Physiological dissection of various effects of ruthenium red dye on *Paramecium* cells

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Summary. Polycationic dye ruthenium red, but not alcian blue, if externally applied to *Paramecium* cells quickly inhibits their phagocytosis. Ruthenium red combined with the cell surface diminishes frequency and duration of ciliary reversals and gradually inactivates the Ca^{++} gating mechanism. This effect persists for 1-3 after ruthenium red removal from the culture medium.

In this paper, some functions of *Paramecium* cells have been studied in the presence or after pretreatment of cells with ruthenium red RR dye. RR is a cationic chloride[3] which combines with the surface coat of *Paramecium* cell[4], but it does not penetrate into living cells[5].

The energy-dependent process of phagocytosis[6] depends on the clonal age of *Paramecium* cells[7] and on ionic composition of the medium[8]. It was observed in other systems that this process begins with slight depolarization of the cell membrane through the reaction of food particles or specific dyes with surface coat[9]. On the other hand, it is known that the energy-dependent uptake and transport of Ca^{++} is inhibited by RR on or within the mitochondrial membrane[10-12] and in sarcoplasmic membrane fraction[13]. In both of the latter instances, the membrane system has no surface coat. Hence the question arises whether the externally applied RR will inhibit phagocytosis when combined with surface coat. To discern the specific activity of RR, the tests have been repeated with another cationic dye Alcian blue AB which also combines with surface coat.

Paramecium is an excitable cell. It starts swimming backwards if its membrane is steadily depolarized by cationic stimulation in the presence of Ca^{++}. In *Paramecium* external Ca^{++} plays an essential role in the coupling between membrane depolarization and the reversal response of cilia[14]. The ciliary reversal is brought about by an influx of Ca^{++} through voltage sensitive Ca^{++}-channels[15,16] of ciliary membrane[17] down to the existing electrochemical gradient. Onimaru[18] has reported the inhibition of the ciliary reversal response of *Paramecium* in the presence of RR. The question arises whether inhibition of ciliary reversal by RR will persist after RR removal and whether bound RR will reduce the membrane permeability to other ion Ba^{++}, which probably enters through voltage sensitive Ca^{++}-channels. In mutant *Paramecium* of group pawn, the inability to move backwards is strongly correlated with low membrane permeability for Ba^{++}[19]. Both effects are due to suppression of the gating through Ca^{++}-channels[19,20]. In some instances, pawn cells are able to change of their shape[21] and to reveal the characteristic behavioral reaction with chlorpromazine[19]. All these tests have been made with RR- treated *Paramecium* cells.

Materials and methods. Organism and culture: *Paramecium tetraurelia*[22] stock 51S wild type and pawn A d4-94 have been routinely handled[24]. Cells were kept at 22 or 27 °C depending on experiment. Their clonal cycle has been partially synchronized by the feeding regime[24]. Wild type cultures with the following characteristics have been used: 12-18 cell divisions after mass autogamy, their mean rate of food vacuole formation during 6-min test was 4.6 ± 1.3, all cells manifested clear backwards swimming after immersion to the test solutions, all were killed in Ba^{++}-paralyzing medium[19] in less than 1 min. In tests of phagocytotic activity, cells were not washed in equilibration medium. In other tests, cells were either directly introduced into tested media, or they were equilibrated about 20 min in equilibration medium 1 mM KCl and 1 mM $CaCl_2$ Tris HCl, pH 7.2, and then tested. pH of culture medium was 7.2-7.4.

Test for food vacuole formation after Preer's method[25] with about 20 specimens used per sample excluding dividers. Behavioral test solutions: Na/Ca and K/Ca test solutions[26] and Ba^{++}-paralyzing solution[19]. Test for cellular shape change after the method of Kaczanowska[21]. The assay for functional ability of the ciliary structures responsible for reversal of ciliary beat as prescribed by Schein[19]. Drugs: ruthenium red standard Fluka, Buchs SG, No. 174535; alcian blue 8 G Hopkins and Williams C.I. 7420; chlorpromazine phenactil POLFA No. 20274 have been used without further purification.

