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Suiko, Masahito
Laboratory of Pesticide Chemistry, Faculty of Agriculture, Kyushu University

Maekawa, Kazuyuki
Laboratory of Pesticide Chemistry, Faculty of Agriculture, Kyushu University

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Repression of the Propagation of Ehrlich Ascites Tumors by Means of Attenuating Tumor Cells. III

Masahito Suiko and Kazuyuki Maekawa

Laboratory of Pesticide Chemistry, Faculty of Agriculture,
Kyushu University 46-02, Fukuoka 812

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Aminoethyl ether of amylose (AEA) and aminoethyl ether of glycol cellulose (AEC) in which the degree of polymerization was about 20-25 were mixed respectively with yeast RNA to produce stable complexes. The complexes dissociated about 5 % at pH 4, while they were almost insoluble at neutral pH. The complexes were hydrolyzed about a half by pancreatic RNase A. When Ehrlich ascites tumor cells were incubated with these complexes, the transplantability of those cells in mice were almost completely abolished. The mice pretreated thus became strongly resistant to a challenge inoculation afterwards. The effectiveness of AEA and AEC as a combining moiety ***was*** similar.

It has already been reported that treatments with an MA: y-RNA complex or clay: y-RNA complex etc. are useful for attenuating Ehrlich ascites tumor cells (Maekawa and Kushibe, 1972; Maekawa and Momii, 1972; Takakuwa ***et al.,*** 1975). However, the results were sometimes unsteady.

The present experiment was therefore undertaken to find a more suitable and reliable method for attenuating tumor cells. As such a compound like that the complex formed with the RNA moiety is set free for the first time on the surface of tumor cells to reveal the cytotoxicity, aminoethylamylose and aminoethylcellulose were chosen and scrutinized in regard to their attenuating effects and other biological activities on E-cells.

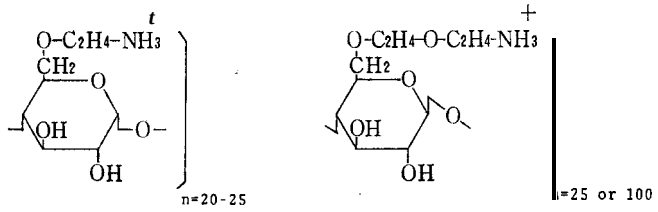
The complexes were composed of both polysaccharide derivatives and y-RNA dissociated about 5 % at pH 4.1, which was the isoionic point of the tumor surface (Straumfjord and Hummel, 1959), so that these substances were likely to be suitable for investigating mild attenuation of E-cells.

EXPERIMENTAL

Materials and Methods

1) AEA and AEC

AEA and AEC were synthesized according to the procedure of Higuchi ***et al.*** (1970). The average degree of polymerization(n) was estimated by a colloid titration method as follows: AEA, 20-25; AEC, 25 and 100. The degree of substitution(DS) was 1.3 for the former, 1.7 and 1.25 for the latter. y-RNA **was** prepared as described previously (Maekawa and Kushibe, 1972).



AEA AEC

Scheme 1. AEA and AEC.

2) Complex formation of ***AEA*** *or* ***AEC*** *with y-RNA*

A preparation of AEA and y-RNA complex (AEA: y-RNA) or AEC and y-RNA complex (AEC: y-RNA) was carried out as follows. Both AEA and AEC were soluble in water, their aqueous solutions being pH 10-11. These solutions were initially adjusted to pH 7.0 with 0.1 N acetic acid. The complexes were then formed by gradually adding neutralized AEA or AEC solution to excess amounts of y-RNA solution which was previously dissolved in 0.9 % NaCl. After stirring for 30 min. at room temperature, the mixtures were centrifuged. The precipitates were washed thoroughly with 0.9 % NaCl. From the difference between A_{260nm} of the supernatant (including washed solution) and that of the starting quality, the amount of RNA which took part in the complex formation was estimated. The combining ratio was evaluated as described in the previous paper. The results are given in Table 1.

Table 1. Complex formation of AEA or AEC with y-RNA.

Polymer	Mol. wt. of hexose unit	Degree of polymerization	KNA Mol. wt. of polymer	KNA combined with polymer (g/g)	Combined RNA (g)	Combined mole of nucleotide* / Mole of polymer
AEA	205	20-25	4100-5125	1.65	6765-8456	21-26
AEC (a)	249	25	6225	1.70	10583	33
(b)	,,	100	24900	7.30	181770	566

* Calculated as average molecular weight 321.

