Mechanism of Enhancement of Polynucleotide Binding to Cells by Mutagens†

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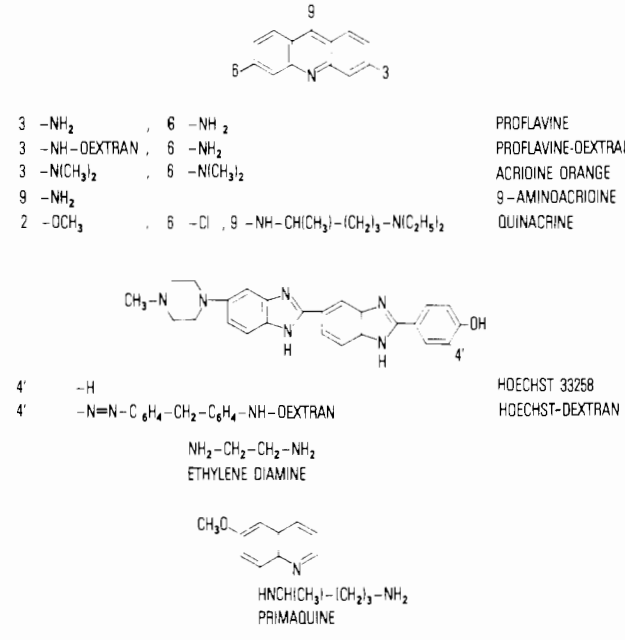
ABSTRACT: The binding of polyuridylate to cells is substantially increased by proflavine. This enhanced binding is saturable with respect to time and to the concentration of both proflavine and polyuridylate. Enhancement is observed only when cells are exposed to both proflavine and polyuridylate together and depends cooperatively on the proflavine concentration. The resulting complex formed between the cell, proflavine, and polyuridylate can be dissociated with salt but not with sucrose solutions. An increase in the binding of polyuridylate to cells similar to that observed with proflavine was also obtained with cationic dyes such as acridine orange, 9-aminoacridine, and Hoechst 33258, while the introduction of a bulky polysaccharide residue, dextran, into the dyes cancels these effects. Similarly, cationic aromatic compounds such as primaquine and quinacrine which carry bulky nonplanar substituents or aliphatic cationic compounds like ethylenediamine do not enhance binding. Proflavine is unable to augment the binding of a basic macromolecule, diethylaminoethyldextran, to cells. The model proposed for the enhanced binding of polyuridylate is based on the cooperative formation of stacked complexes of cationic dye located between the cell surface and the bound polyuridylate.

The cell surface is a selective barrier which protects the interior of the cell from a number of potentially harmful compounds. For example, nucleic acids are known to bind poorly to the cell surface and consequently cells are well protected against foreign genes (Ledoux, 1972; Pitha, 1979); however, this binding can be increased greatly by the presence of carcinogenic agents (Kubinski et al., 1976). The practical significance of this enhancement is unclear since toxic concentrations of carcinogenic compounds are required to achieve substantial augmented binding. Still, the phenomenon is of interest because very diverse carcinogens produce this increase in the binding of nucleic acids while a number of similar, harmless compounds do not (Kubinski et al., 1976). In this work we investigated the mechanism of enhanced binding of poly(U)[1] to cells in the presence of compounds which are known to bind to deoxyribonucleic acid. The use of these compounds and their structural variants (Scheme I) has led to a clarification of the mechanism of enhancement. Furthermore, it was found that only the components of the cell surface are involved in the phenomenon; consequently, the system may be used as a potential probe to investigate differences in various cell surfaces.

Scheme I



Materials and Methods

Materials. Eagle's minimal essential medium, trypsin, fetal bovine serum, glutamine, and antibiotics were purchased from Grand Island Biological Co., Grand Island, NY. (^{3}H)Poly(U) (18–22 μCi/μmol) was obtained from Schwarz/Mann Corp., Orangeburg, NY. DEAE-dextran (M_r 500 000; N content 3.2%; from Pharmacia Co., Sweden) was made radioactive by alkylation with labeled chloroacetic acid (Pitha et al., 1974). The dye, Hoechst 33258, was obtained from Dr. Loewe, Hoechst Co., Frankfurt, West Germany, and ethylenediamine, proflavine hemisulfate, primaquine, quinacrine, acridine orange, and 9-aminoacridine were from Sigma Chemical Co., St. Louis, MO. Attachment of proflavine and Hoechst 33258 by stable covalent bonds to a dextran fraction of average molecular weight 170 000 was described previously (Pitha, 1978). Cover glasses (11 × 22 mm) were purchased from Arthur H. Thomas Co., Philadelphia, PA.

