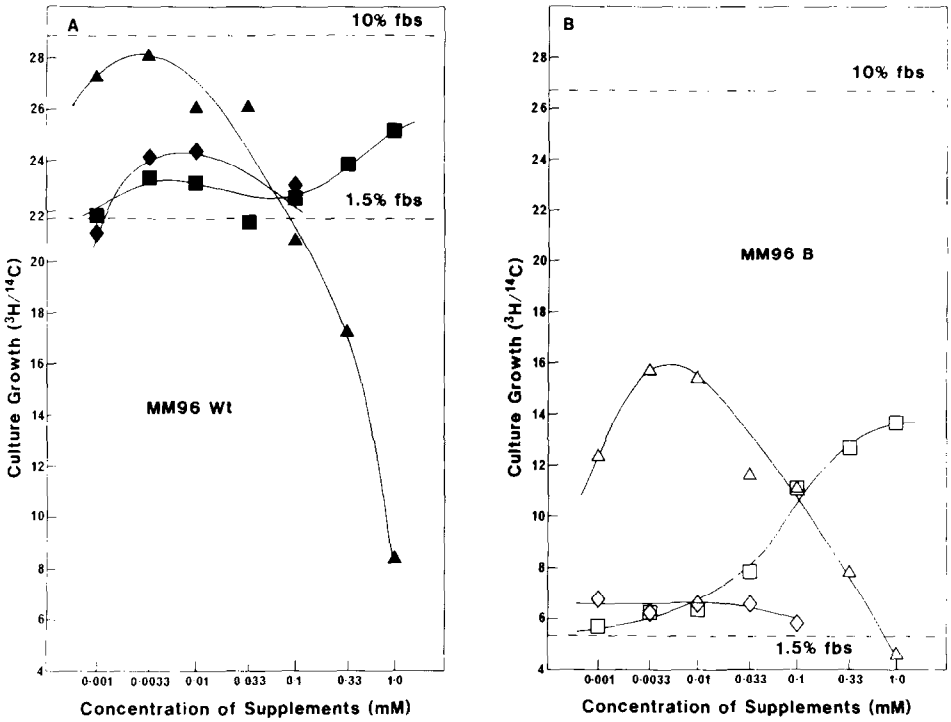

Figure 1. Dose response curves of MM96 wt (A) and its B subline (B) to medium additives. 10 cells cm , prelabelled for 4 days with ^{14}C-thymidine (0.2 μc/ml), were seeded into RPMI 1640 containing 1.5% fbs with or without the indicated supplements (▲ △) potassium ferricyanide (◆ ◇) ferric chloride (■ □) sodium pyruvate. After 3 days 0.4 μc^3H thymidine (final concentration 600 nM) was added and incubation continued for 24 hours. The cultures were then fixed, solubilized, and counted in a liquid scintillation spectrometer. The ^3H dpm provides a measure of the size of the replicating population while the ^{14}C dpm indicates the degree of attachment of the cells. The $^3H/^{14}C$ gives a normalized measure of the replicating population and thus of cell growth. All methods have been detailed elsewhere (3,4).

Toxicity may limit the effectiveness of ferricyanide, and thus dose-response data such as those presented here may underestimate the potential effectiveness of the ferricyanide. To check this possibility we tested whether different doses of ferricyanide when simultaneously present caused inhibition of the pyruvate stimulation. Figure 2B demonstrates that concentrations of ferricyanide progressively greater than that found to produce the maximal stimulatory effect (0.03mM), caused increasing inhibition of the pyruvate stimulation. While a concentration of 0.3mM ferricyanide virtually abolished the pyruvate effect, below 0.03mM, the ferricyanide and pyruvate effects were additive (c.f. figure 2A and 2B).

