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INTERACTION OF INFLUENZA AND PARAINFLUENZA VIRUSES WITH POLYCATIONS, ORGANIC OLIGOCATIONS AND CHROMOSOME PREPARATIONS

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Summary. — As evaluated by light scattering at 90°, natural organic oligocations such as putrescine, spermidine and spermine interfered with myxovirus aggregates which were induced by the histone H2A and strongly amplified by shaking during incubation. In contrast, the synthetic oligocation 1.7-diamino heptane itself aggregated the virus particles, its action being unmodified by adding polycationic H2A in abundance. When human chromosome preparations treated with protamine solution and shaked during incubation were covered with a stable polycationic molecular layer, the chromosomes had become unstainable by the Giemsa method even if the dye was used in excess. Nevertheless, the affinity of influenza virus particles for protamine was so high that they were able to dissociate the protamine molecules from the preformed complexes reconstituting the affinity of chromosome preparations to Giemsa stain. The virus-caused shift in the staining ability of chromosomes did not occur when bacterial suspension was added instead of the viral one. The model of oligocationic relaxation and of polycation condensation accounting for the modulatory effects of polyamines is discussed.

Key words: histone H2A; protamine; polyamines; chromosomes; Giemsa stain affinity; influenza virus

Introduction

Cytocidal effects of the natural polycations such as histones and protamines and their ability to inactivate also some viruses were generally mentioned many years ago (Becker and Green, 1960). The influenza and parainfluenza viruses interact strongly with natural polycations (Antohi *et al.*, 1983), the primary alterations consisting in viral surface pycnosis and formation of interviral aggregates. They lead to a significant increase in optical density (OD) values of viral suspensions accompanied by a decrease in virus haemagglutination ability and structural modifications. Among different polycations assayed, the most active were the histones H2A and H2B; however the protamine was also significantly effective at least in interaction with influenza virus particles. In these interactions the viral surface displayed an

expected electrostatical polyanionic behaviour very similar to that of cell surface, chromatin and chromosomes, e.g. it had affinity for basic compounds such as macromolecular polycations and interacted with them giving together surface condensations and viral aggregations. Formation of pycnosis observable on the cell surface could be abolished by 1.7-diamino heptane, a cationic analogue of the natural polyamines (Antohi and Brumfeld, 1983). We describe here the interactions between influenza virus particles and protamine-charged chromosome preparations resulting in shifts of Giemsa staining and the interfering effects of putrescine, spermidine and spermine on viral aggregation induced by the histone H2A. Such a polycation-oligocation interference in the interaction with polyanionic complexes could account for the mechanism of a well-documented modulatory action exerted by polyamines on different template requiring biosyntheses (Goldemberg and Algranati, 1981) and on cell division (Herbst and Elliot, 1981).

Materials and Methods

Viruses. Parainfluenza virus type 1 (Sendai) was propagated in chorioallantoic membrane (CAM) fragments (Samuel *et* al., 1981) and influenza A/Rom 3/83 (H3N2) virus was grown in chicken embryos. Both viruses were purified and concentrated by differential centrifugation of infected allantoic fluids (at 2,500 g for 60 min and at 75,000 g for 30 min) in Beckman L5-65 ultracentrifuge, rotor Ti60. The pellet was washed twice and finally resuspended in phosphate buffered saline (PBS) by sonication. The initial virus suspensions had a haemagglutination titre of about 1/10,000 estimated as described (WHO Techn. Res., 1973). Portions of 0.1 ml each of initial virus suspension and 1.8 ml aliquots of acetate-saline (natrium acetate 30 mmol/l, natrium chloride 150 mmol/l, pH 6.0) were distributed in 18/180mm tubes; the mixtures, having an OD_{430} of 0.19 for different series of both viruses, were immediately used. This λ-value was prefered since it had occurred within the range of the domains used for light scattering evaluations.

Initial virus-free allantoic mixture. In order to evaluate the degree of intervening participation of the allantoic impurities in virus-polycation interactions, an initial virus-free allantoic mixture was prepared as follows: 1.0 g of washed wet allantoic membranes was disrupted by manual grinding for 15 min at 18 °C, with 1.0 g Quartzsand (Merck) and 10 ml PBS; the resulting product was centrifuged at 2,500 g for 60 min and the supernatant was then mixed 1/5 (v/v) with clear blood-free allantoic fluid prediluted 10% in PBS.

