INHIBITION OF IN VITRO TRANSLATION BY ANTIBODIES DIRECTED AGAINST N^6-METHYLADENOSINE

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1. Introduction

Methylation of cellular and viral mRNA seems to be a general event necessary for the expression of a genetic message. This post-transcriptional modification of mRNA is not restricted to m^7Guo at the 5′-end of a polynucleotide chain since m^6Ado can also be present at the 5′-terminus [1]. Furthermore, numerous mRNAs contain m^6Ado as internal methylated nucleotides [1–3].

Methylation of mRNA has been extensively studied. However, the precise role of internal methylated nucleotides in mRNA still remains unclear. Antibodies specifically directed against m^7Guo or m^6Ado allow specific interaction to be achieved with these minor constituants of the mRNA molecule.

The preparation of antibodies directed against methylated nucleosides has been reported [4]. These antibodies allowed retention of nucleosides, cap structures and tRNA on immunoadsorbant columns [5–7] and it has been shown that, in cell-free protein synthesizing systems, antibodies directed against m^7Guo can inhibit specifically the in vitro translation of capped mRNA [8,9].

Electron microscopic experiments demonstrated the binding of such antibodies to the 5′-end of the ASV RNA [10].

We report here, the effect of antibodies directed against m^6Ado on a wheat germ cell-free protein synthesizing system programmed with RNA extracted from cells 4 h after infection with herpes simplex virus type 1. We chose this time of infection because it had been reported that the cellular and viral mRNAs were highly methylated at this time [11–14]. Such mRNAs should be a good target for evaluating the effect of antibodies directed against m^6Ado on an in vitro translation system.

Abbreviations: m^6Ado, N^6-methyladenosine; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; Temed, *N,N,N′,N′*-tetramethylethylenediamine; enzyme, creatine kinase (EC 2.7.3.2)

2. Materials and methods

2.1. *Chemicals*

ATP, GTP, creatine kinase, creatine phosphate were from Boehringer (Mannheim). Adenosine, N^6-methyladenosine, bovine serum albumine, dithiothreitol, Hepes were from Sigma Chemicals Co. (St Louis MO). Amino acids and sodium dodecyl sulphate (SDS) were from BDH Chemicals (Poole). Carboxylmethyl-cellulose was from Carl Schleicher and Schüll (Kenne, USA). Caprylic acid, enzacryl AH and PPO were from Koch Light (Colnbrook). Crystallized DNase free from RNase was from Worthington Biochemicals (Freehold NJ). $NaIO_4$ and $NaBH_4$ were from Fluka (Muttenz Switzerland). POPOP was from Packard Instruments (USA). ^{14}C-Labelled uridine (53.9 Ci/mmol) from CEA (Saclay). ^{35}S-Labelled methionine (932.5 Ci/mmol) and ^{3}H-labelled adenosine (35 Ci/mmol) were from New England Nuclear (USA). N^6-Methyladenosine (96 Ci/mmol) was ^{3}H-labelled by isotopic exchange by CEA (Saclay). Acrylamide, bisacrylamide and Temed were from Eastman Kodak (Rochester NY). All other chemicals were from Merck (Darmstadt).

2.2. *Preparation of antigens and immunization of rabbits*

Antigens were prepared by coupling nucleosides to albumin according to [15]. All steps were performed

at 4°C. The molar ratio of conjugation of nucleosides to bovine serum albumin was 15:1 for m^6Ado:albumin. Rabbits were immunized according to [15].

2.3. *Preparation of immunoglobulins*

Immunoglobulins were extracted from antisera according to [16]. To eliminate any nucleolytic activity, immunoglobulins were purified according to [17].

Purified immunoglobulins were tested for possible nucleolytic activity. No degradation of ^{3}H-labelled poly(U) was observed after 10 min incubation at 37°C, with purified IgGs [18]. No degradation of ^{14}C-labelled rRNA was observed on sedimentation profiles on sucrose gradients after 1 h incubation at 30°C with purified IgGs.

2.4. *Immunoabsorbant columns*

m^6Ado and Ado were coupled to Enzacryl AH; 30 mg nucleoside oxidized with $NaIO_4$ were added to 2 mg Enzacryl AH in acetate buffer (pH 5.2). The mixture was kept 24 h at 4°C with gentle agitation. Columns were extensively washed, then aliquots of antiserum (2 ml) were adsorbed on the immunoabsorbant. Immunoglobulins were prepared from the non-adsorbed fractions.

2.5. *Radioimmunoassays*

The specificity of immunoglobulins was determined by the membrane filter assay [19] as modified in [20].

