

THE RESPONSE OF MAMMALIAN CELLS
IN THE PLATEAU PHASE TO TREATMENT
WITH MANGANESE

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The toxicity of manganese chloride was evaluated for V79 Chinese hamster cells in asynchronous culture during two different periods of their life: the period of exponential growth and the plateau phase.

The samples of control and manganese-treated cells were microscopically examined and their proliferation rate was estimated.

The inhibition of the cells' colony forming ability served for assessing the toxic potential of three concentrations of manganese chloride during different exposure times. After treatment the cells were seeded at low density. Seven days later the colonies formed by the surviving cells were stained by Giemsa solution, counted and expressed as percentages of the controls considered as hundred per cent. The reduced colony forming ability exhibited by the plateau phase cells proves that they are more susceptible to manganese than the cells growing exponentially, particularly to manganese concentrations above 5×10^{-5} mol and in exposures over 10 hours.

The difference in susceptibility to manganese of proliferative and plateau phase V79 Chinese hamster cells is similar to that shown by the same phases in response to various chemicals and drugs such as bleomycine and nitrosourea derivatives.

The number of chemical agents from the environment which daily contaminate living organisms is constantly increasing. Heavy metal ions are among the most toxic cell contaminants (1). Although the effects of exogenous metals at the cellular level have been a much investigated topic, the interpretation and the understanding of the metal action are becoming more and more intricate. Metals rarely act singly; other environmental agents usually interfere additively or by potentiating or diminishing the toxic effect. The ultimate consequence of intoxication by metals may be lethal, but the appearance of mutant or tumour cells, or even early aging of the cells are also possible (2, 3).

Mammalian cells in culture offer potential advantages for assaying the cytotoxicity of many chemicals including drugs and metals (4). The established cell lines are easily available and not too difficult to cultivate. It is possible to control the composition of the nutritive medium, to select a determined phase of the cell cycle and to follow by appropriate parameters the consequences of the addition of different toxic agents to the medium (5).

After harvesting from a trypsinized confluent culture followed by incubation in a nutritive medium, the cells first enter a *lag phase*. Growth proceeds steadily with the population doubling several times during the *logarithmic exponential phase* (dividing or proliferative cells). Then the cells enter the *stationary plateau phase* (non-dividing quiescent cells) under diverse suboptimum conditions (6, 7). The cells in this phase incorporate tritiated thymidine very slowly in their DNA (8).

Four phases can be recognized in the life cycle of most eucaryotic cells. Two easily distinguishable events *mitosis* and S phase (DNA synthesis) are usually separated by a pre-DNA synthesis (G_1) period and a post-DNA synthesis (G_2) period (9). In asynchronous populations the cells do not go through the cycle phases all at the same time. After several days of proliferation they stop dividing and remain in the plateau phase. In this stage known as G_0 the metabolic state of the cells is unique and distinct from that of the cells actively traversing the cell cycle. Their response to treatment with chemical and drugs is comparable (10, 11). Drugs such as bleomycine and 1,3-bis (2-chloroethyl)-1-nitrosourea are more effective on Chinese hamster ovary cells when applied during the plateau phase than on dividing cells. Hydroxyurea and arabinosylcytosine, on the contrary, exert a stronger effect on growing than on plateau phase cells (7, 8). Although it was suggested that some of these results could be artefacts, it was confirmed that the non-dividing CHO cells are significantly more sensitive to 1,3-bis (2-chloroethyl)-1-nitrosourea (12, 13).

Recently it has been found that HeLa cells in the plateau phase are more sensitive to methylnitrosourea than exponential phase cells but less sensitive to adriamycin (14).

Frequently the cells are most susceptible during the plateau phase which therefore offers an appropriate stage for assaying the cytotoxicity of various drugs and chemicals (14).

Our first investigations of the biological effects of heavy metals on cells in culture were performed with HeLa exposed to lead chloride. The inhibition of macromolecular syntheses and its reversibility were followed (15). The experiment was extended to different cell lines (16, 17). Lead, cadmium and manganese chlorides slowed down not only DNA synthesis but also cell growth and reduced their colony forming ability. The intensity of the effect was dependent on the metal, dose and duration of exposure (18).