Stock solutions of polycationic polypeptides and oligocationic polyamines. Different aqueous solutions of 2 mg/ml of protamine sulphate and histone H2A (Sigma), and 50 mg/ml putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride (Fluka) and 1,7-diamino-heptane (Merck) were separately distributed in 3 ml aliquots, kept at −10 °C and used within 30 days.

Conditions of oligocation — virus — histone H2A interactions evaluated by light scattering at 90°. To separate series of virus-acetate saline mixtures of each virus (Table 1) 0.1 ml stock solution of each oligocation was added and shaken for 10 min at 37 °C (120 strokes/min). The sample tubes had a slanted angle of 20° and shake path length of 30 mm. In experiments presented in the Table 1, stock solution of H2A was distributed as to reach the final polycation concentrations of 50 μg/ml for influenza virus samples and 100 μg/ml for Sendai virus samples. The following controls were prepared: oligocation-free and oligocation-polycation- free samples completing the final 2 ml volumes with 0.1 ml and 0.2 ml of distilled water, respectively; samples with 0.1 ml initial virus-free allantoic mixture and histone H2A in acetate saline (Table 1). All the samples were shake-incubated for 20 min as above and then examined by the light scattering method. The light scattering measurements were performed at 90° and $\lambda = 400$, 450 and 500 nm using a computer-attached Aminco SPF-500 corrected spectrofluorometer. These measurements evaluating the light scattering dependence on λ-values resulted in a reciprocal well-documented control of the modifications appearing during the virus-oligocation and polycation interactions.

At each λ-value at least 50 measurements were done, the resulted mean values of scattered light (Table 1, R_{90}) being independent of sample fluctuations (the relative error was $<0.5\%$.

Chromosome preparations and the shifts in Giemsa stain affinity induced by protamine and influenza virus treatments with shake-incubation. Chromosome preparations were obtained from total human blood cultures employing the method of Moorhead *et al.* (1960) with some modifications: after spreading the cell suspension on chilled slides, these were kept for 10 min at 60 °C in a drying incubator and then fixed in methanol-glacial acetic acid 3/1 (v/v) for 72 hr at room temperature; the slides were stained according to Giemsa.

The treatments of the chromosome preparations with protamine and influenza virus suspension preceded Giemsa staining. To make sure of efficient reciprocal shaking during these treatments, each slide was sustained in horizontal position by attaching it, with its ends, to the nicks cut laterally in rubber plugs; these sustained the slides with the surface containing chromosome preparation immersed in the solutions and viral suspension. All slides attached in this way were immersed into different 70/140 mm cylindric glass boxes containing: (1) 150 ml of buffer (66 mmol/l KH_2PO_4 — 53 volumes, 66 mmol/l Na_2HPO_4 — 47 volumes, pH 6.8) as control sample; (2) 144 ml of buffer and 6 ml of initial virus suspension; (3) and (4) containing 135 ml of buffer and 15 ml of protamine stock solution. All sample series were incubated for 20 min at 37 °C and shaken at 80 strokes/min and a shake-path length of 30 mm. The solutions and suspensions from containers were removed, the slides immediately washed once with 150 ml of distilled water and the series were then recharged with 150 ml of buffer for each of (1) and (3) series and 144 ml of buffer + 6 ml of initial influenza virus suspension for each of (2) and (4) series; the shake-incubation as described above was continued for 20 min. The slides were detached from the nicked rubber plugs, rewashed with distilled water and stained with Giemsa solution (Merck) 1/20 (v/v) in buffer for 18 hr at room temperature. In a parallel group of series, the initial virus suspension was replaced by an initial bacterial suspension prepared by suspending in buffer at $OD_{430} = 1.8$ an overnight culture on slant tryptose agar (Merck) of *Escherichia coli* strain PRC 399.

