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A MISALIGNED DOUBLE-STRANDED RNA, POLY(I)·POLY(C_{12},U), INDUCES ACCUMULATION OF 2',5'-OLIGOADENYLATES IN MOUSE TISSUES

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2',5'-Oligoadenylate synthetase was induced 3-2000-fold in spleen, liver, kidney and brain of NIH Swiss mice injected intravenously with 2-200 μg of the misaligned dsRNA, poly(I)·poly(C_{12},U). Levels of 2',5'-oligoadenylates extracted from these tissues were also elevated, although the amount of 2',5'-oligoadenylates extracted did not correlate directly with the amount of enzyme present. These results suggest that double-stranded portions of the misaligned polymer survived intracellularly and activated the 2',5'-oligoadenylate synthetase, and that the level of dsRNA may contribute to the control of 2',5'-oligoadenylate metabolism.

Double-stranded RNA[1] is a potent inducer of interferons α and β, and it is also involved in the mechanism of interferon action (reviewed, 1). An interferon-induced enzyme, 2',5'-oligoadenylate synthetase, requires double-stranded RNA for activity in vitro and polymerizes ATP into 2',5'-oligoadenylates, pppA2'(pA)$_n$,(2-5A), where n=1-3 in most systems (2). This unique oligoadenylate activates a latent endoribonuclease to degrade RNA and thereby, presumably, block translation and viral replication (reviewed, 3). 2',5'-oligoadenylates (2-5A) have also been implicated in the control of cell differentiation and growth (reviewed, 4). Although interferons induce production of 2-5A synthetase, there is no evidence that increased amounts of enzyme lead to increased levels of enzyme product in the absence of a suitable dsRNA activator.

Because of its ability to induce interferon and mediate interferon action, the therapeutic potential of double-stranded RNA is under active

[1] Abbreviations: dsRNA, double-stranded RNA; 2-5A, (p)$_n$A(2'pA)$_m$ where n = 0 - 3, m > 1; EAT, Ehrlich ascites tumor; ELISA, enzyme-linked immunoassay; poly(I)·poly-(C_{12},U), poly(inosinic acid) poly(cytidylic, uridylic) acid in which the latter strand contains one uridine for every 12 cytidines.

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exploration (reviewed, 5). Poly(I)·poly(C) is extremely toxic and efforts have been directed toward designing a nontoxic dsRNA that retains biological activity. Carter et al. (6) reported that mismatched analogs of poly(I)·poly(C), in which the dsRNA was interrupted by introduction of unpaired bases, induced interferon if the frequency of random insertions was not greater than 1 residue in 12. Although poly(I)·poly(C_{12},U) was less toxic, less pyrogenic, and less antigenic than poly(I)·poly(C) (7-10), and although it activated 2',5'-oligoadenylate synthetase in cell extracts (11), it had a reduced effective half-life and increased susceptiblity to nuclease degradation *in vitro* compared to poly(I)·poly(C) (5, 6). Activation of the interferon-induced 2-5A synthetase *in vivo* by exogenous dsRNA would require that the dsRNA be present substantially longer than the 5 minutes required for interferon induction (12). We demonstrate here that poly(I)·poly(C_{12},U) elicited increased levels of 2',5'-oligoadenylates in mouse tissues and discuss the implications of this finding.

MATERIALS AND METHODS

Four to six week old pathogen-free NIH Swiss mice were purchased from the National Cancer Institute, National Institutes of Health. At 6-8 weeks of age, the mice were injected intravenously with saline, 2 μg, 20 μg, or 200 μg poly(I)·poly(C_{12},U) in saline, kindly provided by Dr. Carl Dieffenbach (Department of Pathology). After 24 hours, the mice were anesthetized by metofane enhalation and sacrificed by cervical dislocation. Tissues were extracted and submerged immediately in liquid nitrogen. Tissues from 3-5 mice were pooled and pulverized under liquid nitrogen. Portions were removed and weighed prior to assay for 2-5A and 2-5A synthetase. Protein was determined by the method of Bradford (13). Sera were treated with RNase A at 50 μg/mL for 1 hour at 37°C (14) prior to assay for interferon by a dye uptake method on mouse L cells using vesicular stomatitis virus as the challenge virus (15).