Cell Culture. WI-38 human fibroblasts obtained from Dr. V. Cristofalo, Philadelphia, PA, were grown at 37 °C in minimal essential medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air. Routine monitoring for mycoplasma infection was performed (Levine, 1972).

Assay for Polynucleotide Binding to Cells. Confluent monolayers of human fibroblasts grown on cover glasses (about 1×10^5 cells/cover glass) were used in all experiments. Cell counts were determined by using a hemocytometer. Cells were

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[1] Abbreviations and compositions of buffers used: PBS, isotonic-buffered saline containing NaCl (8 g/L), $Na_2HPO_4 \cdot 7H_2O$ (2.2 g/L), KCl (0.2 g/L), and KH_2PO_4 (0.2 g/L), pH 7.2; Dulbecco's phosphate-buffered saline containing NaCl (8 g/L), KCl (0.2 g/L), $CaCl_2$ (0.1 g/L), $MgCl_2 \cdot 6H_2O$ (0.1 g/L), and $Na_2HPO_4 \cdot 2H_2O$ (1.2 g/L), pH 7.2; poly(U), polyuridylate.

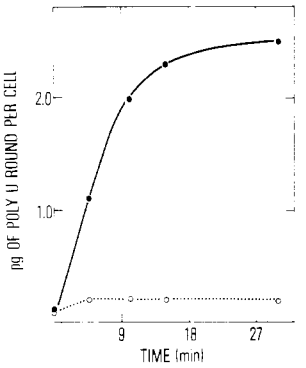


FIGURE 1: Binding of poly(U) to confluent human fibroblasts in the presence of proflavine (100 μg/mL) (●) and in its absence (○).

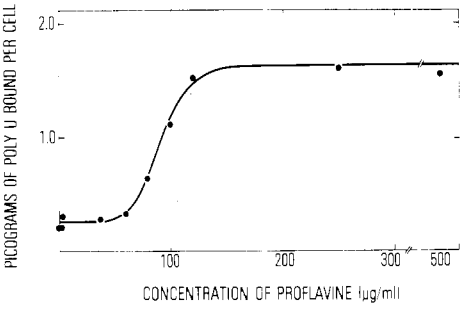


FIGURE 2: Dependence of poly(U) binding to cells on proflavine concentration. The solid line represents theoretical values derived from the Hill equation, $v = Vs^{nh}/(K + s^{nh})$, where $V = 1.36$, $nh = 7.2$, and $\log K = 14$. The solid circles represent experimental data where the amount of poly(U) bound to cells in the presence of varying concentrations of proflavine was measured after 30 min and then corrected for binding which occurs in the absence of proflavine. As observed, the data closely fit the theoretical curve for positive cooperativity.

washed once with PBS and pulsed with 10 μL of a solution of (^{3}H)poly(U) with or without proflavine at 37 °C in the same buffer. Except for Figure 2, the final concentration of proflavine used in all the experiments was 100 μg/mL. Saturating concentrations of poly(U) (about 61 μg/mL) were used in all experiments except Figure 3. Following the pulse, the cover glasses were rapidly washed by immersion in a series of beakers containing ice-cold Dulbecco's phosphate-buffered saline, and radioactivity was measured by scintillation counting in a toluene-based scintillant after overnight digestion with Nuclear Chicago Solubilizer (NCS). Binding of (^{3}H)DEAE-dextran was measured after 30 min at a concentration of 34 μg/mL as described above for poly(U).

The experimental results were influenced by less than 10% when different batches of (^{3}H)poly(U) were used. Similarly, different cultures of nonsenescent human fibroblasts consistently gave closely similar enhancement values. In addition to WI-38 cells, stimulation of poly(U) binding by proflavine has also been observed in several other eucaryotic mouse and human cell lines; the level of enhancement is dependent on cell density (unpublished experiments).

Results

Figure 1 shows that fibroblast monolayers exposed to a solution of poly(U) bind approximately 0.2 pg/cell of polynucleotide and that saturation occurs within a few minutes. However, when cells are exposed to poly(U) and proflavine (100 μg/mL) together, poly(U) binding is dramatically increased (10–20 times) in a time-dependent manner which saturates within 20 min. The observed binding occurs to cells rather than to the cell substrate as an area of glass equivalent

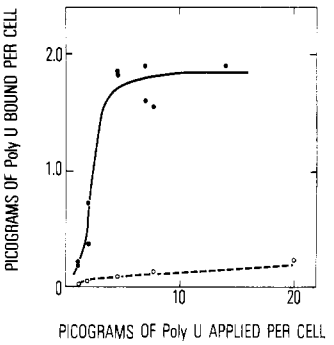


FIGURE 3: Dose–response relationship between the amount of poly(U) applied and the amount of poly(U) bound to human fibroblasts in the presence of proflavine (●) and in the absence of proflavine (○) after 30 min at 37 °C.