Table 1 indicates that 21-26 units of mononucleotides of y-RNA combined with 20-25 hexose units of AEA. In the case of the AEC : y-RNA complex, the combining amount of nucleotide was a little higher, but close to that of AEA. This might be explained by assuming that in addition the y-RNA combined with NH_3^+ groups bound at other positions than the primary hydroxyl group of the hexose units. From this result it was estimated that to some extent the complex contained a different complex with 3 molecules of AEC(n=

Abbreviations: AEA, Aminoethyl ether of amylose; AEC, aminoethyl ether of glycol cellulose; E-cell, Ehrlich ascites tumor cell; MA, methylated bovine serum albumin; PBS(-), phosphate buffered saline (without Ca^{2+}, Mg^{2+}); RFL, rat fibroblast cells; y-RNA, an RNA fraction isolated from yeast.

25) linked with one chain of y-RNA. However, in the case of the complex composed of AEC(n=100) the amount of combining RNA was in a remarkably high concentration, so that the linkage in this complex was presumably different from that of the complex composed of the low polymer. For example, it may have contained the complex in which only one side of y-RNA combines with the polymer. This fact also implies that for the complex formation it is necessary to mix AEA or AEC with excess RNA. In the contrary situation it is likely to build up something like the complex formed from AEC(n=100).

3) ***Mice***

Female mice (ddN) of an inbred strain, weighing 18-26 g, were obtained from the Animal Center, Kyushu University.

4) ***Tumor cells and their treatments***

Ehrlich ascites tumors were given i.p. and maintained in this inbred strain, as described previously (Maekawa and Kushibe, 1972). The conditions of tumor cell treatment with the complex and of the challenge inoculation were the same as those already reported.

5) ***Dissociation of AEA : y-RNA complex in acidic solution***

An AEA : y-RNA complex was prepared from 3 ml (34.2 mg) of AEA (at pH 7.0) and excess y-RNA (113 mg), (combining ratio of AEA: y-RNA was 1: 1.7), washed thoroughly with water. The washed complex was suspended in buffer solution (AcOH-AcONa and HCl-AcONa), then repeatedly washed with the same buffer solution, until A_{260nm} of the supernatant became constant. From the A,,,,, of all washed solutions, the quantity of dissociated RNA was calculated. The results were as illustrated in Fig. 1.

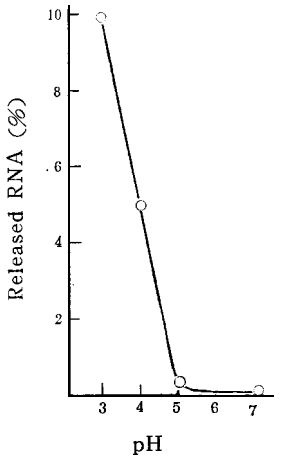


Fig. 1. Apparent dissociation of **AEA: y-KNA complex.**

6) ***Hydrolysis of AEA : y-RNA complex by RNase A***

An aqueous suspension (mg/ml) of AEA : y-RNA complex (1 :1.7 wt./wt.) was used. To this suspension (1 ml), 0.5 ml (1.4 mg) of bovine pancreatic

RNase A (Mann Research Lab.), which had been dissolved in 0.1 M tris-HCl buffer (pH 7.5), was added and incubated for 2 hours at 37°C with shaking. Then 0.5 ml of trichloroacetic acid (2.5 %) was added, and the solution was allowed to stand for 30 min. at 0°C. The acid soluble fraction and the precipitate were separated after centrifugation. The phosphate content of the former was determined by the method of Allen (1940). The results showed that the digestivity of AEA : y-RNA complex decreased 49 %, as compared with that of the RNA.

The phosphate content of AEA : y-RNA complex ..... 4.5 %
The phosphate content of the acid soluble fraction separated from the complex after RNase A treatment ..... 3.1 %
Phosphate liberated on a control experiment (without RNase A) . . . . . 0.9 %
Therefore, it was calculated that the RNA composed of the AEA: y-RNA complex was hydrolyzed to $\left(\frac{3.1-0.9}{4.5}\times 100 =\right)$ 48.8 %.

AEC(n=25): y-RNA complex was estimated in the same manner, the digesting ratio being similar to that of the AEA : y-RNA complex. The AEC(n=100): y-RNA complex was not estimated.

RESULTS

1) Toxicity of AEA and AEC

The AEA : y-RNA and AEC : y-RNA complexes scarcely exhibited any toxicity to mice, whereas injection of AEA alone revealed considerable toxicity. That is, when 4.0 mg of AEA initially adjusted to pH 7.2 was injected intraperitoneally into 5 mice, all of them died in 1-2 hours. The effects of AEC(n=25) were similar to those of AEA.