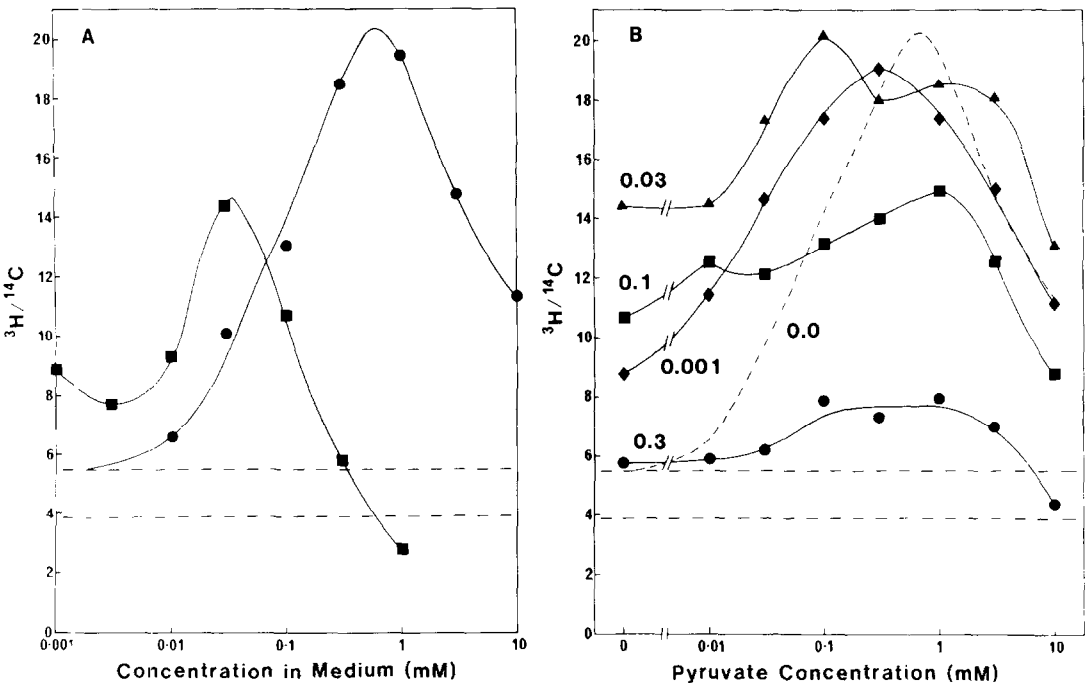

Figure 2. Inhibitory effects of ferricyanide on pyruvate stimulation at higher concentrations. (A) Dose response of MM96B growth to each additive independently. Ferricyanide (■), pyruvate (●). (B) Dose response of MM96B to pyruvate in the presence of different concentrations of ferricyanide, as indicated. The two dashed straight lines indicate the range of basal level values for $^3H/^{14}C$ with 1.5% fbs alone at the beginning and end of the experiment. The value for 10% fbs was 46.9. Experimental conditions as for figure 1.

The higher concentrations of ferricyanide also cause cell detachment, as do the 2-oxocarboxylates (4). Figure 3 shows a plot of the growth state of the culture, measured as $^3H/^{14}C$ (ordinate) against cell attachment, measured as ^{14}C dpm from the prelabelling procedure (abscissa). The lower pair of lines define the growth-attachment relationship for the B subline in low serum (1.5% fbs) in the presence of varying concentrations of pyruvate or ferricyanide. As previously described (4) increasing concentrations of pyruvate increase both parameters so that increase in growth is directly proportional to increase in attachment, yielding a straight line plot. A similar phenomenon was found in other experiments with ferricyanide but unlike the case of the 2-oxocarboxylates, which yield an hysteresis effect, the inhibitory doses of ferricyanide also produce a striking linear relationship between growth ($^3H/^{14}C$) and attachment (^{14}C).