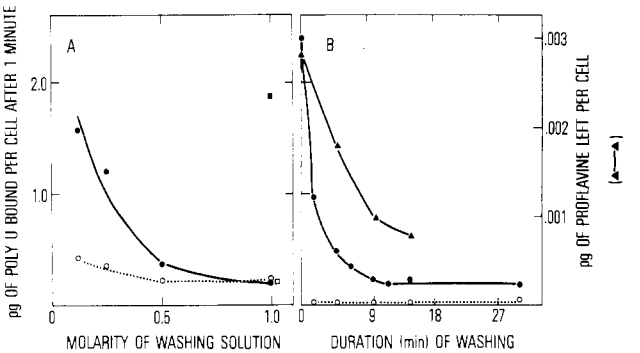


FIGURE 4: Desorption of poly(U) and proflavine from the cell surface. (A) Cells exposed for 30 min to a mixture of poly(U) and proflavine (●) or to poly(U) alone (○) were washed routinely in Dulbecco's phosphate buffer and then immersed for 1 min in different concentrations of NaCl. When cells are instead immersed in 1 M sucrose after treatment with poly(U) + proflavine (■) or poly(U) (□), no release of the polynucleotide from the cell surface is observed. (B) Cells exposed to poly(U) and proflavine together (●) or to poly(U) alone (○) for 30 min were first washed rapidly in ice-cold Dulbecco's phosphate buffer and then immersed in 1 M NaCl for the times indicated. Alternatively, cells were exposed to proflavine alone (100 μg/mL) (▲) for 30 min and then washed with isotonic saline for varying lengths of time. The amount of proflavine left bound to cells was determined by extraction with Me_2SO, 1:1, and absorbance was measured at 420 nm.

to one cell adsorbs only about 0.1 pg of poly(U) in the presence of proflavine (data not shown).

The amount of poly(U) bound to cells is dependent on the concentration of proflavine. Figure 2 shows that a minimum amount of proflavine is required for this augmented binding and that the association is cooperative, since the experimental data can be well fitted by the Hill equation (Atkins, 1973). Figure 3 shows that the binding of poly(U) to cells can be saturated both in the presence and in the absence of proflavine. At the point of saturation, proflavine causes as much as one-third of the polynucleotide that is applied to remain bound to the cells.

Poly(U) bound to cells in the presence of proflavine can be released from cells by a brief treatment with isotonic saline. The pattern of this desorption is exquisitely sensitive to time and to the salt concentration. Figure 4B shows that the proflavine-mediated enhancement effect is completely abolished by a 10-min washing with saline. Figure 4A shows that solutions of higher ionic strength dissociate poly(U) from cells more effectively than does isotonic saline. This effect is not due to the hypertonic nature of the solutions since a 1 M sucrose solution is incapable of releasing poly(U) from the cell surface (Figure 4A). The data clearly point to the ionic character of the proflavine-augmented binding of poly(U) to

Table I: Requirement for the Simultaneous Presence of Poly(U) and Proflavine

	poly(U) bound per 1.3×10^5 cells in 30 min (cpm)
pretreatment (min)[a]	
PBS (10)	1306, 1271
PBS (15)	1473, 1958
PBS (30)	1941
proflavine (10)	1073, 1029
proflavine (15)	1441, 1505
proflavine (30)	1765
cotreatment	
poly(U) + proflavine	9968, 9407 9968, 9409

[a] Cells were pretreated with PBS or with proflavine (100 μg/mL) for the times indicated in parentheses, washed once rapidly in PBS, and pulsed with radioactive poly(U) (approximately 28×10 10^3 cpm/1.3×10^5 cells) for 30 min. Control cells were cotreated with poly(U) and proflavine for 30 min. Duplicate determinations are shown in some cases.

the cell surface. Also, since $MgCl_2$ and NaCl were equally effective, dissociation appears to be independent of the component ions (data not shown).