Extraction of 2-5A was essentially as described by Stark and coworkers (16). Neutralized acid-soluble samples were subjected to fractionation on silica and C18 Sep-paks and reverse phase HPLC as described elsewhere (Hearl and Johnston, submitted for publication). Radiolabeled 2-5A, pppA2'pA2'pA2'pA(^{32}P)3'pCp, provided by Dr. Robert Silverman (Department of Pathology), and NADP were used to characterize each column. Radiolabeled 2-5A was included in each run. Fractions were concentrated to dryness on a Roto-Vac and assayed for 2',5'-oligoadenylates. Samples expected to contain 5'-phosphorylated forms of 2-5A, based on their elution time off the HPLC column, were analyzed in a competition ELISA with a mouse monoclonal antibody specific for $(p)_nA(2'pA)_n$, where n=1-3 (17). Samples expected to contain 2-5 cores, were analyzed with a rabbit antiserum specific for 2-5A cores (18). As reported previously, the rabbit and mouse antibodies reacted well with trimer and longer forms of 2-5A, but were 40- and 170-fold less reactive, respectively, with dimer forms. Concentrations of 2-5A were calculated by comparison of dilution curves of unknowns with

that of pA2'pA2'pA or A2'pA2'pA (18), or by using the HP-Genechem Titercalc Computer program to accomplish a four parameter statistical analysis. ELISA results were the mean of triplicate determinations in which dilutions of each sample were assayed in duplicate. Results were expressed as moles 2-5A per gm tissue.

Percent recovery of 2-5A was estimated by addition of known amounts of 2-5A to frozen liver and/or spleen prior to extraction. Recovery was about 50% when 1 pmole 2-5A was added per gram of tissue and over 90% when 50 pmole per gram was added (not shown). Percent recovery was not taken into consideration in the values presented. Further, when 5'-phosphorylated 2-5A (pppA2'pA2'pA) was added prior to extraction, over 90% was recovered as 5'-phosphorylated 2-5A, suggesting that the high level of core extracted from certain tissues was not an artifact of the extraction procedure (not shown).

Tissues from 3-5 mice were pooled and pulverized as described above and then extracted and assayed for 2-5A synthetase essentially as described by Stark and coworkers (20) except that samples were analyzed for 2-5A with an ELISA that employed a mouse monoclonal antibody (Hy21-3AC9) (17). Freshly thawed portions of extract were bound to poly(I)·poly(C)-cellulose on three separate occasions and dilutions of each reaction mixture were assayed in triplicate in the ELISA. The ELISA results are the mean of the three separate determinations and were expressed as moles 2-5A per hour per gm tissue. An alternative solution assay for 2-5A synthetase was performed as described previously (19).

RESULTS

In control, saline-injected NIH Swiss mice, basal levels of 2-5A synthetase were highest in spleen and kidney, with much lower levels found in liver and brain (Table 1). Even though serum interferon was not exceptionally high at 24 hours post injection of poly(I)·poly(C_{12},U), 2-5A synthetase was induced up to 2000-fold in liver (Table I). The degree of 2-5A synthetase induction depended on the tissue and the dose of dsRNA. Injection of 2 μg poly(I)·poly(C_{12},U) resulted in measurable induction of synthetase only in liver and kidney. Enzyme levels were

Table 1. Levels of 2',5'-Oligoadenylate Synthetase in Tissues of Mice Treated with Poly(I)·Poly(C_{12},U)

Poly(I)·poly(C_{12},U) (μg)	2',5'-Oligoadenylate Synthetase[a] (pmole hr-1 gm-1)				Interferon (units/ml)
	Spleen	Liver	Kidney	Brain	
0	48	0.3	26	2.0	<1 (800)[b]
2	30	3.0	280	1.0	<1
20	160	700	1,100	21	3
200	14,000	10	73	120	20

[a] Enzyme levels were determined 24 hr post injection as described under Materials and Methods. Standard deviations were < 50%.

[b] The level of circulating interferon in one control mouse of this group of 5 mice was 800 units/ml.