These results were obtained with exponentially growing cells. In the present work we decided to extend research to the plateau phase cells. We tested the relative sensitivity of the proliferative and plateau phase V79 Chinese hamster cells after exposure to different concentrations of manganese chloride.

MATERIAL AND METHODS

The cells in asynchronous culture growing exponentially

The cell line used was a clone derived from V79-379, a Chinese hamster lung fibroblast line. The surface attached cells growing in monolayer were maintained in Eagle's minimal essential medium MEM supplemented with antibiotics and serum (10% calf or fetal calf serum). The doubling time was about 11 ± 1 h at 37° C. The cells in the logarithmic phase of growth were treated with 0.25% trypsin (DIFCO) and harvested. The cell concentration per millilitre of suspension was adjusted to the experimental purpose.

All other conditions were the same as before (17, 18).

The plateau phase cells

Several plastic Falcon tissue culture flasks were seeded at the same time at an initial concentration of 5×10^4 cells/ml. One flask was trypsinized daily, the cells were counted in a haemocytometer and the increase in cell number was recorded.

The plateau phase was considered to be reached when the doubling time began to increase, usually after 5 days.

The experiment was continued a day after the plateau phase had been reached.

Chemicals

Manganese chloride was purchased from Kemika, Zagreb and stocked in a sterile solution at a concentration of 10^{-2} mol. Plastic culture flasks were from Falcon. Nutrient media and solutions were prepared with chemicals supplied by Nutritional Biochemical Corporation, USA and Flow laboratories, Scotland.

Cell treatment

Before the experiment, there were two categories of cell samples kept in flasks: the cells growing exponentially and the cells seeded 6 days earlier, which had reached the plateau phase.

At the same starting time three concentrations of $MnCl_2$ were added to half of the flasks of each cell category.

The following four groups of cells were analysed:

1. Control non-treated asynchronous culture in the exponential phase of growth.
2. Several asynchronous cultures exposed to three different concentrations of MnCl_2 (10^{-5} , 5×10^{-5} and 2.5×10^{-4} mol) and returned to the metal free medium after 2, 4, 6, 10 and 20 hours respectively.
3. Control non-treated plateau phase cells, seeded 6 days before the beginning of the treatment.
4. Plateau phase cells exposed to the same MnCl_2 concentrations as Group 2.

Microscopic examination

One sample of each cell group was observed daily to detect possible morphological changes produced by manganese chloride.

Proliferation rate

At regular intervals, one sample from each group was trypsinized, the cells were counted and their number was recorded. The aim was first to establish the time of occurrence of the plateau phase in non-treated cells growing asynchronously and second to evaluate the effect of MnCl_2 on the growth rate of the cells.

Cell survival

For assaying the survival of the cells their colony forming ability was tested. After being seeded at low density (50—100 cells/ml) a cell which has recovered normal functions in optimum conditions gives rise to a colony visible after several days. After manganese treatment the cells were rinsed, trypsinized and seeded at low density in a fresh manganese-free medium. Seven days later when they were large enough but not confluent, the colonies were stained with Giemsa solution. The survival was determined by scoring the number of colonies containing more than 50 cells. The surviving fraction of the treated cells was estimated as a fraction of the control colonies originating from exponential and plateau phase cells considered as 100 per cent. The plating efficiency was 70—80 per cent.

RESULTS

Microscopic observations

Twenty hours after the beginning of the treatment with manganese all exponentially treated cells had a normal appearance. One day later the cells exposed to the highest manganese concentration seemed to be

damaged. Some of them appeared swollen and the membrane was often shapeless.

After a four-day incubation many cells both in the highest and in the medium manganese concentration lost their original shape. After six days in the highest manganese concentration almost all were swollen. Those exposed to the lowest concentration began to detach and their cytoplasm became granulous.

When manganese chloride was added to the cells which already were in the plateau phase cell destruction occurred about one day earlier than with the treated cells growing exponentially.

Proliferation rate

The first curve in Fig. 1 which represents the growth rate of an asynchronous population of V79 Chinese hamster cells shows the onset of the plateau phase about 5 days after the beginning of subculture.