2.6. *Cell culture, extraction of RNA and assays in vitro*

The procedure for propagation and maintenance of human epidermoid carcinoma no. 2 (HEp2) cells and production of the F strain of herpes simplex virus, HSV-1 (F) was performed as in [21].

Cells were infected with 25 p.f.u. of HSV-1/cell, and 4 h after infection, RNA was extracted. Assays of protein synthesis in vitro were performed as in [22].

2.7. *Polyacrylamide gel electrophoresis*

Analysis of products of assays in vitro was performed by electrophoresis on SDS–polyacrylamide gel slabs according to [23]. Treatment of gels for fluorography was performed as in [24].

3. Results

3.1. *Specificity of immunoglobulins*

The specificity of immunoglobulins was evaluated in ligand binding experiment by the radioactivity retained by antibodies incubated with various amounts of ^{3}H-labelled nucleosides (fig.1). The amount of m^6[^{3}H]-Ado retained in these experiments was dependent on the concentration of m^6[^{3}H]Ado, while [^{3}H]Ado was not significatively retained. It can be concluded that the methyl group in position N^6 is immunodominant in the hapten response.

3.2. *Effect of purified immunoglobulins on protein synthesis in vitro*

The RNA extracted from cells after 4 h infection with HSV-1 was preincubated 1 h at 30°C, and 1 h at 0°C, with increasing amounts of immunoglobulins. The translation of this RNA in a wheat germ cell-free protein synthesizing system, evaluated by the incorporation of ^{35}S-labelled methionine in the acid-insoluble fraction, was inhibited by 35% with 0.4 μg IgG to m^6Ado and by 61% with 0.8 μg (table 1, assays 2, 3). When similar amounts of immunoglobulins from a preimmune serum were used, no effect was observed. Therefore the inhibitory effect of antibodies to m^6Ado could not be attributed to a non-specific effect of immunoglobulins.

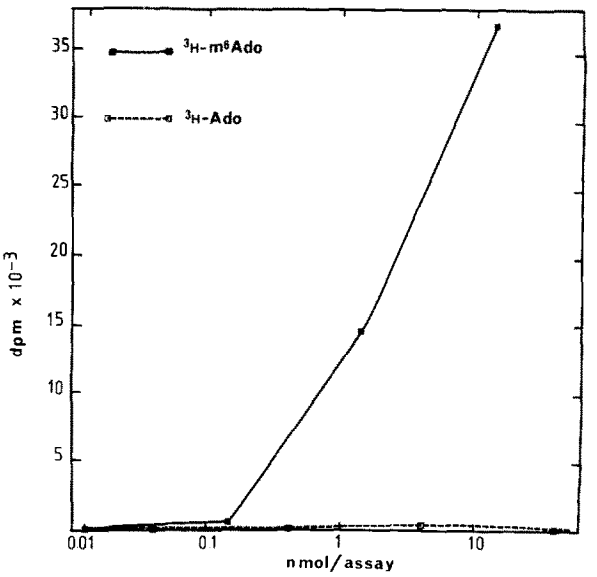

Fig.1. Radioimmunoassays. IgG (70 μg) were incubated in a total volume of 120 μl with various amounts of m^6[^{3}H]Ado (96 Ci/mmol) or [^{3}H]Ado (35.2 Ci/mmol) in Tris–NaCl buffer; 100 μl were then filtered on pre-wet Millipore filters (22 mm, 0.45 μm). Filters were washed with 3 × 2 ml buffer, dried and counted in a standard scintillation mixture; (□) Ado; (■) m^6Ado.

Table 1
Effect of antibodies to m^6Ado translation in vitro of RNA from cells infected with HSV-1

Addition	[^{35}S]Methionine (dpm) incorp. in 5 μl assay	Inhibition (%)
(1) None	25 702	
(2) 0.8 μg IgG	10 092	61%
(3) 0.4 μg IgG	16 778	35%
(4) 0.8 μg IgG adsorbed cn m^6Ado	25 212	2%
(5) 0.4 μg IgG adsorbed on m^6Ado	24 922	3%
(6) 0.8 μg IgG added without preincubation	26 308	0%

Assays were done as in section 2: total volume of assays was 50 μl; assays (2–5), RNA was preincubated for 1 h at 30°C and 1 h at 0°C with IgG; assay (6) 0.8 μg IgG was added as a beginning of a translation reaction without preincubation with RNA

The lack of degradation of either ^{3}H-labelled poly(U) or ^{14}C-labelled rRNA, after incubation with immunoglobulins demonstrated the absence of nucleolytic activity from the preparation.