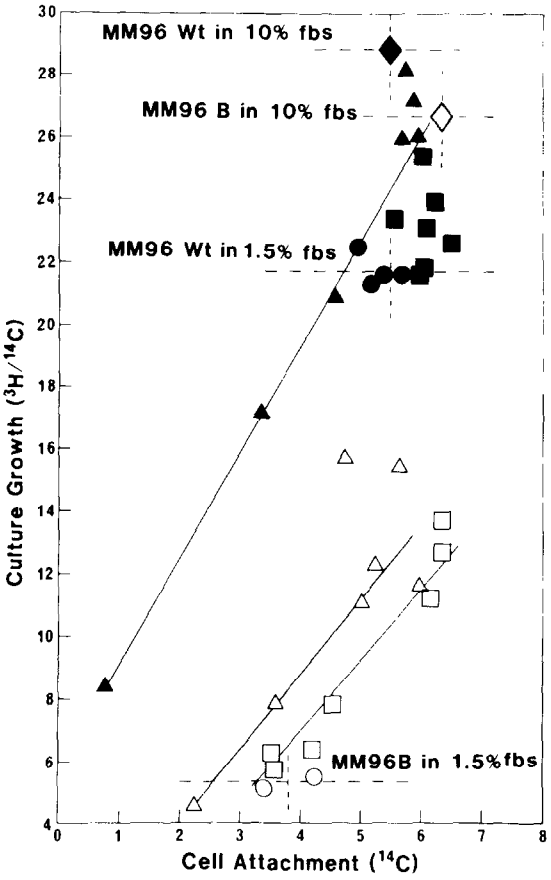

Figure 3. Growth vs attachment plot of MM96 wt (upper line and data) and MM96B (lower lines and data), in the absence (● ○), or presence of ferricyanide (▲ △) or pyruvate (■ □). Experimental conditions as in figure 1.

Interestingly, the inhibitory effect of the ferricyanide differs between the wt and B subline cultures in low serum. Whereas the B subline shows only an inhibition of the stimulatory effect (i.e. returns to base line for $^3H/^{14}C$ and ^{14}C) the wt shows gross inhibition below the values of these parameters in low serum alone.

Even though ferricyanide has one of the lowest dissociation constants of all coordination complexes, it was thought prudent to examine the effects of the ferric ion on the cells. Addition of $Fe(NO_3)_3$ or $FeCl_3$ from 1μM to 1mM did not result in significant reversal of the serum limitation effects, as can be seen for ferric chloride in figure 1. It proved difficult to check the effects of ferrocyanide in aerobic solution since it

Table 1

Ferricyanide Potentiation of Cloning Efficiency

Final Concentration (mM)	Pyruvate	Ferri-cyanide	Ferro-cyanide
0 (10% fbs)[a]	131.5[b]		
0 (2.5% fbs)[a]	5.5		
0.0001	2.5	6.5	6.0
0.00033	2.0	18.0	16.5
0.0033	2.0	37.0	31.5
0.01	21.5	33.0	23.0
0.033	63.0	-	8.5
0.1	57.0	26.0	2.0
0.33	90.5	0.5	0
1.0	107.5	0.5	0.5
3.3	44.0	2.0	0.5

[a] Control cultures without additives

[b] Values are the average number of colonies in duplicate petri dishes (35mm diameter) from 500 cells seeded in RPMI 1640 + 2.5% fbs containing the indicated concentration of additive. Cultures were incubated for 14 days then fixed and stained.

undergoes spontaneous oxidation to the ferri-form, and these cells will not grow in an anaerobic environment. However, we found that it takes 24 hours for 77% of the ferro to oxidize to the ferri form in RPMI 1640 in air/5% CO_2 at 37°C, at a concentration of 0.3mM. This delay in conversion would lead to a lower effective concentration of the ferri form than when the latter was added, and we would thus anticipate that if the ferro form were inactive that the response to its oxidation product would be diminished. Comparison of the $^{3}H/^{14}C$ response to ferri- and ferro- cyanide after 3 days at concentrations between 0.001mM and 1mM showed that at all concentrations the stimulation occurring with ferrocyanide was less than half that with ferricyanide (data not shown).

To check that the stimulation of growth measured by $^{3}H/^{14}C$ was paralleled by a more direct measure of cell growth, we explored the effects of ferricyanide on cell colony forma tion. The data in Table 1 clearly indicate that ferricyanide (ferrocyanide will be virtually all in the ferri form during the long cloning assay) increases the cloning efficiency of MM96 wt under serum limiting conditions, although not as well as pyruvate or 10% fbs.

The fact that the ferricyanide anion can reproduce the effects of a variety of 2-oxocarboxylates in reversing the serum requirement for growth and attachment of certain cell types, is consistent with a determinative role for membrane events alone in these phenomena. The rationale for testing ferricyanide was based on the notion that local membrane energization might be effected by electron flow from NADH to appropriate substrates, and that such activation might be important in cell committment to a particular set of functions. However, alternative explanations could be that ferricyanide may act differently from the 2-oxocarboxylates e.g. by oxidizing an inhibitory component of the medium, or perhaps by oxidizing functionally critical thiol groups at the cell surface. It is necessary to test a variety of compounds capable of modulating cell membrane oxidoreductases as well as other strategies to enhance or falsify the basic argument of this study.

ACKNOWLEDGEMENTS

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