Protection of Mice Against Encephalomyocarditis Virus Infection by Preparations of Transfer RNA

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SUMMARY

Preparations of bacterial transfer RNA (tRNA), give dose-dependent protection of mice against encephalomyocarditis (EMC) virus infection at up to 1 mg tRNA per mouse with maximum response when the tRNA is administered around 6 h before infection. Protection occurs with intraperitoneally and intravenously administered tRNA against infections by both these routes. In some experiments significant protection occurs by single treatments of tRNA up to 24 h after infection with virus doses of $1 \times 10^2$. Some tRNA preparations of eukaryotic origin do not give significant protection. Protection is not a feature of all species of bacterial tRNA; partially purified valine, tyrosine and phenylalanine tRNAs from *Escherichia coli* are not protective. tRNA treatment does not induce circulating interferon nor does it ‘hypo-reactivate’ the protective effect of poly (I).poly (C) treatment of mice. Humoral and cell mediated immune responses do not seem to be involved in tRNA mediated protection since first, cytosine arabinoside treatment does not affect protection by tRNA; second, serum from mice treated with tRNA and an EMC vaccine does not protect other mice against infection, and third, mice that survive normally lethal infections as a result of tRNA treatment are generally just as susceptible to re-infection as previously untreated, uninfected mice. Silica treatment abolishes protection of mice by tRNA implying that macrophages are necessary. However, tRNA does not seem to act by clearance of virus particles since vaccination of mice by inactivated EMC virus is not affected by tRNA treatment. These results are considered in relation to the presence of a tRNA-like structure in EMC virus RNA and protection of mice by other single stranded polynucleotides.

INTRODUCTION

Replication of RNA viruses is dependent, initially at least, on interactions between host cell factors and nucleotide sequences within the virus genome. The existence of many quite specific interactions between an RNA molecule and various proteins is well illustrated by the extensive work on the assembly of ribosomes from their constituent proteins and nucleic acid moieties (Nomura & Held, 1974). Thus the existence of specific nucleotide sequences in virus RNA could allow a virus to utilize host proteins during virus replication. Since Mengo virus and encephalomyocarditis (EMC) virus contain a tRNA-like structure within the virus
genome (Salomon & Littauer, 1974; Lindley & Stebbing, 1976) we examined the antiviral properties of tRNA preparations and their mode of action in the case of EMC virus infection of mice.

METHODS

**Virus.** Encephalomyocarditis (EMC) virus was grown and stored as previously described (Stebbing, Grantham & Carey, 1976). An EMC vaccine was prepared by incubating a virus stock that titrated at \(10^6\) LD100/ml at 37 °C for 7 days in the presence of 0·1 % formalin. This material was stored at −20 °C.

**Chemicals.** Virus dilutions and polynucleotides were all made up in 0·89 % (w/v) NaCl, 10 mM-HEPES, pH 7·5 (HBS) and injected intraperitoneally or intravenously into one of the lateral tail veins of mice in a vol. of 0·1 ml, or as described. Poly(I), poly(C) was from P-L Biochemicals (International Enzymes), Windsor, Berks., U.K. and was made up in water to a concentration of 1 mg/ml. Cytosine arabinoside HCl, micrococcal nuclease (grade VI from *Staphylococcus aureus*) and trypsin (type III) were obtained from Sigma Chemical Co., London. Bovine pancreatic ribonuclease was obtained from Worthington Biochemical Corp., Cambrian Chemicals Ltd, Croydon, U.K. Silica (Dorentrup 12) was a gift from Dr R. Norpoth, Institut für Arbeitsmedizin, Munster, W. Germany.

*Escherichia coli* tRNA was obtained from British Drug Houses (BDH) Ltd, Poole, U.K. Yeast tRNA type I and *E. coli* strain W tRNA XXI were obtained from Sigma Chemical Co. Yeast tRNA was also obtained from Miles Laboratories Ltd, Stoge Poges, U.K. Total tRNA from *E. coli* strain K12 CA265 and partially purified preparations of valine, phenylalanine and tyrosine tRNAs were obtained from the Microbiological Research Establishment (MRE), Porton, U.K. The partially purified preparations of tRNA were obtained by benzoylated diethylaminoethyl (BD)-cellulose column fractionation. The valine, phenylalanine and tyrosine fractions were the ones that eluted at 0·45 M-NaCl, 0·8 to 0·9 M-NaCl and in the ethanol/NaCl gradient at 0·55 M-NaCl, respectively. A tRNA preparation which we designate the ‘serine-rich’ fraction was the one that was eluted at 0·55 M-NaCl but this fraction, since it is not homogeneous, did show some acceptor activity for many other amino acids. Yeast tRNA was also prepared by phenol and salt extraction using a modification of the method of Parish & Kirby (1966): the dried ethanol precipitate of the aqueous phenol phase was extracted three times with 3 M-sodium acetate, pH 6·0, and tRNA precipitated from these bulked extracts with 2 vol. of ethanol. Mouse (LACA) liver tRNA was prepared by the same procedure. The tRNA preparations used most extensively in the present work were the *E. coli* tRNAs from BDH and MRE. All commercially obtained tRNA preparations are specified in the text by the source in parentheses.