2) The effectiveness of AEA to E-cells (*in vitro*)

E-cells were suspended in PBS($-$) to obtain a concentration of 8×10^7 cells/ml. 0.01 ml of the E-cell suspension and AEA 0.5 ml of variable concentrations were mixed and incubated for 2 hours at room temperature. Afterwards, by adding 1.5 ml of nigrosine to the mixture, living and dead cells were estimated by using the Bürker-Türk haematometer. The results are displayed in Table 2.

Table 2. Cytocidal activity of AEA in *vitro*.

Amount of AEA in testing mixture (0.51ml)	Relative conc. of AEA	Cytocidal activity (%)
0.114 μg	0.01	0.8
0.507 μg	0.05	0.9
1.14 μg	0.1	7
2.8 μg	0.2	15
5.7 μg	0.5	54
11.4 μg	1	100
114.0 μg	10	100*

* E-cells cohered at a high concentration of AEA.

3) **The action of AEA : y-RNA complex on E-cells and RFL-cells**

The procedure employed was similar to that described in 2), except the incubation time was variant. The results are given in Table 3. A **20** min. incubation period and an amount of 3.2 mg of AEA : y-RNA complex seemed to be insufficient to attenuate E-cells, while cytocidal activity was clearly apparent at twice the dose and incubation for **2** hours. It was therefore, assumed that the longer the incubation time, the more complex that dissociated, and the more cytocidal activity of AEA that appeared. On the other hand, the complex exhibited cytocidal activity against rat fibroblast cells (RFL-cell line) to a much lower extent than against E-cells.

Table 3. The effects of mixing amounts and duration of incubation on cytocidal activity of AEA : y-RNA complex.

Amounts of AEA : y-RNA complex in the testing mixture (0.51ml)	Duration of incubation (at 37°C)	Cytocidal activity* (%)	
3.2 mg	20 min.	1.3	E-cells
	120 min.	2.1	
6.4 mg	20 min.	14.3	
	120 min.	38.4	
6.4 mg	120 min.	9.7	RFL-cells (fibroblast of rat)

* $\text{Living } \frac{\text{cells in control-living cells in test}}{\text{living cells in control}} \times 100$

4) **Inhibition of tumor transplantation by treatment with AEA (or AEC) : y-RNA complex**

(a) The experimental results relating to the treatment with AEA : y-RNA complex are shown in Table 4. Thus, the effects of attenuating E-cells with the complex were examined in the dose range of 3.2 mg/animal to 26.7 mg/animal. Each concentration was effective in causing 85-100% inhibition in the transplantation. Moreover, the mice treated were resistant against challenge inoculation afterwards.

From Table 4, it is clear that a high concentration of AEA : y-RNA complex

Table 4. Effects of AEA : y-RNA complex on transplantability of Ehrlich ascites tumor cells.

Amounts of AEA : y-RNA (mg)	Inoculated E-cells[1)]	Survival ratio (S/T)		Survival ratio after challenge[2)]	
		Control[3)]	Test	Control[3)]	Test
26.7	5.6×10^4	1/ 8	10/10	0/5	9/10
15.2	1.1×10^5	0/ 8	7/ 8	1/9	7/ 7
9.9	1.0×10^5	0/10	10/10	0/4	10/10
3.6	1.2×10^5	1/10	9/10	0/4	8/ 9

1) Inoculated after incubation for 20 min. at 25°C with AEA : y-RNA complex.
2) In 31 days after treatment, 1.0×10^5 of E-cells were challenged.
3) PBS(-) alone was applied.
S, survival mice; T, test mice.

Table 5. Effects of AEC(n=25) : y-RNA complex on transplantability of Ehrlich ascites tumor cells.

Group	Amounts of complex applied	Inoculated E-cells[1]	Survival ratio (S/T)	Survival ratio after challenge[2]
AEC(n=25): y-RNA	15.2 mg	1.5X105	10/11	8/10
Control	—		0/10	0/4

[1] Inoculated after incubation for 30 min. at 25°C with AEC(n=25): y-RNA complex.
[2] In 30 days after treatment, 1.5×10^5 of E-cells were challenged.

is not always necessary, and that there is no bad effect by using large quantities. (b) The effects of attenuating E-cells by AEC(n=25): y-RNA complex are shown in Table 5. A 90 % attenuation was achieved, that is, ten to eleven mice survived. Furthermore, eight to ten mice were resistant to the challenge inoculation afterwards. Compared with the results given in Table 4, it is clear that there are little differences between the two methods, that is, the effects did not differ much by changing the configuration of the constituent saccharide (steric structure).