Table 2. Levels of 2',5'-Oligoadenylates in Tissues of Mice Treated with Poly(I)·Poly(C_{12},U)

Poly(I)·poly(C_{12},U) (μg)		2',5'-Oligoadenylates[a] (pmole gm-1)			
		Spleen	Liver	Kidney	Brain
0	5'-phosphorylated	3.1	0.1	2.4	1.2
	cores	2.9	1.2	0.2	1.7
2	5'-phosphorylated	2.7	0.5	6.0	1.8
	cores	1.2	1.3	0.3	0.7
20	5'-phosphorylated	4.6	2.1	5.1	5.0
	cores	7.7	5.8	14	4.0
200	5'-phosphorylated	2.0	1.7	0.3	0.1
	cores	62	9.1	32	20

[a] 2',5'-oligoadenylate levels were determined 24 hr post injection as described under Materials and Methods. Standard deviations were < 50%.

highest in liver and kidney after a dose of 20 μg, whereas enzyme levels were highest in spleen and brain after a dose of 200 μg dsRNA. An alternative assay for 2-5A synthetase in which the enzyme is not pre-bound to poly(I)·poly(C)-cellulose gave similar results (data not shown).

Low levels of 2-5A were extracted from tissues of control mice injected with saline (Table 2). These levels were, however, slightly higher than those extracted from pathogen-free mice sacrificed within 24 hours of receipt (21). In control liver, most 2-5A was found as non-5'-phosphorylated core forms, (A2'pA(2'pA)$_n$), whereas in control kidney, most 2-5A was 5'-phosphorylated (p_nA2'pA(2'pA)$_n$, where n=1-3). 2-5A from control spleen and brain were equally divided between core and 5'-phosphorylated forms.

Mice injected with poly(I)·poly(C_{12},U) contained elevated levels of 2-5A, particularly at the higher doses of dsRNA (Table 2). The highest levels of 2-5A were detected in spleen after injection of 200 μg poly(I)·poly(C_{12},U). At the two lower doses of dsRNA, 2-5A levels were higher in kidney than in spleen, brain or liver. In spleen and brain, the dose response for 2-5A accumulation in the tissues roughly paralleled but was not directly proportional to the change in 2-5A synthetase levels. In kidney and liver, the highest levels of 2-5A occurred after injection of 200 μg dsRNA, even though the levels of 2-5A synthetase in those tissues were lower than after administration of 20 μg dsRNA.

DISCUSSION

Several laboratories reported previously that injection of interferons α or β or an interferon inducer such as dsRNA led to increased levels of 2',5'-oligoadenylate synthetase in a variety of tissues (reviewed, 12). While interferon levels peaked about 12 hours after injection of dsRNA, 2-5A synthetase peaked at about 24 hours (results not shown). We detected elevated levels of 2',5'oligoadenylates, or 2-5A, in tissues of mice injected 24 hours previously with the mismatched dsRNA, poly(I)·poly(C_{12},U). Since interferon treatment alone did not result in substantial elevation of 2-5A in cultured cells (16, 22), it is likely that the increased levels of 2-5A in tissues of animals injected with dsRNA resulted from activation of synthetase by exogenous dsRNA, though this has not been demonstrated conclusively. The implication is that the mismatched polymer remained in the tissues long enough after interferon induction to accomplish synthetase activation. Further, since a high percentage of 2',5'-oligoadenylates were found as biologically inactive 'cores', (A2'(pA)$_n$), 5'-phosphatase activity may be a major mechanism by which 2-5A is inactivated *in vivo*.

The level of accumulated 2-5A was not directly proportional to the level of 2-5A synthetase present in each tissue. Increases in enzyme activity of up to several hundred-fold , as measured *in vitro*, resulted in only a 7-12-fold increase in intracellular 2-5A levels. In general, the ratio of 2-5A extracted per unit of enzyme activity varied over 200-fold.

Lengyel and coworkers (23) demonstrated that the dose-response curve of 2-5A synthetase purified from mouse EAT cells differed slightly for different dsRNA's, and that maximal enzyme activity occurred at an optimal dsRNA to enzyme ratio. In our study, variations in the amount of 2-5A extracted per unit of enzyme present may have resulted from the presence of differing ratios of dsRNA to enzyme in the various tissues. Accumulation of 2',5'-oligoadenylates relative to the levels of 2-5A synthetase, intracellular dsRNA, and 2-5A degradative enzymes must be explored further to clarify

the critical features that control the level of 2',5'-oligoadenylates in intact cells. Further, the source of dsRNA for activation of 2',5'-oligoadenylate synthetase in control, saline-treated mice remains a mystery.

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