All tRNA preparations were examined on 5·0 % polyacrylamide gels by the method of Loening (1969) and their ability to accept ³H-labelled amino acids in the presence of tRNA synthetases was also checked using the procedure described previously (Lindley et al. 1976). In all the tRNA preparations 92 % to 94 % of the 260 nm absorbing material migrated in the 4S position and they showed approx. equal incorporation of a standard ³H-amino acid mixture (TRK 440 from the Radiochemical Centre, Amersham, U.K.).

Some of the more rare nucleosides that occur in tRNAs which were tested for antiviral activity were: pseudouridine, di-hydroxyuridine, 5-methyl-ctyosine, 7-methyl-guanosine, 1-methyl-adenosine, inosine, 1-methyl-inosine, 6-amino-3-dimethyl-allylurine, 6-furfurylaminopurine and the riboside of the last, all obtained from Sigma Chemical Co.

**Mice.** Female LACA mice 6 to 10 weeks old, weighing between 18 and 24 g were obtained from Bantin and Kingham Ltd, Hull, U.K. Mice were maintained at 22 °C with unlimited
Protective effect of tRNA against EMC virus

Fig. 1. Protective effect of various doses of tRNA (BDH), administered intraperitoneally 6 h before infection, against 10 × LD_{100} EMC virus. •, infected only; ○—○, 50 μg/mouse; ▼—▼, 100 μg/mouse; □—□, 500 μg/mouse; △—△, 1000 μg/mouse.

access to water and standard rat and mouse breeding diet from Grain Harvesters Ltd, Wingham, Kent, U.K.

Interferon assay. Mouse serum interferon was assayed by determining that serum dilution which gives 50 % plaque reduction of EMC virus on L-929 cells as previously described (Stebbing et al. 1976).

Statistical methods. The survival time (t) of mice in hours was obtained from records prepared twice daily. An average survival time for a group of mice was obtained by calculating the mean of the reciprocals of the survival times taking the reciprocal of survivors to be zero and multiplying this mean by 100. These calculations are carried out on the data at 18 days post-infection (p.i.) by which time no further deaths occur (Stebbing et al. 1976) and the values obtained are designated as values of (1/t) × 10^2. Since mice infected with more than 1 × LD_{100} virus die between 96 h and 125 h after infection the (1/t) × 10^2 value for infected control groups is around 1.04 to 0.80. Where treatment of mice causes some to survive and/or delays the time of death, the (1/t) × 10^2 value is lower. Detailed comparisons of survival data by various methods are to be found in our previous publication (Stebbing et al. 1976). Values of (1/t) × 10^2 are used here as comparative measures of the survival of different groups of mice. However, significant differences in the survival times of different groups of mice were tested for by calculating χ^2 values by the logrank method of Peto & Pike (1973) on the survival data up to 18 days p.i. The significance levels of χ^2 values are indicated by asterisks as follows: ***, P < 0.001; **, P < 0.01; *, P < 0.05. No asterisk indicates P > 0.05 and is taken as non-significant.
RESULTS

Protection of mice by single treatments of E. coli tRNA at various times relative to infection

We show in Fig. 1 the protective effect of various doses of E. coli tRNA (BDH) administered intraperitoneally 6 h before infection of LACA mice with 10 × LD$_{100}$ EMC virus. For convenience of presentation these mortality curves show deaths occurring in any one day at one time only. The data in Fig. 1 are part of a more extensive investigation of the dose dependence of protection by E. coli tRNA at various virus doses 6 h before infection or 2 h after infection. The complete data for significantly protected groups ($P < 0.05$) are presented.
Protective effect of tRNA against EMC virus

![Graph](image)

Fig. 3. Protective effect of tRNA (BDH), 400 µg/mouse, administered intraperitoneally at various times relative to infection with 10 × LD_{100} EMC virus (●●●) and 1 × LD_{100} EMC virus (■■■).

in Fig. 2 in which the value of (1/t) × 10^3 for each group of treated mice is plotted against the dose of tRNA used. Doses of 50 µg and 100 µg per mouse were tested against each virus dose but significant protection with these doses only occurred at the lower virus doses. It is clear that the degree of protection of mice by *E. coli* tRNA (BDH) increases with increasing doses of the tRNA up to 1 mg/mouse and decreases as the virus challenge increases. Above 1 mg/mouse there appears to be a decrease in protection against infection (see Fig. 2) but on no occasion was this decrease statistically significant.