Results

As shown in Table 1, the light scattering values given by control polycation-free virus samples were enhanced at all wavelengths of incident light, when the histone was added alone. The polycation was used in different concentrations of 50 μg/ml and 100 μg/ml for influenza and Sendai viruses, respectively,

Table 1. Light scattering at 90° (R_{90}) values of different histone H2A-virus-oligocation mixtures at three different wavelengths

Histone H2A μg/ml in samples of:			Oligocation 2.5 mg/ml	R_{90}					
				S.v. at λ =			I.v. at λ =		
S.v.	I.v.	F.a.		400	450	500	400	450	500
—	—	—	—	47.8	45.5	43.7	74.3	61.1	52.7
—	—	—	spermidine	44.6	42.0	37.0	64.1	53.6	46.6
—	—	—	spermine	40.2	38.0	35.2	63.8	54.3	48.0
—	—	—	putrescine	51.7	48.6	47.1	65.0	55.8	48.1
100	50	—	—	86.3	78.6	74.0	104.0	89.8	81.6
100	50	—	spermidine	38.9	37.5	36.2	76.6	65.5	58.5
100	50	—	spermine	40.0	37.9	36.4	74.8	63.7	58.1
100	50	—	putrescine	59.2	55.2	54.1	82.3	68.4	65.0
—	—	—	1,7-diamino heptane	Tight precipitation in a single block with both virus samples					
100	50	—	1,7-diamino heptane	Precipitation in 5—6 large aggregates per sample					
—	—	50	—	$R_{90} < 5\%$ of the lowest value given by virus samples					
—	—	100	—						

S.v. = Sendai virus; I.v. = Influenza virus; F.a. = free allantoic mixture

to allow a good light scattering evaluation of the effect exerted by oligocationic polyamines. Spermine, spermidine and putrescine, generally caused a slight decrease in the scattering values of histone-free virus suspensions, while their preaddition to histone — virus samples evidently impeded the enhancement of light scattering values constantly induced with the polycationic H2A. The oligocations caused also similar changes of the increased light scattering values, even if they were added after the polycationic treatment of the viral suspensions. At different λ-values of the incident light, there was a clear-cut linearity of the light scattering values given by Sendai virus samples, while the figures provided by influenza virus samples showed slight slope modifications displayed particularly by spermine and putrescine series of histone--virus mixtures. The control series of allantoic extract and fluid mixed with histone H2A provided R_{90} values between 0.7 and 1.5, suggesting the significant predominance of virus particles in the isolates used as well as in the interactions with polycations and oligocations (Table 1).

The treatment of the chromosome preparations with protamine (200 μg/ml) completely impeded the subsequent Giemsa staining even on conditions of overstaining exposure; the slides (cell nuclei and metaphase chromosomes) became, in other words, Giemsa negative. The subsequent exposure of protaminized Giemsa-negative slides to the treatment with influenza virus suspension rendered the chromosome affinity for Giemsa staining, i.e. the slides became Giemsa positive. The chromosomes undergoing the protamine--viral treatment cycle showed structural modifications (Figs 1, 2) including chromosome swelling, chromomerization and puffing; these structural modifications also occurred, but with lower intensity and frequency, in the control samples.

Discussion

Previous investigations on the interactions between macromolecular polycations and bacteria, mammalian cells and enveloped viruses have shown that the surface structures such as bacterial wall, plasma membrane and envelope membrane, respectively, are drawn by polycation molecules into pycnotic processes strongly amplified by shake-incubation (Antohi, 1982; Antohi and Brumfeld, 1983; Antohi *et al.*, 1983). This polycation-cell surface condensation, apparently of electrostatic nature, was amplified also by using synthetic polycations of high molecular weight such as poly-L-arginine and poly-L-lysine, and displayed a surface multizonal distribution. It induced high molecular distorsions on cell surfaces revealed as globular pycnosis (Antohi, 1982) finally resulting in bacteriolysis (Antohi and Popescu, 1979), haemolysis and nucleate cell disruptions (Antohi and Brumfeld, in press). The viral surface pycnosis was accompanied by formation of stable interviral aggregates, which resulted in the increase of optical density displayed most significantly with slight lysine rich histones H2A (Antohi *et al.*, 1983).