The increased binding of poly(U) to cells in the presence of proflavine occurs only when all three of the components are simultaneously present. Data in Table I show that pretreatment of cells with proflavine alone, followed by immediate exposure to poly(U), does not result in enhanced polynucleotide binding, whereas cotreatment does. During the pretreatment, a considerable amount of proflavine (about 0.0028 pg/cell) is bound to the cells and internalized. Upon washing with isotonic saline, proflavine is eluted, and the amount which remains cell bound is gradually decreased. This elution pattern is, however, slower than the loss of poly(U) from the cell surface (Figure 4B), suggesting that poly(U) is mainly surface bound. Furthermore, poly(U), once dissociated from the cell surface by washing, can be re-bound to the same cells in the same manner (unpublished experiments).

While proflavine mediates a (10–20)-fold increase in the binding of poly(U) to cells, the binding of a basic macromolecule, diethylaminoethyldextran, is, in contrast, influenced only slightly by proflavine. About 1.4 pg of diethylaminoethyldextran is bound per cell; this amount decreases by 9% when 100 μg of proflavine is present in the solution and by 65% when the concentration of the latter is 1 mg/mL.

To determine what chemical features were essential for a compound to enhance the binding of poly(U), a number of compounds differing in basicity or aromaticity were tested (Figure 5). Ethylenediamine, primaquine, and quinacrine were unable to mediate enhancement; 9-aminoacridine is less efficient than proflavine while acridine orange and Hoechst 33258 were both very effective. However, when proflavine or Hoechst 33258 was attached by firm chemical bonds to the soluble polysaccharide, dextran, their ability to augment the binding of poly(U) to cells was completely lost (Figure 6).

Discussion

The binding of poly(U) to cells can be increased by three aromatic compounds: proflavine, acridine orange, and Hoechst 33258. A common denominator of these compounds is their basicity and their ability to bind to deoxyribonucleic acid (Muller & Crothers, 1975; Muller & Gautier, 1975). However, these two properties alone apparently are not sufficient to stimulate binding, since ethylenediamine, which is a strong aliphatic base, or quinacrine and primaquine, both

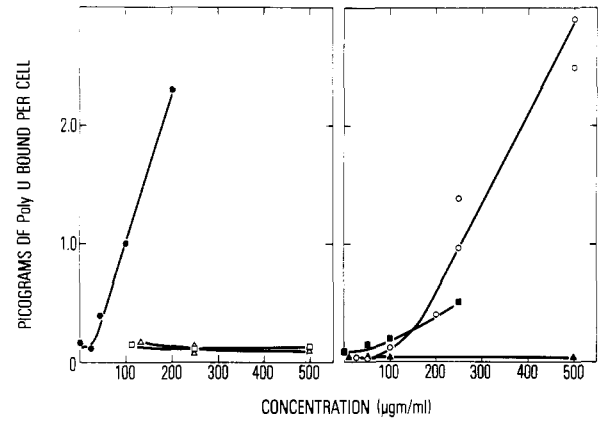


FIGURE 5: Comparison of the effect of varying concentrations of basic compounds on the binding of poly(U) to cells. All compounds were incubated with poly(U) (61 μg/mL) at 37 °C for a few minutes before cells were exposed to the mixtures. The effects of acridine orange (○), 9-aminoacridine (■), ethylenediamine (▲), primaquine (□), quinacrine (△), and Hoechst 33258 (●) are shown.

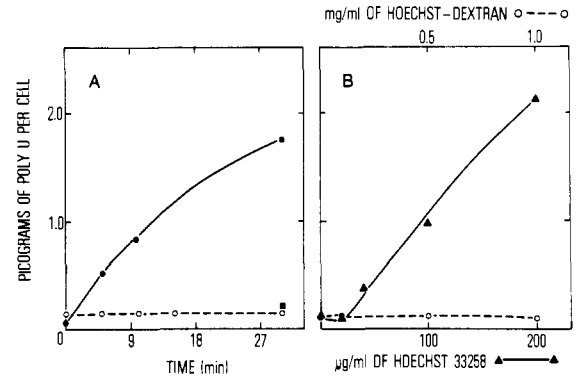
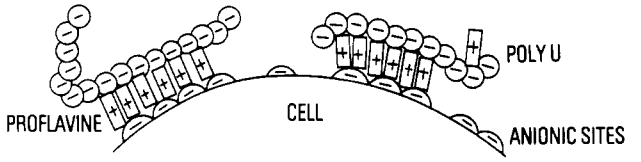


FIGURE 6: (A) Comparison of the enhancement effect of proflavine (●) and proflavine–dextran (○) on the binding of poly(U) to cells. The concentration of proflavine–dextran used was 2 mg/mL, which corresponds to an effective proflavine concentration of 100 μg/mL. No enhancement of poly(U) binding was observed after 30 min even when the concentration of proflavine–dextran was increased to 60 mg/mL (■). (B) Effect of varying concentrations of Hoechst 33258 (▲) or Hoechst–dextran (○) on binding of poly(U) to cells after 30 min at 37 °C. The composition of Hoechst in Hoechst–dextran amounts to 7.2% by weight.

