THE EFFECT OF ANTI-SPERMINE ANTIBODIES ON A WHEAT GERM CELL-FREE PROTEIN-SYNTHESIZING SYSTEM

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1. Introduction

The polyamines spermine, spermidine and putrescine have been reported to play a significant role in many biological systems [1–3]. When added to cell-free protein synthesizing systems, polyamines stimulate protein synthesis by mechanisms involving all the steps of in vitro translation [4,11]. Polyamines are found associated with ribosomes [12] and the cytosol and, as such, are present in all cell-free systems. An interdependence between added spermine and Mg^{2+} for maximum stimulation of protein synthesis in vitro has been described [7,8]. However as spermine could not completely replace Mg^{2+} it is difficult to differentiate between its effect as a polycation and its effect as a specific component necessary for maximum efficiency in protein synthesis.

One way to answer this question would be to specifically sequester spermine using antibodies directed against it. The results of such an investigation will be reported here.

2. Materials and methods

2.1. *Chemicals*

Wheat germ was obtained from Choay (France). ATP, GTP, creatine kinase (EC 2.7.3.2), creatine phosphate were from Boehringer Mannheim (GmbH), dithiothreitol (DTT), Hepes, spermine, spermidine and putrescine as the hydrochloride salts from Sigma Chem. Co. (USA), amino acids and sodium lauryl sulfate from BDH Chemicals Ltd, Poole (England), [^{35}S]methionine (spec. act. > 400 Ci/mmol) from the Radiochemical Center, Amersham (England), acrylamide, bis-acrylamide, *N,N,N′,N′*-tetramethylenediamine (TEMED) from Eastman Kodak Co. (USA), Enzacryl AH from Koch Light, Colnbrook (England). All other chemicals were purchased from Merck, Darmstadt (FRG).

Abbreviations: ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; Hepes, 4-(2-hydroxyethyl)piperazine sulfonic acid; DTT, di-thiothreitol; SDS, sodium dodecylsulfate; p.f.u., plaque forming unit.

2.2. *Preparation of antibodies*

Antibodies directed against spermine were prepared as in [13]. Immunoglobulins were extracted according to [14]. Specific immunoglobulins directed against spermine were prepared as in [15]. Briefly, total immunoglobulins from an immune serum were loaded on an immunoadsorbent column prepared by linking spermine to Enzacryl AH. The column was washed with 0.14 M NaCl, 0.01 M phosphate buffer (pH 7.2) until the effluent showed no absorption at 280 nm. Anti-spermine antibodies were then eluted with 0.1 M glycine–HCl buffer (pH 2.3). Eluates were immediately neutralized with 1 M Tris–HCl (pH 7.6) dialysed against 0.14 M NaCl, 0.01 M Tris–HCl (pH 7.2) and finally against water before lyophilisation. Antibodies were redissolved in water at 2 mg/ml and stored at −70°C.

2.3. *Cell culture, virus growth and extraction of RNA*

Vero cells were grown in Eagle's minimal essential medium containing 10% inactivated foetal calf serum. Confluent monolayer cultures were infected with

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measles virus (Halle strain) at a multiplicity of infection of 1 p.f.u./cell. Total RNA was extracted with phenol 24 h after infection, precipitated with 66% ethanol and redissolved in water at 1 mg/ml. Aliquots (50 μl) were kept frozen at −70°C.

2.4. *In vitro protein synthesis*

Wheat germ extracts were prepared as in [16]. Standard reaction mixtures contained, in a total vol. 25 μl, 1 mM ATP, 0.02 mM GTP, 8.5 mM creatine phosphate, 23 mM Hepes–KOH (pH 7.6), 110 mM K acetate, 2 mM DTT, 25 μM each amino acid except methionine, 5 μCi [^{35}S]methionine (spec. act. > 400 Ci/mmol), creatine kinase (final conc. 40 μg/ml), 7.5 μl wheat germ extract and 5 μg RNA. Magnesium acetate, polyamines and immunoglobulins were added in amounts indicated in the figure legends. Incubation was at 23°C for 90 min. Aliquots of 5 μl were precipitated with cold 5% trichloroacetic acid containing 1% unlabelled methionine. After boiling for 15 min, samples were cooled in ice and filtered on Millipore filters. Filters were dried and counted in an Intertechnique liquid scintillation spectrometer.