The respective surface condensing phenomena, induced with the polycations, may be considered as belonging to the same class of natural condensations

occurring in the chromatin DNA package (cf. Laskey and Earnshaw, 1980), in the ribosomal organization of rRNAs (cf. Nierhaus, 1982) and even in the microtubule assembly by microtubule associated protein in the cytoskeleton morphopoiesis (Haskins *et al.*, 1981). Thus, alterations in condensing aggregations between natural polycations and viruses induced by some factors of physiological interest, could constitute an experimental basis for modelling the molecular mechanism concerning the role of these factors. For example, oligocationic polyamines including putrescine, spermidine and spermine, have physiological stimulatory effects on all macromolecular template requiring biosyntheses (Knutson and Morris, 1978; Algranati and Goldemberg, 1981), on cell division (Herbst and Elliot, 1981) and even on cell differentiation (Heby and Emanuelson, 1981). On the other hand, the sites of these general processes are the natural polycation condensing structures mentioned above, i.e., chromatin, ribosomes and cytoskeleton. Although the stimulatory effect of polyamines is apparently well-documented, except the hypothesis of oligocationic interference (Antohi and Brumfeld, in press) there does not seem to be any suggestion or experimental findings leading to the possible connection between oligocationic modulatory stimulation and changes in polycation condensation level. As described above, spermine, spermidine and putrescine interfere with the condensing ability of histone H2A, this effect being evidently illustrated by the decrease of light scattering values of virus-polycation aggregations in the presence of oligocations. Analogically, we can suggest that the natural enhancement of intracellular concentration of polyamines accompanying cell proliferation during embryogenesis and tissue regeneration, would change the level of polycation condensation. The oligocations, spermidine and spermine, being less condensing than their polycationic counterparts (histones, ribosomal proteines and cytoskeletal polycation), would cause reversible relaxations of chromatin, ribosomes and cytoskeletal microtubules. Interfering the polycation condensations, the oligocations open the pathways for template-requiring biosyntheses and for tubulin reorganization, favouring the initiations of DNA synthesis, RNA synthesis, protein translation, and of construction of mitotic apparatus, respectively. This model, that we name "oligocationic relaxation of polycation condensation" seems to match the stimulatory action exerted by oligocations on many biosynthetic pathways closely related to the cell proliferation and cell growth.

The high aggregating effect of 1,7-diamino heptane exerted on viral particles (Table 1), which did not occur with any other oligocations, would point out the importance of the chain-intermediate amino groups of the natural oligocations, and of chain interval between two amino groups in the molecules of these physiologically active factors. The natural polyamines, structurally are 1,4-diamino butane (putrescine), 3-amino propyl-1,4-diamino butane (spermidine) and N,N-bis-3-amino propyl-1,4-diamino butane (spermine), having intermediate amino-bridges between propyl and butyl residues. It appears that the carbon stretches between two amino groups could not be longer than four carbon atoms, otherwise the oligocationic amine would be harmful causing disturbancies in membrane lipids by its greater apolar reactivity; this may account for the high interviral aggregations caused by

1,7-diamino heptane in viral suspensions. The large quickly sedimenting aggregates did not allow any light scattering evaluation (Table 1).

The differences between the slopes of scattering values of influenza and parainfluenza viruses, particularly displayed by spermine and putrescine series of virus-histone mixtures, appear difficult to be explained; however, they seemingly become significant by applying the relationships between light scattering values, the size of dispersed particles and the wavelength of the incident light (Fabelinsky, 1969; Hörer, 1973). Accordingly, these slope differences are to be expected because of the size differences between influenza and parainfluenza virions.

The ability of influenza virus particles to reestablish the Giemsa stain affinity to the protaminized chromosome preparations suggests that the viral envelope surfaces have a high affinity for protamine molecules; this results in the dissociation of protamine layers from chromosome preparations. However, the size of viral particles, their absorbtion capacity and the surface distribution of reactive (anionic) groups would be important since bacterial suspensions were not able to make stainable the protaminized chromosome preparations in spite of the high affinity for protamine of the *E. coli* cells (Antohi, 1982). This virus-bacterium discrepancy in the staining shift of the protaminized chromosomes outlines once more the importance of the size of dispersed particles occurring in interactions with polycation molecules and seems to match the slope differences between influenza and parainfluenza series discussed above.