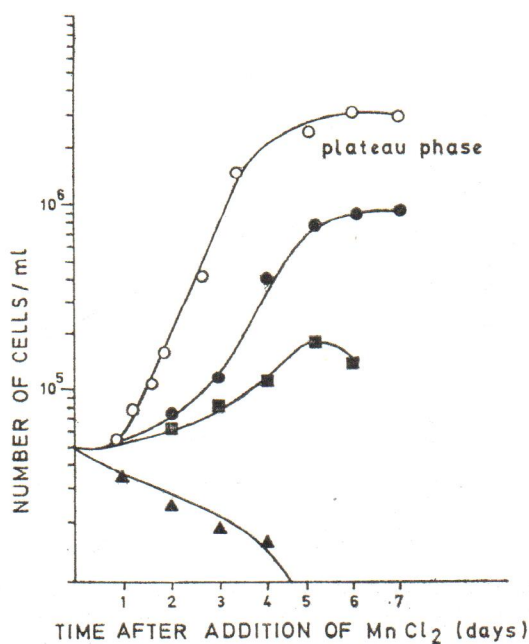


Fig. 1. The proliferation rate of asynchronous populations of V79 Chinese hamster cells showing that the plateau phase is reached after 5 days. The growth rate of asynchronous populations exposed to 3 concentrations of $MnCl_2$ is shown. ○ control cells, ● cells incubated with 10^{-5} mol $MnCl_2$, ■ cells incubated with 5×10^{-5} mol $MnCl_2$, ▲ cells incubated with 2.5×10^{-4} mol $MnCl_2$.

The three other curves illustrate the effect of manganese chloride on the proliferation rate. The lowest concentration slows down the growth which stops at a lower cell density while with the other two the plateau phase is never reached and the cells are destroyed earlier.

Colony forming ability

The main results of our investigation are given in Fig. 2. The six curves represent the fraction of the colonies formed as a function of time for three manganese concentrations, parallelly for proliferative

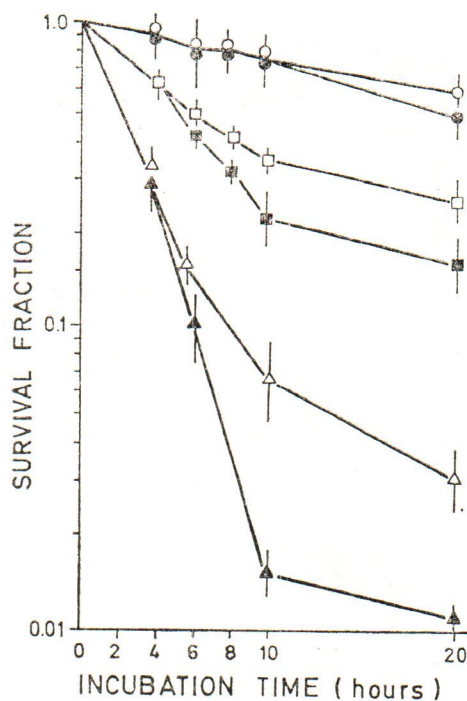


Fig. 2. The effect of 3 concentrations of $MnCl_2$ on the colony forming ability of proliferative and plateau phase V79 cells. The different incubation times with $MnCl_2$ are plotted on the abscissa. The survival fraction of treated cell colonies relative to the control ones is shown on the ordinate. Each point represents the mean \pm standard error of triplicate flasks from two separate experiments. \circ , \square , \triangle fractions of colonies formed by exponentially growing cells incubated with 10^{-5} , 5.10^{-5} and 2.5×10^{-4} mol $MnCl_2$, respectively. \bullet , \blacksquare , \blacktriangle fractions of colonies formed by plateau phase cells incubated with 10^{-5} , 5.10^{-5} and 2.5×10^{-4} mol $MnCl_2$, respectively.

and plateau phase cells. The number of colonies formed by the control groups is considered to be 1.

For a concentration of 10^{-5} mol $MnCl_2$ differences in the percentage of colonies between the two populations are slight and do not depend on the incubation time.

With the intermediate concentration of the metal salt, differences are very clear after a 10 hour-interval when practically all cells in each phase of the cell cycle have been in contact with manganese.