We also examined the protective effect of single doses of 400 µg *E. coli* tRNA (BDH) at various times before and after infection. The data in Fig. 3 show that maximum protection occurs around 6 h before infection when tRNA is administered intraperitoneally. Significant protection occurred against both virus doses up to 6 h after infection. Similar patterns of protection were obtained with both higher and lower doses of tRNA although 100 µg tRNA/mouse only gave significant protection at 6 h before infection and with 800 µg tRNA/mouse the degree of protection at all times was greater and was significant even at 24 h after infection.

**Features of tRNA preparations necessary for antiviral activity**

Polyacrylamide gel analyses of *E. coli* tRNA (BDH) samples fractionated on a Sephadex G200 (Pharmacia) column showed that about 8% of the material was less than 4S in size. This smaller material had no significant antiviral activity against EMC infection of mice even at a dose of 600 µg/mouse 6 h before infection or 2 h after infection.

To confirm that the polynucleotide material migrating in the position of 4S is necessary for the antiviral effects observed with *E. coli* tRNA (BDH) and not due, for example, to a contaminating endotoxin, we tested its antiviral activity after treatment with trypsin or various nucleases. The results in Table 1 show that trypsin treatment has no effect on the antiviral activity of the tRNA (Table 1B) but that nuclease treatments completely destroy its ability to protect mice against EMC virus infection (Table 1A). Trypsin and nuclease treatments of tRNA were carried out in solutions of *E. coli* tRNA (BDH) at 4 mg/ml.
Table 1. The effect of pancreatic and micrococcal nucleases (A) and trypsin (B) on the antiviral effect of E. coli tRNA (BDH) administered 6 h before intraperitoneal infection of mice with 10 × LD_{100} EMC virus

Values of (1/t) × 10^8 and logrank χ² comparisons.

(A) Treatment of tRNA prior to administration

<table>
<thead>
<tr>
<th>Treatment of tRNA prior to administration</th>
<th>(1/t) × 10^8</th>
<th>χ²_{inf}</th>
<th>χ²_{i}</th>
<th>χ²_{e}</th>
<th>Infected only (1/t) × 10^8</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.91</td>
<td>0.80</td>
<td>7.43**</td>
<td>0.06</td>
<td>0.95</td>
</tr>
<tr>
<td>Pancreatic ribonuclease</td>
<td>0.86</td>
<td>1.31</td>
<td>6.48**</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>Micrococcal nuclease</td>
<td>0.91</td>
<td>0.80</td>
<td>7.43**</td>
<td>0.06</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Number of mice per group = 20.
Amount intact or degraded tRNA administered per mouse = 400 µg, intraperitoneally.

χ²_{inf} = logrank comparisons with the infected only group.
χ²_{i} = logrank comparisons between the tRNA treated group and the groups given nuclease treated tRNA.
χ²_{e} = logrank comparisons between the groups given nuclease treated tRNA and control groups given just the heated nucleases without tRNA ((1/t) × 10^8 values not given, see text).

Asterisks indicate significance levels. See Methods.

(B) Treatment of tRNA prior to administration

<table>
<thead>
<tr>
<th>Treatment of tRNA prior to administration</th>
<th>(1/t) × 10^8</th>
<th>χ²_{inf}</th>
<th>χ²_{i}</th>
<th>χ²_{e}</th>
<th>Infected only (1/t) × 10^8</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.38</td>
<td>5.11**</td>
<td></td>
<td></td>
<td>0.17</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0.44</td>
<td>4.31**</td>
<td></td>
<td></td>
<td>0.82</td>
</tr>
</tbody>
</table>

Number of mice per group = 10.
Amount of tRNA administered per mouse = 400 µg, intravenously.

χ²_{inf} = logrank comparisons with the infected only group.
χ²_{i} = logrank comparison between the group given untreated tRNA and the group given trypsin treated tRNA.

Asterisks indicate significance levels. See Methods.