Results. 1. Rate of food vacuole formation. The results of experiments with RR are presented in figure 1. At moderate concentration of RR one may distinguish 3 sequential periods of phagocytotic activity: a) short period of enhancement of phagocytosis during elapse of 6 min, which is the minimal time required to record responses; this phase is followed by b) an abrupt and concentration of RR dependent decrease of phagocytosis, which finally levels at c) some residual activity. In experiments with high concentration of RR (not shown) and with 0.3×10^{-4} M RR, a beginning of the rate of food vacuole formation is not detected and the residual activity largely disappears.

In high doses of AB, time- and concentration-dependent drop of phagocytosis is also observed. However in a broad range of concentrations 0.1×10^{-4} to 0.6×10^{-4} M AB, the rate of food vacuole formation fluctuates slightly but remains insignificantly different with the rate of food vacuole formation of untreated wild type control cells throughout 6 h of experiment.

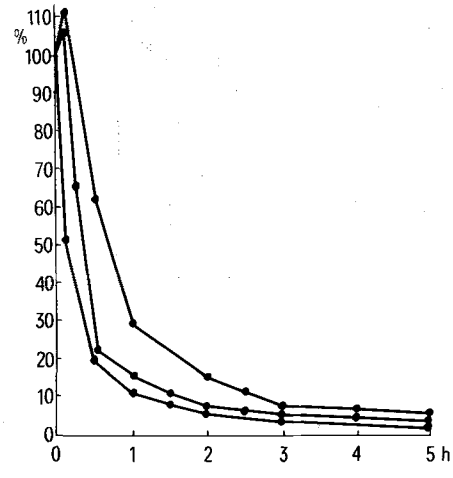

Fig. 1. Effect of ruthenium red dye on the rate of food vacuole formation in *Paramecium* at 22 °C. Ordinate: percentage of the rate of food vacuole formation in control cells. Abscissa: time of incubation of cells in RR culture medium. Upper curve: cells incubated in 0.1×10^{-4} M, middle curve: 0.2×10^{-4} M, and low curve: 0.3×10^{-4} M. Each point is a mean of 3 experiments compared with 3 controls. The SD is not shown; it ranges from about 5% to about 20%, but the latter only at the steep slopes of curves.

2. Survival and cellular behavior in the permanent presence of RR and AB. Survival of cells: Time of death of 50% of cells has been plotted against the RR concentrations at 2 temperatures (figure 2).

Behavioral reactions: In all concentrations used, cells immersed into RR media displayed at first very short ciliary reversals and fast forwards swimming, and next the reaction of reversion largely disappeared. Periodically the behavioral reaction was tested with Na/Ca and K/Ca media of RR-treated cells. Cells were isolated to Na/Ca and K/Ca media either directly from RR-medium or after 8 min equilibration in equilibration medium. At the start of the experiments, cells still maintained an ability to reverse after chemical stimulation if RR was removed.

However, cells which had been incubated in RR media for long periods, progressively lost their ability to reverse after RR removal. Relationship between concentration of RR

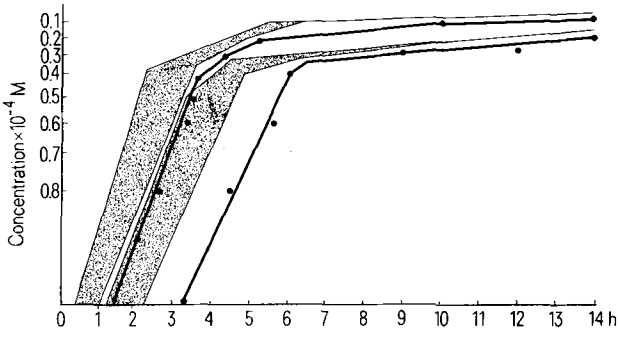

Fig. 2. Lethality of 50% of cells (LD_{50}) and the loss of excitability of cells incubated in RR culture medium and then tested in Na/Ca test solution. Ordinate in log system: concentration of RR. Abscissa: time of incubation in RR. LD_{50} curves: heavy lines: left for experiments performed at 27 °C and right at 22 °C. Each point is a mean of 3 experiments and curves are approximated. Dashed areas to the left of both LD^{50} curves represent fields occupied by the points resulting from particular tests when loss of excitability of about 20 RR incubated cells have for the first time been revealed in Na/Ca test solution. Left dashed area results from 3 experiments performed at 27 °C, right dashed area results from 5 experiments performed at 22 °C. Concentrations used: 1.0×10^{-4}–0.1×10^{-4} with intervals of 0.1×10^{-4} M RR and 0.15×10^{-4} M.