2.5. *Polyacrylamide gel electrophoresis*

Samples were treated with denaturing buffer for 3 min at 90°C. Electrophoresis was carried on 10% acrylamide slab gels containing SDS, with the discontinuous Tris system buffer [17]. Gels were dried and treated for fluorography as in [18]. Absorbance traces of the autoradiograms were carried out on a Vernon densitometer Model PHI 6.

3. Results

Purified antibodies did not show any nucleolytic activity when tested as in [19] in that no degradation of [^{3}H]poly(U) was observed after 30 min incubation at 37°C with these purified antibodies.

When incubated with [^{14}C]spermine, [^{14}C]spermidine, and [^{14}C]putrescine, antibodies showed an absolute specificity for spermine as determined by equilibrium dialysis (table 1).

In the absence of any added spermine the optimum concentration of Mg^{2+} was found to be 1.5 mM. Addition of spermine alone at final conc. 30 μM, 80 μM or 130 μM brought about a 2-fold increase in the incorporation of [^{35}S]methionine but did not change the optimum concentration of Mg^{2+}. The overall efficiency of the system was drastically reduced at $Mg^{2+} < 1$ mM or > 2 mM, in the presence or in the absence of added spermine. Therefore all assays were performed in the range of 1–1.5 mM magnesium.

Table 1
Retention of [^{14}C]polyamines by anti-spermine antibodies

Polyamines	Picomoles retained/ 100 μg antibodies
Putrescine	–
Spermidine	–
Spermine	76

Aliquots (4 μg) of immunopurified antibodies were incubated with 50 000 dpm each of [^{14}C]putrescine (1200 pmol) [^{14}C]spermidine (425 pmol) and [^{14}C]spermine (500 pmol) for 1 h at 37°C, and for 16 h at 4°C. At the end of this period, incubation mixtures were dialysed against 0.14 M NaCl until the dialysis liquid was free of radioactivity

The addition of increasing amounts of purified antibodies to the protein synthesizing incubation mixture resulted in an inhibition of [^{35}S]methionine incorporation as shown in fig.1. When similar amounts of immunoglobulins from a non-immune serum were

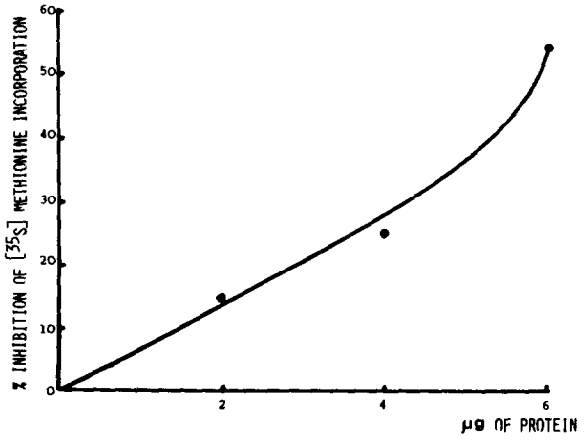

Fig.1. Inhibition of [^{35}S]methionine incorporation in the wheat germ extract programmed with RNA from Vero cells infected with measles virus. Incubations were performed as in section 2. Antibodies were added at the beginning of the incubation. [Mg^{2+}] 1.5 mM. No polyamines added.

used, no effect was observed. Therefore the inhibitory effect of anti-spermine antibodies cannot be attributed to a non-specific effect of immunoglobulins.