Trypsin was added to a concentration of 0.25 mg/ml and the mixture incubated at 37 °C for 60 min and then frozen until the time of administration to mice. Micrococcal nuclease was added to a concentration of 80 units/ml and pancreatic ribonuclease was added to a concentration of 50 µg/ml and each was then incubated at 37 °C for 45 min and then heated at 100 °C for 3 min and stored at −20 °C until injected. 10 % polyacrylamide gel analysis of the products obtained after digestion of the tRNA with micrococcal or pancreatic nuclease showed no material migrating at 4S and most of the 260 nm absorbing material migrated in the position of mononucleotides or oligomers less than 10 nucleotides in length. We have observed that treatment of mice with nuclease-treated tRNA delays the time of death of EMC virus infected mice by about 36 h, although none survived infection. Nevertheless this feature complicated the analysis of the results and led us to heat inactivation of nucleases after incubation with tRNA. The data in Table 1A show that heated, nuclease treated tRNA does not significantly affect the time of death of mice compared with mice treated with the same doses of the nucleases alone, which had been heat inactivated (χ²_{i} values in Table 1A). The loss of antiviral activity of E. coli tRNA (BDH) with nuclease treatment also indicates that the effect is not due to the minor modified bases present in the tRNA. However, we checked 7 such bases (the first 7 listed in Methods) at doses of 500 µg per mouse 6 h before and 2 h after infection with 1 × LD_{100} EMC virus and found no significant protection. Because of their lower solubility the last 3 bases listed in Methods were tested only at 50 µg/mouse but these also showed on antiviral activity. Since the amounts of these bases in tRNAs are only a
Table 2. Protective effect of E. coli tRNA (BDH) administered intraperitoneally (i.p.) and intravenously (i.v.) at various times relative to EMC virus infection of mice

Values of \((1/t) \times 10^4\) and \(\chi^2\) comparisons with infected controls in brackets.

<table>
<thead>
<tr>
<th>Time (h), relative to infection, at which tRNA administered to mice</th>
<th>Route of administering tRNA</th>
<th>Virus control</th>
<th>(LD_{50}) Route of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>i.p.</td>
<td>0.36 (9.32***)</td>
<td>0.31 (9.61***)</td>
</tr>
<tr>
<td>6</td>
<td>i.v.</td>
<td>0.52 (6.89***)</td>
<td>0.18 (12.37***)</td>
</tr>
<tr>
<td>+2</td>
<td>i.p.</td>
<td>0.41 (9.30***)</td>
<td>0.44 (11.73***)</td>
</tr>
<tr>
<td>i.v.</td>
<td>0.56 (9.27***)</td>
<td>0.37 (9.92***)</td>
<td>0.37 (9.92***)</td>
</tr>
</tbody>
</table>

Number of mice per group = 10.
Amount of tRNA administered per mouse = 400 \(\mu\)g.
Asterisks indicate significance levels. See Methods.

Table 3. Protective effect of various types of tRNA at different times relative to infection of mice with EMC virus

Values of \((1/t) \times 10^4\) and \(\chi^2\) comparisons with infected controls in brackets.

<table>
<thead>
<tr>
<th>tRNA/mouse (mg)</th>
<th>Type of tRNA</th>
<th>Time (h) of treatment relative to infection</th>
<th>Virus dose controls ((LD_{50}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>(E. coli) tRNA (BDH)</td>
<td>0.59 (1.60) 0.47 (6.13***) 0.67 (0.69)</td>
<td>0.74 10</td>
</tr>
<tr>
<td>0.5</td>
<td>(E. coli) tRNA (strain W, Sigma)</td>
<td>0.55 (3.21) 0.47 (5.76***) 0.59 (4.05***)</td>
<td>0.71 10</td>
</tr>
<tr>
<td>0.4</td>
<td>Yeast tRNA (Miles)</td>
<td>0.71 (0.50) 0.72 (0.24) 0.71 (0.01)</td>
<td>0.71 10</td>
</tr>
<tr>
<td>0.4</td>
<td>Mouse liver tRNA</td>
<td>0.94 (3.91*) 0.94 (3.69*) 0.71 (0.50)</td>
<td>0.71 10</td>
</tr>
<tr>
<td>0.4</td>
<td>Yeast tRNA</td>
<td>0.77 (0.19) 0.71 (1.60) 0.68 (2.31)</td>
<td>0.87 10</td>
</tr>
<tr>
<td>0.4</td>
<td>Yeast tRNA (Sigma type I)</td>
<td>0.70 (2.17) 0.65 (2.44) 1.11 (0.11)</td>
<td>1.02 5</td>
</tr>
<tr>
<td>0.4</td>
<td>Yeast tRNA (Sigma type I)</td>
<td>0.76 (3.99*) 0.76 (3.99*)</td>
<td>0.98 10</td>
</tr>
</tbody>
</table>

Number of mice per group = 10.
All tRNA preparations administered intraperitoneally.
Asterisks indicate significance levels. See Methods.

little over 1 %, our tests were carried out at doses at least 50 times greater than those occurring in doses of the intact tRNA preparations that are antiviral.