(c) The effects of attenuating E-cells by AEC(n=100): y-RNA complex are indicated in Table 6.

This complex floated in 0.9 % NaCl solution, and was difficult to mix with E-cells. The molecular weight of the complex was so large that the viscous liquid was difficult to inject into mice. The results were therefore uncertain. It seems that the effects of AEC(n=100): y-RNA complex were inferior to those of AEA or AEC(n=25): y-RNA complex, probably because of the difficulty in using the former.

Table 6. Effects of AEC(n=100): y-RNA complex on transplantability of Ehrlich ascites tumor cells.

Group	Amounts of complex applied	Inoculated E-cells	Survival ratio (S/T)	Survival ratio after challenge[1]
AEC(n=100): y-RNA complex	14.7mg	2 heads 1.2X105 2 heads 9.3X 104 8 heads 1.3×10^5	10/10	4/10
Control	—	1.2×10^5	0/10	0/5

[1] Mice were inoculated with 1.3×10^5 E-cells in 31 days after the treatment.

5) **Therapeutic effects of AEA (or AEC) : y-RNA complex**

In attenuating E-cells as described, the effects of the AEA : y-RNA complex injection were examined to elucidate the mechanism of action of the complex. After 1.3×10^5 cells/mouse were implanted intraperitoneally, one group was injected with 5 mg AEA: y-RNA complex/mouse daily for 5 days from the first day onwards; a second group was injected daily for 5 days from the 3rd day onwards; and a third group was treated daily for 5 days from the 5th day

onwards. Each 5 mg AEA : y-RNA complex was suspended in 0.2 ml of physiological saline solution (0.9 % NaCl) and injected i.p. The results are shown in Table 7.

As seen from Table 7, the complex displayed certain inhibitory effects on the implantation and multiplication of E-cells. Especially in the group that received the complex within *24* hours after E-cell transplantation, prevention was almost complete. Furthermore, the inhibiting effect was mainly apparent following early treatment, there being no appreciable effect on the mice in which the multiplication of E-cells had proceeded and the ascites emerged.

Table 7. Effects of AEA : y-RNA on transplantability and repression of Ehrlich ascites tumor cells.

Group*	Survival ratio (S/T)	Average life span (days) of dead mice
1	8/8	survived
2	7/8	20
3	4/8	30
Control	1/8	20

* Mice were inoculated with 1.3~105 E-cells.
1.: 5 mg of AEA : y-RNA (in 0.2 ml of 0.9 % NaCl solution) was administered i.p. once daily starting 24 hours after inoculating tumor cells (total: 5 times).
2: Same procedure was done from 72 hours after inoculating tumor cells (total: *5* times).
3: Same procedure was done from 120 hours (total: 5 times).

DISCUSSION

AEA or AEC in which the degree of polymerization was about 20-25 and consequently the molecular weight was 5-6000, was mixed with y-RNA in a region of excess y-RNA to obtain a complex of an approximately constant molar ratio. The combining ratio was 1 :1.65—1.7 g/g in the AEA as well the AEC: y-RNA complex. On the other hand, AEC(n=100) showed an abnormally high combining ratio, suggesting that the mode of complex formation was different from the other two.

These complexes dissociated 5 % at pH 4.1 which was the isoionic point of the tumor surface, and were hydrolyzed about 49 % by pancreatic RNase A.

When E-cells were incubated with these complexes the transplantability of E-cells in mice was almost completely inhibited. Thus, pretreated mice becamc strongly resistant to a challenge inoculation afterwards. There was hardly any difference by changing the configuration of saccharide. The effects of AEC : y-RNA complex seemed to be slightly better than that of AEC : y-RNA complex, because the viscosity of AEA was lower than that of AEC, and the reaction proceeded homogeneously.

Some published approaches involve the conversion of an inactive drug to an active metabolite in the more acidic microenvironment surrounding tumor cells (Friedkin, 1973; Papanastassiou *et al.*, 1966; Stevens and Mosteller, 1969).

It appeared that RNA was linked to AEA or AEC by ionic linkages, and

that the toxicity of the latter was masked by linking with RNA. Therefore, it might be inferred that the attenuation of E-cells caused by incorporation of these complexes mainly ascribed to AEA or AEC.

Furthermore, it is suggested that a shorter AEA or AEC molecule, or a more dissociable one at pH 4.1 than the present compounds might be more effective. We believe this approach warrants further investigation.

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