To ascertain the specificity of this inhibition, the same antiserum was adsorbed on m^6Ado or Ado covalently linked on insoluble matrixes and IgGs were prepared from the eluates. IgGs prepared from the serum adsorbed on Ado columns retained the inhibitory activity (table 2, assays 2,3), whereas IgGs prepared from antiserum adsorbed on m^6Ado lost their capacity to inhibit in vitro translation (table 1, assays 4,5).

It can be concluded that inhibition of the in vitro translation of the RNA extracted from cells infected by HSV-1 was due to a reaction between the antibodies and the m^6Ado of the RNA. Since m^6Ado is also present in tRNA and rRNA [24], in order to determine the target of the antibodies, we have added the IgGs to m^6Ado at the beginning of in vitro translation reaction. No inhibitory effect was observed when the RNA was not preincubated with IgGs (table 1, assay 6).

The formation of an immunocomplex between

Table 2
Effect of antibodies to m^6Ado extracted from serum adsorbed on Ado column

Addition	[^{35}S]Methionine (dpm) incorp. in 5 μl assay	Inhibition (%)
(1) None	58 157	–
(2) 0.45 μg IgG	34 458	41%
(3) 0.25 μg IgG	48 445	17%

Total volume of the assays was 50 μl: assays (2,3), RNA was preincubated for 1 h at 30°C and 1 h at 0°C with IgG

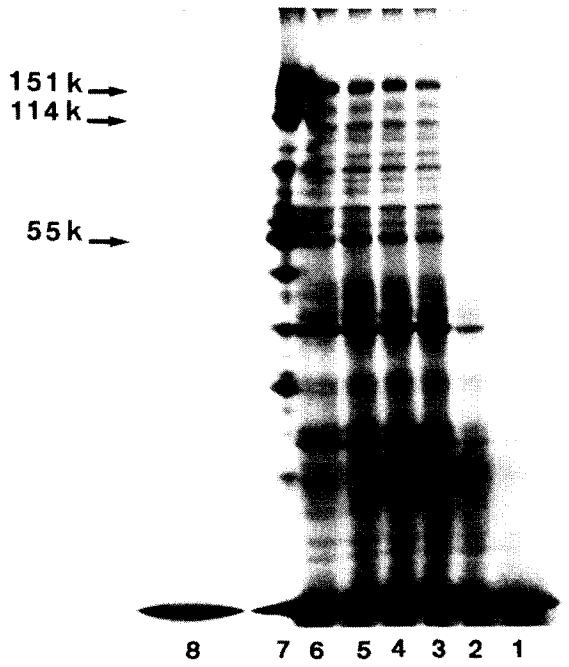

Fig.2. SDS–polyacrylamide gel electrophoresis of products synthesized in vitro. Lanes (1–6) assays programmed with RNA extracted from cells infected with HSV-1: (1,2) RNA was preincubated, respectively, with 0.8 and 0.4 μg IgG to m^6Ado 1 h at 30°C and 1 h at 0°C (table 1 assays 2,3); (3,4) RNA was preincubated with 0.8 and 0.4 μg of IgGs adsorbed on m^6Ado column (table 1 assays 4,5); (5) RNA not preincubated (table 1 assay 1); (6) RNA + 0.8 μg IgGs added without preincubation (table 1 assay 6); (7) Infected cells labelled in vivo with [^{14}C]valine, [^{14}C]isoleucine and [^{14}C]leucine; (8) no RNA.

RNA and immunoglobulins seems to be necessary to obtain the effect described. Furthermore, this inhibition is not due to interaction of immunoglobulins with ribosomes since no antibodies were found to cosediment with 80 S ribosome on sucrose gradients (not shown).

To visualize the dose-dependent inhibition of in vitro translation by antibodies, aliquots of incubation mixtures were analyzed on SDS–10% acrylamide slab-gel.

The dose-dependent inhibition of incorporation of ^{35}S-labelled methionine into polypeptides synthesized, when RNA was preincubated with IgGs was confirmed (fig.2 lanes, 1,2 cf. lane 5). The migration pattern of the products obtained by translation of RNA extracted from infected cells was not modified when IgGs were added at the beginning of the incubation (fig.2, lane 6). Furthermore, the in vitro products in assays programmed with RNA preincubated with IgGs extracted from antiserum adsorbed on m^{6}Ado column gave the same migration profile (fig.2 lanes, 3,4 cf. lane 5).

Thus the observed inhibition of in vitro translation could be attributed to the specific interaction of the antibodies with mRNA translated in our system.