It could be argued that protection by intraperitoneally administered tRNA before infection could be due to a local reaction limiting the passage of the virus inoculum from the peritoneal cavity. We therefore examined the protective effect of intraperitoneally and intravenously administered tRNA at various times relative to infection by both the intraperitoneal and intravenous routes. The results, in Table 2, show that good protection is achieved with tRNA administered by both routes at \(-24\) h and \(-6\) h relative to infection and logrank \(\chi^2\) analyses showed no significant differences between the two routes at any of the treatment times.

Bacterial tRNA preparations consistently showed antiviral activity but significant protection has not been found with all tRNA preparations from eukaryotic sources. The data in Tables 3 and 4 show that tRNA from \(E. coli\) strain W (Sigma) and \(E. coli\) strain CA 265 (MRE) show antiviral activity essentially similar to that of \(E. coli\) (BDH). However, the mouse-liver and the yeast tRNA preparations except for, Sigma type I, do not show significant protection, even at doses of 5 mg/mouse (see Table 3). In fact, the mouse-liver tRNA
Table 4. Protective effect of partially purified E. coli tRNA species compared with unfractionated tRNA preparations against EMC virus infection of mice

<table>
<thead>
<tr>
<th>Type of tRNA</th>
<th>(1/t) x 10^2</th>
<th>( \chi^2 )</th>
<th>Infected control</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli tRNA (MRE)</td>
<td>0.40</td>
<td>6.05*</td>
<td></td>
</tr>
<tr>
<td>Serine-rich tRNA</td>
<td>0.19</td>
<td>9.89**</td>
<td>0.92</td>
</tr>
<tr>
<td>Valine tRNA</td>
<td>0.85</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Tyrosine tRNA</td>
<td>0.79</td>
<td>1.16</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine tRNA</td>
<td>0.70</td>
<td>3.16</td>
<td></td>
</tr>
</tbody>
</table>

Number of mice per group = 10.
tRNA preparations administered intraperitoneally 6 h before infection, 400 \( \mu \)g/mouse.
Virus dose = 5 \( \times \) LD_{50}.
Asterisks indicate significance levels. See Methods.

appears to be deleterious (\( \chi^2 \) significant and \( (1/t) \times 10^2 \) values greater than the infected control, see Table 3).

The antiviral activity of some partially purified E. coli tRNA (MRE) preparations obtained by fractionation on BD-cellulose (see Methods) are shown in Table 4. From the results it is clear that the valine, phenylalanine and tyrosine containing fractions are without antiviral activity and that the antiviral activity is present in the serine-rich fraction. However, \( \chi^2 \) analysis showed that the antiviral activity of this fraction is not significantly greater than that of the unfractionated starting material.

Interferon inducing and ‘hypo-reactivation’ capacity of tRNA

Since E. coli tRNA (BDH) preparations consistently show good antiviral activity against EMC virus infection of mice we tested the ability of this tRNA to induce interferon by analysing mouse serum samples obtained 4, 8, 12 and 24 h after administration of the tRNA at two doses. Serial 0.5 log_{10} dilutions of the sera were prepared and assayed for inhibition of plaque formation by EMC virus on L-929 monolayers, the highest concentration tested being 0.5 log_{10} dilutions of the sera. At this level of sensitivity no interferon was detectable in serum at any of the times tested after intraperitoneal administration of 400 \( \mu \)g or 800 \( \mu \)g tRNA per mouse, although poly (I). poly (C) (100 \( \mu \)g/mouse) produced readily detectable amounts of serum interferon 4 and 8 h after treatment of the mice. We also examined the ability of serum, prepared 2, 4, 8 and 24 h after treatment of mice with 400 \( \mu \)g E. coli tRNA (BDH), to protect other mice against EMC virus infection. Such serum was not found to confer protection at any of these times, even at 0.5 ml serum per recipient mouse, although serum from poly (I). poly (C)-treated donor mice was highly protective.