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References

Algranati, I. D., and Goldemberg, S. H. (1981): Initiation, elongation and termination of polypeptide synthesis in cell-free systems from polyamine-deficient bacteria. *Biochem. Biophys. Res. Comm.* **103**, 8–15.

Antohi, S. (1982): Polycation — bacterium interactions and wall subunits as endocytosis factors. Topoisomerase-like action of basic polypeptides suggesting a 7th class of enzymes, the stereases. *Z. Naturforsch.* **37c**, 985–994.

Antohi, S., Samuel, I., Dumitrescu, S. M., Petrescu, A., Brumfeld, V., and Cajal, N. (1983): Polycation induced alterations of the envelope surface of influenza and parainfluenza viruses. *Rev. Roum. Med.-Virol.* **34**, 163–166.

Antohi, S., and Brumfeld, V. (1983): Polycation — cell surface interactions and plasma membrane compartments in mammals. Interference of oligocation with polycationic condensation. *Z. Naturforsch.* in press.

Antohi, S., and Popescu, A. (1979): Protamine and polyarginine bacteriolysis; similarities in its mechanism with chromatin DNA pyknosis. *Z. Naturforsch.* **34c**, 1144–1150.

Becker, E. F., and Green, H. (1960): The effects of protamines and histones on the nucleic acids of ascites tumor cells. *Exp. Cell Res.* **19**, 361–375.

Fabelinsky, D. C. (1969): *Molecular scattering of light.* Plenum Press, New York.

Goldemberg, S. H., and Algranati, I. D. (1981): Polyamines and antibiotic effects on translation. *Med. Biol.* **59**, 360–367.

Haskins, K. M., Donoso, J. A., and Himes, R. H. (1981): Spirals and paracrystals induced by Vinca alkaloids; evidence that microtubule-associated proteins act as polycations. *J. Cell Sci.* **47**, 237–247.

Heby, O., and Emanuelson, H. (1981): Role of polyamines in expression of the differentiated phenotype of chondrocytes in culture. *Med. Biol.* **59**, 417–422.

Herbst, E. J., and Elliot, Q. D. (1981): Role of polyamines in HeLa cell proliferation. *Med. Biol.* **59**, 410–416.
Hörer, O. (1973): *The structure of biopolymers.* Abacus Press, Kent.
Knutson, J. C., and Morris, D. R. (1978): Cellular polyamine depletion reduces DNA synthesis in isolated lymphocyte nuclei. *Biochim. Biophys. Acta* **520**, 291–301.
Laskey, R.A., and Earnshaw, W. C. (1980): Nucleosome assembly. *Nature (Lond.)* **286**, 763–767.
Moorhead, P. S., Nowell, P. C., Mellman, W. J., Battips, D. M., and Hungeford, D. A. (1960): Chromosome preparations of leucocytes cultured from human peripheral blood. *Exp. Cell Res.***20**, 613–616.
Nierhaus, K. H. (1982): Structure, assembly and function of ribosomes, pp. 81–155. In Henle W. *et al.* (Eds): *Current Topics in Microbiology and Immunology* vol. 97, Springer Verlag, Berlin–Heidelberg–New York.
Samuel. I., Tomas, E., and Bârnaure, F. (1981): Investigation concerning cultivation of myxo- and paramyxo viruses on chorioallantoic membrane fragments. *Rev. Roum. Med.-Virol.* **32**, 145–154.
WHO Committee on Influenza. (1973): *Techn. Rep. nr.* **64** Geneve.

Explanation of Micrographs (Plate XLVIII):

Recovery by influenza virus treatment with reciprocal shaking of Giemsa stainig affinities of the chromosome preparations previously made Giemsa unstainable by protamine treatment. — Figs 1 and 2 = metaphase of different chromosome preparations undergoing the protamine-virus shake-incubation treatment. The structural modifications (chromosomal alterations) also appeared, though at lower intensity and frequency, in control nonprotaminized slides, where they could be caused by shake-incubation.