Aliquots of the incubation mixtures were analysed by polyacrylamide gel electrophoresis and the amount of [^{35}S]methionine incorporated was estimated by fluorography. A progressive decrease of incorporation of the labelled precursor was observed in those samples to which anti-spermine antibodies were added (fig.2). Absorbance traces of the autoradiograms showed that the synthesis of high molecular weight

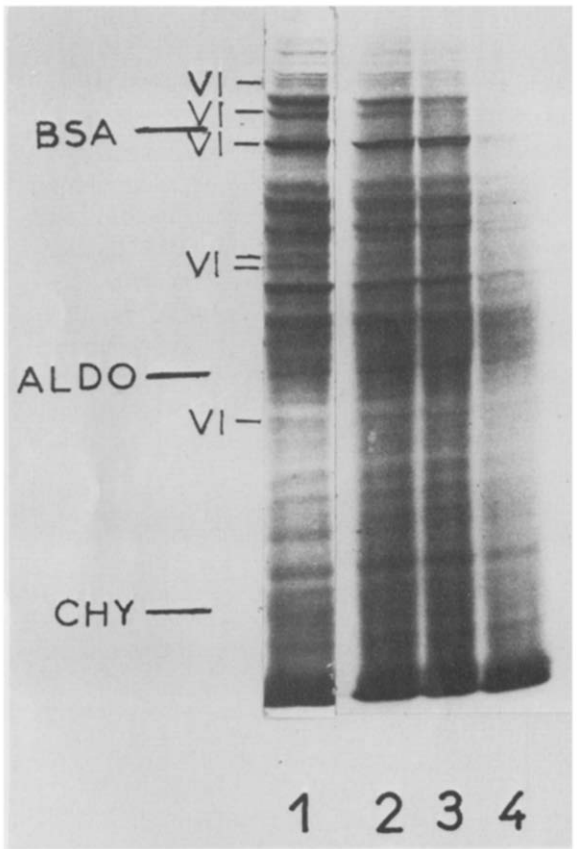

Fig.2. Gel electrophoresis of in vitro synthesis products. Incubation and electrophoresis were carried out as in section 2. [Mg^{2+}] 1.5 mM. No polyamines added. Lane 1, no antibodies added; lane 2, 2 μg anti-spermine antibodies added; lane 3, 4 μg antibodies added; lane 4, 6 μg antibodies added. Antibodies were added at the beginning of incubation. Standard proteins for molecular weight determination: bovine serum albumin (BSA); chymotrypsinogen (chy); aldolase (ALDO). Viral polypeptides: VI.

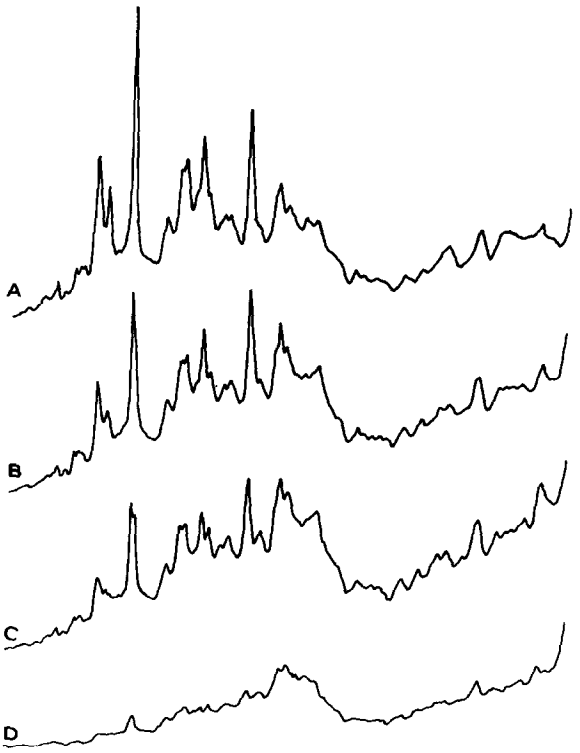

Fig.3. Absorbance traces of autoradiograms of in vitro products in absence or in the presence of increasing amounts of antispermine antibodies. (A) No antibodies added; (B) 2 μg anti spermine antibodies added; (C) 4 μg anti spermine antibodies added; (D) 6 μg anti spermine antibodies added.

products was preferentially reduced by addion of antibodies (fig.3).