The ability to induce interferon and cause additional protection of mice against infection is ‘hypo-reactivated’ after repeated stimulation by polynucleotide interferon inducers (Ho, 1973). Thus, if the protective effect of tRNA is by induction of interferon, daily treatment with tRNA for 5 days should be protective and should block further protection by poly (I). poly (C). The data in Table 5 show that daily treatment for 5 days with tRNA at two different doses does not give significant protection against virus challenge on the 7th day after commencing treatment and that after these treatment regimes mice were still protected by a single dose of 100 \( \mu \)g poly (I). poly (C) administered 8 h before the virus challenge. In contrast, treatment with 40 \( \mu \)g poly (I). poly (C) daily for 5 days, while protective, did block further protection by the 100 \( \mu \)g dose of poly (I). poly (C) administered 8 h before the virus challenge (see Table 5). We conclude, since tRNA does not cause a ‘hypo-reactivated’ res-
Protective effect of tRNA against EMC virus

Table 5. Effect of repeated tRNA treatment on the ability of poly (I).poly (C) to induce protection of LACA mice against EMC virus infection

<table>
<thead>
<tr>
<th>Pre-treatment daily for 5 days</th>
<th>Treatment 8 h before infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBS</td>
<td>None</td>
</tr>
<tr>
<td>400 µg tRNA</td>
<td>100 µg poly (I).poly (C)</td>
</tr>
<tr>
<td>800 µg tRNA</td>
<td></td>
</tr>
<tr>
<td>40 µg poly (I).poly (C)</td>
<td></td>
</tr>
</tbody>
</table>

Values of (t/t) x 10^3 and χ^2 comparisons.

Number of mice per group = 10.
Poly nucleotides administered intraperitoneally in the amounts per mouse shown.
Log rank χ^2 values are from comparisons of groups treated with 100 µg poly (I).poly (C) 8 h before infection and the control groups not treated.
Asterisks indicate significance levels. See Methods.

Table 6. Effect of cytosine arabinoside treatment before and after infection on the protective effect of tRNA administered 2 h after infection of LACA mice with EMC

<table>
<thead>
<tr>
<th>Cytosine arabinoside treatment</th>
<th>tRNA treatment</th>
<th>(t/t) x 10^3</th>
<th>χ^2_{inf}</th>
<th>χ^2</th>
<th>Infected control</th>
<th>Infected and tRNA treated control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>After</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>0.90</td>
<td>(0.97)</td>
<td>–</td>
<td>0.82</td>
<td>0.30</td>
</tr>
<tr>
<td>–</td>
<td>+</td>
<td>0.91</td>
<td>(0.99)</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>0.34</td>
<td>(0.71**)</td>
<td>(0.30)</td>
<td></td>
<td>(11.69***</td>
</tr>
<tr>
<td>–</td>
<td>+</td>
<td>0.36</td>
<td>(8.53**)</td>
<td>(0.12)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Number of mice per group = 10.
Virus dose was 5 x LD_{100}, intraperitoneally.
Amount E. coli tRNA (BDH), injected intravenously 2 h after infection = 400 µg.
Cytosine arabinoside treatment was 4 mg intraperitoneally daily for 4 days before or after the day of infection, as indicated.
χ^2_{inf} = log rank comparisons with the infected control group.
χ^2 = log rank comparisons with the infected and tRNA treated control group.
Asterisks indicate significance levels. See Methods.

Response to protection by poly (I).poly (C), that tRNA does not cause interferon production in mice.

Requirement for macrophages but not immune systems for protection by tRNA

We investigated the involvement of mouse immune systems on the antiviral effect of tRNA by examining the effect of cytosine arabinoside treatment; the protective effect of serum from mice treated with tRNA and an EMC vaccine; and the susceptibility to re-infection of mice that survived lethal doses of EMC virus as a result of tRNA treatment. Treatment every day for 4 days with cytosine arabinoside is known to be immunosuppressive (Connors, 1973) and prolongs survival of skin and kidney allografts in rabbits (Beyer & Friedman, 1975) but such a treatment regime was found to have no effect on protection of mice with tRNA whether cytosine arabinoside treatment was given before or after infection (see Table 6).

On several occasions we have re-infected the surviving mice from groups that were infected with EMC virus doses in excess of 1 x LD_{100} and treated with tRNA 6 h before infection. These surviving mice were re-infected with virus doses greater than 1 x LD_{100} 15 days after
the first infection and were generally found to be just as susceptible to re-infection as control groups of mice that had not previously been treated or infected. In some experiments the re-infected mice showed a significant but small degree of protection but this was never greater than that of mice surviving EMC virus infections as a result of poly (I).poly (C) treatment.