The highest concentration of $MnCl_2$ during 10 hours highly depressed the colony forming ability of all cells, especially in the plateau phase cells. Between 10 and 20 hours of exposure, the level of the colony forming ability slowly decreases.

The toxicity of manganese chloride can also be shown by comparing the percentages of colonies formed by cells during the plateau phase and those formed by an exponential population after different treatments. The ratios are given in Table 1.

Table 1.

Comparison of manganese toxicity for V79 Chinese hamster cells in the plateau and exponential phase: ratios of colony forming ability

$MnCl_2$ concentrations	Ratios calculated after 3 incubation times		
	6 h.	10 h.	20 h.
10^{-5} mol	0.90	0.90	0.83
5×10^{-5} mol	0.83	0.64	0.55
2.5×10^{-4} mol	0.62	0.23	0.36

The sensitivity of plateau phase cells relative to exponential cells is seen to increase with the concentration of the metal salts and with the time of exposure.

DISCUSSION

Metals differ from other pollutants in that some of them, including manganese, are present in biological systems as essential trace metals. These metals play an active part in cell metabolism. They are involved in enzyme catalysis, oxydation-reduction process, transport, functioning of membrane and mitochondria and also in stabilizing or modulating the structure of proteins and nucleic acids (19). Metal's binding to phosphates stabilizes the DNA structure whereas their binding to bases destabilizes it. Metal ions are preferentially bound to reiterative

DNA sequences where they may induce conformational variations and thus modify the binding or activity of effector molecules such as repressor and polymerases (20).

The addition of foreign metals may seriously disturb this equilibrium. Experimental data have shown that excessive exposure to metals affects those sites in the cell which are responsible for its primordial functioning (20). Several toxic effects may also appear indirectly (21).

It has been shown that besides the essential role of metals in living cells and biological functions, many of their harmful effects are not negligible (Utter, 21). The sensitivity of hamster cells to manganese seems to involve several systems. The diversity of the effects speaks in favour of the hypothesis that the overall toxicity is a summation of multiple intracellular and membrane events (18).

Although the sites of action and the mechanisms concerned are not yet well elucidated, some authors explain the effects of metals by their binding to different cell elements (22). Variations in the nature and content of the metal bound to DNA have been described for different physiological states of the tissues (23). It could be postulated that the quiescent state of V79 Chinese hamster cells favours manganese binding to more sites in the cell thus slowing down their metabolism more than when cells are growing exponentially. This enhanced sensitivity to heavy metals of the plateau phase cells seems comparable to that observed for several agents mentioned previously (7, 8, 10). The intensity of inhibition is also dose and time dependent.

To conclude we agree with other authors that the level of colony forming ability is a good and sensitive parameter for evaluating the toxicity of many agents including heavy metals (24). Owing to their high susceptibility presently shown for manganese, the plateau phase cells offer a convenient model system for assaying metal toxicity.

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Sažetak

REAKCIJA ANIMALNIH STANICA U PLATO FAZI NA UČINAK MANGANA

Proučavani su toksični učinci manganova klorida na dvije faze asinkrone populacije V79 kineskog hrčka: stanice koje rastu eksponencijalno i one koje se nalaze u plato fazi.

Kontrolne i tretirane stanice promatrane su mikroskopski i određivana je brzina njihove proliferacije.

Sposobnost formiranja kolonija iskorištena je za ocjenu djelovanja triju koncentracija manganova klorida u raznim vremenima aplikacije. Nakon inkubacije s manganom, tretirane kulture kao i kontrole, nasadene su vrlo rijetko. Sedam dana kasnije, kolonije su obojene Giemsinom otopinom, izbrojene i njihov broj izražen u postocima od kontrole.

Smanjena sposobnost stvaranja kolonija stanica u plato fazi u odnosu na stanice koje rastu eksponencijalno pokazuje da su te stanice osjetljivije naročito za koncentraciju mangana iznad 5×10^{-5} mol i za ekspoziciju dulju od 10 sati.

Ova razlika u osjetljivosti slična je razlici koju pokazuju stanice u eksponencijalnoj fazi rasta i one koje su dosegle plato fazu ako se tretiraju agensima kao što su bleomicin ili derivati nitrozoureje.

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