4. Discussion

Our results show that antibodies directed against m^{6}Ado inhibit protein synthesis in a wheat germ cell-free system programmed with RNA extracted from cells infected with HSV-1. The specificity of antibodies and the fact that IgGs purified from antiserum adsorbed on m^{6}Ado covalently linked to an insoluble matrix did not show any inhibitory activity provide evidence for a specific interaction of these antibodies with m^{6}Ado in RNA. The site of interaction of antibodies could have been mRNA, ribosomes or tRNA. We showed that inhibition of protein synthesis in vitro is obtained only when the RNA used to program the system is preincubated with the antibodies and that no marked effect on protein synthesis in vitro is observed when immunoglobulins are added at the beginning of the translation reaction.

This suggests that the inhibition of in vitro translation is not due to interactions between antibodies and tRNA or rRNA. In fact, we were unable to detect any significant interaction between labelled antibodies and 80 S ribosomes. Thus we attribute the inhibitory effect of antibodies to m^{6}Ado on protein synthesis in vitro to their interaction with mRNA.

In cellular and viral mRNA extracted from cells infected with HSV-1, m^{6}Ado has been found in the cap structure and as internal methylated nucleotides [11–14]. Our results can not distinguish between these two locations, but the fact that all polypeptides synthesized in vitro were affected similary by the antibodies suggest that all types of translated mRNA molecules have one or more m^{6}Ado.

These results demonstrate the specificity of the antibodies directed against m^{6}Ado and the accessibility of the antigenic site along the polynucleotide chain. Such specific tools should allow us to detect the presence of m^{6}Ado in different kinds of mRNA with the electron microscope.

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References

[1] Shatkin, A. J. (1976) Cell 9, 645–653.
[2] Canaani, D., Kahana, C., Lavi, S. and Groner, Y. (1979) Nucleic Acids Res. 6, 2879–2899.
[3] Salditt-Georgieff, M., Jelinek, W., Darnell, J. E. Furuichi, Y., Morgan, M. and Shatkin, A. J. (1976) Cell 7, 227–237.
[4] Munns, T. W. and Liszewski, M. K. (1980) Prog. Nucleic Acid Res. Mol. Biol. 24, 109–165.
[5] Salomon, R., Fuchs, S., Aharonov, A., Giveon, D. and Littauer, U. Z. (1975) Biochemistry 14, 4046–4050.
[6] Munns, T. W., Oberst, R. J., Sims, H. F. and Liszewski, M. K. (1979) J. Biol. Chem. 254, 4327–4330.
[7] Munns, T. W., Liszewski, M. K., Oberst, R. J. and Sims, H. F. (1978) Biochemistry 17, 2573–2578.
[8] Munns, T. W., Morrow, C. S., Hunsley, J. R., Oberst, R. J. and Liszewski, M. K. (1979) Biochemistry 18, 3803–3810.
[9] Dante, R. and Niveleau, A. (1980) Eur. J. Biochem. 110, 539–544.
[10] Castleman, H., Meredith, R. D. and Erlanger, B. F. (1980) Nucleic Acids Res. 8, 4485–4499.
[11] Garcia, A. (1979) Thesis, University Lyon I.
[12] Bartkoski, M. and Roizman, B. (1976) J. Virol. 20, 583–588.
[13] Bartkoski, M. J. J. R. and Roizman, B. (1978) Virology 85, 146–156.
[14] Moss, B., Gershowitz, A., Stringer, J., Holland, L. E. and Wagner, E. (1977) J. Virol. 23, 234–239.

[15] Erlanger, B. F. and Beiser, S. (1964) Proc. Natl. Acad. Sci. USA 52, 68–74.
[16] Steinbuch, M. and Audran, R. (1969) Arch. Biochem. Biophys. 134, 279–284.
[17] Palaccios, R., Palmiter, R. D. and Schimke, R. T. (1972) J. Biol. Chem. 247, 2316–2321.
[18] Spahr, P. F. (1964) J. Biol. Chem. 239, 3716–3721.
[19] Humayun, M. Z. and Jacob, T. M. (1973) Biochim. Biophys. Acta 331, 41–53.
[20] Vold, D. (1979) Nucleic Acids Res. 7, 193–204.
[21] Kieff, E. D., Hoyer, B., Bachenheimer, S. L. and Roizman, B. (1972) J. Virol. 8, 125–132.
[22] Niveleau, A. and Wild, T. F. (1979) Virology 96, 295–298.
[23] Laemmli, U. K. (1970) Nature 227, 680–685.
[24] Bonner, W. M. and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83–88.