Susceptibility to re-infection of mice surviving 15 days after infection with lethal doses of EMC virus indicates that no long-term stimulation of IgG production or cell-mediated immunity occurs with tRNA treatment but does not eliminate stimulation of production of IgM by tRNA since this immunoglobulin peaks between 3 and 4 days after antigen challenge in mice. We therefore tested serum from mice 3 days after vaccine treatment and looked for an increase in the ability of this serum to protect other mice when the donor mice had also been treated with tRNA 6 h before the vaccine treatment. The dose of EMC vaccine used was one that just gave significant protection against challenge with \( 5 \times 10^9 \) EMC virus 15 days after vaccination and corresponded to a titre of \( 10^3 \times \text{LD}_{100} \) prior to inactivation. Mice treated with such sera (0.8 ml per recipient mouse, i.p.) were challenged half an hour later with \( 1 \times \text{LD}_{100} \) EMC virus but no significant protection was conferred to the recipient mice compared with an untreated, infected control group (\( \chi^2 = 0.77 \)). We have previously shown that the serum transfer protocol used does enable us to detect protective levels of immunoglobulins (Stebbing, Grantham & Kaminski, 1976) and we therefore conclude that tRNA does not stimulate production of specific IgM against EMC virus.

A requirement for macrophages for the protective effect of tRNA to be manifest is shown by the abolition of tRNA mediated protection when mice are also treated with silica which is known to be specifically cytotoxic for macrophages (Allison, Harington & Birbeck, 1966). The data in Fig. 4 show that the protective effect of tRNA, administered intravenously 6 h before infection with \( 10 \times \text{LD}_{100} \) EMC virus, is completely eliminated by intraperitoneal treatment of the mice with 25 mg silica 22 h before infection. For convenience of presenta-
Table 7. The effect of tRNA treatment 6 h before vaccine treatment on the protection of LACA mice against EMC infection 21 days later when the first vaccine dose is followed by a second dose 14 days later

<table>
<thead>
<tr>
<th>Treatment 6 h before first vaccine dose</th>
<th>(t/r) x 10^3</th>
<th>Infected only</th>
<th>Double 'vaccine' treatment only</th>
<th>First 'vaccine' only</th>
</tr>
</thead>
<tbody>
<tr>
<td>400 μg tRNA</td>
<td>2.54</td>
<td>(7.54**)</td>
<td>0.88</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Values of (t/r) x 10^3 with χ² comparisons shown in brackets.

- Number of mice per group = 20.
- Amount E. coli tRNA (BDH) injected intravenously 6 h before first 'vaccine' dose = 400 μg/mouse.
- First vaccine dose and second dose 14 days later were both equal to 300 × LD₅₀₀ before inactivation.
- Virus dose 21 days after first 'vaccine' dose = 5 × LD₁₀₀.
- χ² = logrank comparison with the infected control.
- χ² = logrank comparisons with the double 'vaccine' control.
- Asterisks indicate significance levels. See Methods.

Infection, deaths occurring in any one day have been represented at one time only (see Fig. 4). Logrank χ² comparison of the tRNA treated group and the infected control was 4.87* and there was no significant difference between the infected control group and the group treated with both silica and tRNA. We have also observed that protection by tRNA, administered 2 h after infection, is also abolished by silica treatment 22 h before or 4 h after infection.

The requirement for macrophages for the antiviral effect of tRNA to occur suggests that tRNA may stimulate macrophages to clear virus particles from the mouse. The possibility of virus particle clearance was tested by examining the effect of tRNA treatment on the ability of EMC vaccine to protect mice against a later virus challenge. We first determined a dose of the vaccine that was not protective in a single dose 7 days before challenge by 5 × LD₅₀₀ EMC virus but which did give significant protection when two equal doses were given 14 days apart, the second vaccine dose being 7 days before the virus challenge. Each of these doses of vaccine corresponded to approx. 300 × LD₅₀₀ before inactivation and would therefore have contained only slightly more virus particles than normally used to infect mice.

We then examined the effect of tRNA, given 6 h before the first vaccine dose, on the degree of protection against infection 7 days after the second dose of vaccine. The results, in Table 7, show that the tRNA treatment does not affect the degree of protection conferred by the two vaccine treatments. We therefore conclude that tRNA treatment does not affect the induction of antiviral immunity by small vaccine doses and is therefore unlikely to affect the clearance of virus particles, for example by macrophages; tRNA is therefore unlikely to exert its antiviral effect by such a mechanism.