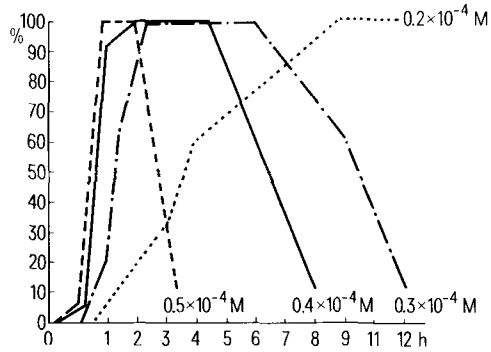

Fig. 3. Kinetics of the change of resistance to Ba^{++} of cells incubated in different concentrations of RR. Abscissa: time of incubation of cells in RR culture medium. Ordinate: maximal time of survival of RR-treated cells since their immersion into Ba^{++} paralyzing test solution expressed in percentage of time of survival of resistant pawn cells (100%). Maximum time of survival is estimated by the time of death of at least 2 most resistant cells out of 20 in sample introduced to Ba^{++}. This restriction of data to the most resistant cells is required since cells incubated in RR culture medium at random approach to LD_{50} parameters. Each point represents the mean of 3 experiments performed at 22 °C.

during incubation and the elapse of time needed for a complete inhibition of reversion of 20 cells introduced to Na/Ca test medium reveals fluctuations from one sample to another and from one experiment to another. However, if all results of experiments performed at a given temperatures are protocolled in 1 graph, they are all dispersed inside the dashed areas in figure 2. The loss of excitability markedly precedes a time of cell death in a regular manner in both temperatures tested.

All cells which still swim actively in RR incubation medium (while their excitability is lost as tested in Na/Ca and K/Ca media) may be rescued if replaced to plain culture medium. Recovery of their excitability after Na/Ca stimulation requires 1–3 h at 27 °C, as has been tested on unexcitable cells pretreated in RR concentrations ranging from 0.6×10^{-4} M to 0.1×10^{-4} M RR.

Exactly comparable experiments performed with cells incubated in the various concentrations of AB failed to reveal the loss of excitability of cells. Pawn cells displayed roughly similar kinetics and concentration dependent lethality in RR as did wild type cells.

3. Test of resistance of cells to Ba^{++} toxic action after their incubation in media with cationic dyes. Cells immersed into RR culture medium of a given concentration of RR were periodically assayed for their resistance to killing action of Ba^{++} test solution (figure 3). Cells of wild type incubated in RR presence gradually become resistant to the toxic action of Ba^{++} and some of them reach the resistance level of pawn cells. This resistance is maintained during farther incubation in RR and spreads to more cells in the sample tested in Ba^{++}, but after long exposure to RR it eventually drops. The increase of resistance ot Ba^{++} strictly corresponds in time to loss of excitability of cells during incubation in a given concentration of RR. Drop of resistance to Ba^{++} observed after long incubation in RR coincides with a period of death of about 50% of cells.

Comparable tests with AB dye applied to wild type cells revealed no change of excitability or resistance to killing action of Ba^{++}. 4. Test for the appearance of invaginated cells in the presence of RR dye. Cells introduced into exhausted medium[21] supplemented with 0.4×10^{-4} M RR revealed about 2–5% of invaginated cells after 2 h of incubation, while none was observed in control cells introduced into plain exhausted medium.

5. Test for an ability of cilia to reverse in unexcitable RR-incubated cells of the wild type. Test followed the method of Schein[19]. In the presence of chlorpromazine unexcitable RR-treated cells did not reverse until the final moment of their death, when they abruptly reversed and died. This reaction is reminiscent to the behavior of pawn cells[19], while the wild type control displayed the frequent ciliary reversals at the start of experiments.