aromatic bases known to bind to DNA (Whichard et al., 1968; O'Brien et al., 1966; Estensen et al., 1969; Krey & Hahn, 1974), were not effective. Further examination of the formulas of the compounds revealed that the effective enhancers have a more planar geometry than those that were inactive (cf. Scheme I). This condition for stimulatory activity was confirmed by synthesizing and testing derivatives of proflavine and Hoechst 33258 into which a bulky substituent, polysaccharide dextran, had been inserted (Scheme I). Although these derivatives retained the basicity of the original compounds and their ability to interact with DNA (Pitha, 1978), the ability to increase poly(U) binding to cells was completely lost.

The enhanced binding of poly(U) to cells involves forces that are ionic in nature since the binding is easily dissociated by salt but not by sucrose solutions. Since poly(U) is electronegative and the overall electric charge of the cell surface is also electronegative (Wallach, 1972; Hubbard & Cohn, 1976), it is conceivable that electropositive enhancers, such as proflavine, act by decreasing the electrostatic repulsion between the polynucleotide and the cell. This may occur by several mechanisms. Enhancers are soluble both in water and in lipids (Albert, 1966), and a considerable amount is capable of

Scheme II



crossing the cell membrane and dissolving in the cell (see Figure 4B), a process which by itself may change the electric charge of the cell. We therefore investigated the effect of proflavine on the surface charge of cells using a basic macromolecule, diethylaminoethyldextran, which by itself binds extensively to the electronegative cell surface; this binding would be expected to decrease due to the electropositive charge conferred on the cell by proflavine. Although this decrease in binding was indeed observed, extraordinarily high concentrations of proflavine (1 mg/mL) were necessary to achieve a reduction of 65%. This finding indicates that the dissolution of the enhancer within the cell is not the main mechanism of enhancement of polynucleotide binding. Other observations support this conclusion as well. When cells were pretreated with proflavine and then immediately exposed to a poly(U) solution free of proflavine, no enhancement was observed in spite of the fact that cells still contained a considerable amount of proflavine. Thus, for enhancement, the presence of proflavine in the medium together with poly(U) is more critical than its presence in the cell.

Another possible mechanism for enhancement consists in the formation of a polynucleotide–enhancer complex, whereby the electronegative charge of the polynucleotide is reduced, and the complex would then be adsorbed to the cell. Although the compounds studied here bind extensively to deoxyribonucleic acid, binding to poly(U) is generally weaker. Thus, this mechanism alone cannot account for the enhancement phenomenon.

To elucidate the mechanism possibly responsible for the enhancement of poly(U) binding to cells by proflavine, we had to consider three aspects of the phenomenon: (1) the necessity for a flat geometry for the enhancer, (2) the requirement for a threshold concentration of enhancer in the medium, and (3) the necessity for cells to be exposed simultaneously to enhancer and polynucleotide. These observations suggest that self-association of the enhancer may be essential for increased binding.

At pH 7.2, proflavine and acridine orange bear a single positive charge and are known to self-associate to form complexes in which the aromatic rings are stacked upon each other; these self-associations thus result in the formation of polycationic complexes (Muller & Crothers, 1975; Schwarz et al., 1970). The self-association of proflavine or acridine orange is considerably increased in the presence of polyanions, like polyglutamate, to which these compounds bind cooperatively (Schwarz et al., 1970; Schwarz & Balthasar, 1970). Consistent with this view of self-association, the enhanced binding of poly(U) to cells thus depends critically on the proflavine concentration and shows the observed cooperative pattern of association. Apparently, stacked proflavine complexes with multiple cationic charges form a bridge between the electronegative surface of the cell and the electronegative poly(U) molecule. This situation, illustrated in Scheme II, is thus somewhat analogous to the enhanced binding of polynucleotides to cells achieved by simple polybases such as diethylaminoethyldextran.

Amongst the large number of compounds which were previously tested for their ability to enhance the binding of deoxyribonucleic acid to cells, only chemical mutagens and ultimate carcinogens were found to be effective (Kubinski et al., 1976). Our results suggest that the mechanism of this enhanced binding may be due in some cases to the ability of certain electropositive compounds to self-associate strongly, or to approach each other closely, in the presence of polyanions. As nucleic acids are polyanionic and are an important target for carcinogens, it is evident that both phenomena share certain common features and factors.

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