**DISCUSSION**

The antiviral properties of tRNA preparations reported here do not appear to be due to interferon mediated protection for the following reasons. Not only is serum interferon not detected but the maximum response time for protection by tRNA is around 6 h before infection and the degree of protection 24 h before infection is low, in contrast to interferon induction by other polynucleotides. Moreover, structural features of tRNAs (small size, low Tₘ) are unlike those recognized as necessary for polynucleotide interferon inducers (De Clercq, 1974). Furthermore, repeated treatment with tRNA does not 'hypo-reactivate' protection by poly (I).poly (C) indicating that tRNA protection is not due to induction of
interferon. It should be noted that testing the ‘hypo-reactivation’ capacity of tRNA showed that 5 daily treatments with poly (I), poly (C) gave considerable protection against a virus challenge 2 days later, while tRNA administered in the same regime did not result in significant protection (Table 5) despite the fact that the doses of tRNA used gave good protection in single treatments 6 h before infection (see Fig. 2). The hypothesis that protection by tRNA is due to induction of interferon does not seem to us to have a sound basis but is difficult to refute and would demand the formation of a novel form of interferon that is unusually short lived and does not circulate.

The variability in the antiviral effect of tRNA preparations of different origin is itself an indication that the antiviral effect is not due to interferon induction since only features of the secondary structure of polynucleotides seem of importance for interferon induction (De Clercq, 1974) and tRNAs are essentially similar in many of their secondary structural features (Philipp, 1969). The marked difference in the antiviral properties of prokaryotic and eukaryotic tRNA preparations also indicates that the effect may be a property of only certain tRNAs. This is further indicated by the observation that some partially purified fractions of E. coli tRNA (MRE) show no antiviral activity (Table 4). It is therefore possible that the antiviral effect is due to only those tRNA species that resemble the tRNA-like structure in EMC-RNA (Lindley et al. 1976) and that at the molecular level these tRNAs act as competitive inhibitors of a virus process dependent on the tRNA-like structure. Although the tRNA-like structure in EMC-RNA may be more like prokaryotic than eukaryotic tRNAs, it is also possible that it is simply heterologous, non-amino acylatable tRNAs that exert the antiviral affect. The effect of other purified and chemically modified tRNAs will have to be investigated in order to elucidate the mechanism of action.

Protection of mice by tRNA treatment does not seem to involve immune responses since mice that survive infection as a result of the polynucleotide treatment show little or no protection against re-infection and the immunosuppressive agent, cytosine arabinoside, does not suppress the ability of tRNA to protect mice. The possibility of a stimulation of IgM mediated protection against infection seems unlikely from the serum transfer experiments reported here.

The abolition of protection by tRNA on treating mice with silica and the maximum response time of tRNA protection relative to infection (−6 h) are features also found with poly (C) and poly (I) mediated protection of mice against EMC virus infection (Stebbing et al. 1976). The apparent involvement of macrophages in the protective effect of tRNA could be for reasons similar to those suggested for poly (C) and poly (I) mediated protection: the virus initially replicates in macrophages and tRNA has its effect primarily in these cells; there is an additional independent macrophage mediated antiviral mechanism such that neither mechanism alone is protective; some direct antiviral macrophage activity is stimulated by tRNA, such as the mechanism of Alexander & Evans (1971) or Bradish, Allner & Fitzgeorge (1975). Macrophages and similar cells do accumulate viruses rapidly from the blood stream (Mims, 1964) and also support the replication of several viruses including EMC virus and Mengo virus (Eustatia et al. 1972; Allison, 1974). However, it would seem unlikely that precisely the same mechanisms are affected by tRNA and by poly (C) or poly (I) in view of the very different structures involved and because different cellular proteins bind to the poly (C) tract and tRNA-like regions in EMC-RNA (Lindley & Stebbing, 1976).

To our knowledge no antiviral properties have previously been attributed specifically to tRNA although Ebel et al. (1968) have described protection by chick embryo cell RNAs after methylation. Takano et al. (1965) have demonstrated antiviral effects of yeast nucleic
acid preparations against influenza and EMC virus infections of mice. Their RNA preparations almost certainly included tRNA but probably as a minor component only and the features of the antiviral effects described are different from those reported here for purified tRNA preparations: protection by the yeast RNAs occurred only prophylactically and although long-lasting (many days) was only achieved when the nucleic acid and virus were administered by the same route (intranasally; Takano et al. 1965). Inhibition of EMC virus production in L cell monolayers has also been observed (our unpublished results).

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REFERENCES


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