Discussion. Results presented here clearly demonstrate that in all cells the energy-dependent process of phagocytosis is specifically inhibited in the presence of RR. Slight stimulation of phagocytosis observed at the start of experiments performed in the presence of weak concentration of RR probably reflects the primary stimulation induced by the combining of RR with the surface coat and is later effaced by the inhibitory RR action on energy supply. This inhibitory action of RR is not observed in the presence of other polycationic AB. This inhibition of phagocytosis is not directly related to mechanism of suppression of ciliary reversals, since unexcitable pawn cells are able to food vacuole formation at the normal rate[21].

The maintenance of cells in RR-culture medium when the rate of food vacuole formation was tested, with omission of washing bath of equilibration medium, though less precise, was applied to avoid any change in the state of membrane

of *Paramecium* caused by starvation in equilibration medium.

Cells maintained in RR-culture medium were not prestarved and they represented an array of stages within the cell cycle. This fact probably accounts for the individual variability of cell resistance to RR and to Ba^{++}.

The cell membrane itself and the membrane potential changes during cell cycle[27,28], and this may influence timing of effects of RR in a particular cell.

The behavioral reaction of wild type cells during their immersion into RR solutions displayed some variability: short lasting reversions, inhibition of reversions and/or fast forwards swimming. All these reactions are known to be under membrane potential control[29], since hyperpolarization decreases the frequency of action potentials and increases ciliary beat frequency while depolarization increases frequency of action potentials and decreases ciliary beat frequency. Therefore it is expected that, at least in the majority of cells, RR solution brings about the temporal hyperpolarization and inhibits depolarization. However, this prediction requires an electrophysiological confirmation.

Cells immersed into RR solution and then washed out are able to reverse; however, after long exposure to RR they gradually lose excitability. These cells become resistant to the toxic action of Ba^{++} and become capable of spontaneous shape transformation. They also behave like pawn cells in the presence of chlorpromazine. All these facts are taken as evidence that RR acts on the Ca^{++} gating mechanism through its gradual inactivation. Since this reaction depends on periods of exposure and concentration of RR, and since its kinetics depends on temperature, some conformational changes in cell membrane are probably involved in this inactivation. This gradual inactivation of Ca^{++} gating mechanism is specific to RR and not to all polycationic dyes, since it does not occur in the presenc of AB.

1 This study is partly supported by grant PAN 22 m/3 76.
2 Thanks are due to Prof. Dr T.M. Sonneborn for providing strains used in this paper, to Prof. Dr D.L. Nanney and Dr Y. Naitoh for instructive discussions and for critical reading of the manuscript. Technical assistance of Miss Studzińska, is also acknowledged.
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Hematocrit as an index of changes in plasma volume in conscious dogs[1]

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Summary. Hematocrit (HCT) measurements were made in intact and splenectomized conscious dogs to determine if observed decreases in HCT were produced by plasma volume expansion or splenic sequestration of erythrocytes. We found that in conscious dogs HCT is a poor indicator of changes in plasma volume.

During the course of experiments on conscious dogs it was observed that hematocrit values obtained from animals immediately after being brought from the kennel area were considerably higher than those obtained subsequently from the same animals after lying unrestrained for 15–30 min in the laboratory. The experiments being conducted required the administration of various amounts of water and it was our desire to use hematocrit as an indication or confirmation of changes in plasma volume. Although such correlations are commonly used with anesthetized animal preparations it was unlikely that the volumes of water used in our experiments would have increased plasma volume by more than 2% and probably could not alone bring about the observed reductions in hematocrit. It is well documented that large changes in hematocrit can occur in dogs during exercise[5–7] due to an increase in sympathetic stimulation and contraction of the splenic reservoir. It would then seem reasonable that a trained dog brought from the kennel environment where the level of exercise and excitement is presumably high might show a reduction in hematocrit when placed in the more controlled and less stimulating laboratory environment. This reduction in hematocrit would most likely be a gradual process accomplished by a decrease in sympathetic tone and splenic dilatation over a period of minutes or perhaps hours. It might also be possible to observe an increase in hematocrit during the course of an experiment if the animal were to become emotionally aroused or if sympathomimetic agents are administered. This posed an interesting question concerning the acceptability of using hematocrit as an index of