In order to test whether anti-spermine antibodies were acting by blocking initiation, purified antibodies were added at various times after the reaction was initiated. Addition of antispermine at any time immediately stopped any further incorporation of the labelled precursor (fig.4).

The inhibitory effect of anti-spermine antibodies was abolished by the subsequent addition of 80 μM spermine to the incubation system. The original activity of the system was then completely restored (table 2). The addition of spermidine and putrescine at 0.8 mM and 3 mM, respectively, at which concentrations maximum stimulation of the system was observed, did not reverse the inhibition due to the anti-spermine antibodies.

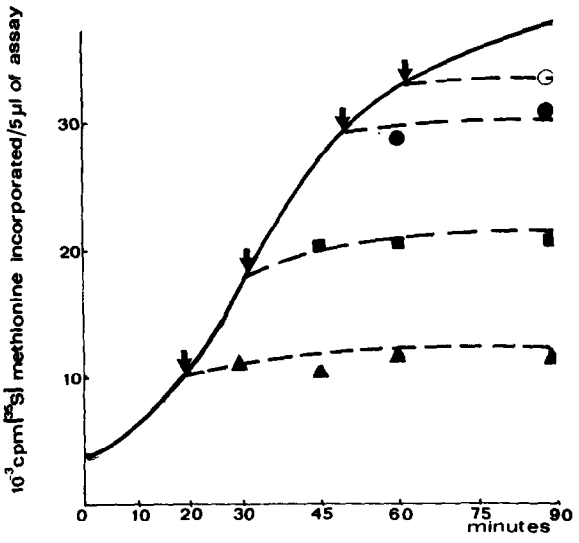

Fig.4. Effect of anti-spermine antibodies on [^{35}S]methionine incorporation, when added at different times during incubation. Incubations were carried out as in section 2. [Mg^{2+}] 1.5 mM. No polyamines added. Aliquots (5 µl) were withdrawn at times indicated by arrows.

4. Discussion

Our results show that antibodies directed specifically against spermine inhibit protein synthesis in the wheat germ cell-free system programmed with exogenous messenger RNA.

The specificity of the antibodies for spermine as shown in table 1 and the reversal of their inhibitory effect on protein synthesis by spermine but not by spermidine or putrescine provide evidence that spermine plays an essential role in protein synthesis. Though this conclusion has already been drawn from experiments in which spermine was added to in vitro synthesizing systems [7,8], it was never clear whether the spermine added was acting specifically as spermine or as a polycation.

The immunochemical approach, in permitting us to differentiate between these two possibilities, provides direct evidence for a role for spermine itself. However, there are two mechanisms by which the antibodies could exert their effect:

(1) they could interact with spermine naturally bound to ribosomes or to tRNA to form complexes and block the translation machinery;
(2) they could deprive the system of spermine by sequestering any free spermine.

As regards the second hypothesis, it seems reasonable to assume that free spermine present in the crude wheat germ extract would have been retained on the Sephadex column and the effluent containing wheat germ ribosomes would be devoid of free spermine.

Experiments are in progress to try to obtain direct evidence for the interaction of anti-spermine antibodies with ribosomes and tRNA.

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Table 2
Inhibition of protein synthesis by anti-spermine antibodies and its reversal by spermine

Addition	[^{35}S]Methionine incorporated (cpm/5 µl)	Effect
1. None	36 000	Incorporation 100%
2. Anti-spermine 4 µg	16 000	Inhibition 54%
3. Spermine 80 µM	70 000	Stimulation 94%
4. Spermine 80 µM + anti-spermine 4 µg	36 000	Reversion 100%

Incubations were performed as in section 2. [Mg^{2+}] 1.5 mM. In assay no. 4, antibodies were first added to the incubation mixture. After 15 min at 23°C the assay was supplemented with spermine

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