JOURNAL OF VIROLOGY, May 1987, p. 1586–1592
0022-538X/87/051586-07$02.00/0

Vol. 61, No. 5

Accumulation of 2′,5′-Oligoadenylates in Encephalomyocarditis Virus-Infected Mice

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Received 1 October 1986/Accepted 28 January 1987

Levels of 2′,5′-oligoadenylates (2-5A) in various tissues of murine encephalomyocarditis virus (EMCV)-infected mice were determined and compared with those found in pathogen-free mice and in mice treated with the interferon inducer poly(I) · poly(C). In control, pathogen-free mice, liver, spleen, brain, and kidney tissues possessed levels of 2-5A below 1 pmol/g of tissue, demonstrating that 2-5A was not a major component of uninfected mouse tissue. All control tissues had low basal levels (0.3 to 2.0 pmol/h per g) of 2-5A synthetase, the enzyme responsible for 2-5A production. After mice were injected intravenously with the interferon inducer poly(I) · poly(C), circulating interferon, 2-5A synthetase, and 2-5A were elevated with increasing doses of double-stranded RNA. The greatest response to poly(I) · poly(C) occurred in the kidney, in which enyme levels increased 5-fold and 2-5A levels increased 24-fold to 15 pmol/g. Mice that were infected with EMCV also possessed elevated levels of 2-5A and 2-5A synthetase in the four tissues examined, although the relative distribution differed from that observed with poly(I) · poly(C), indicating that the interferon inducer affects the concentration and location of intracellular 2-5A. Brain, spleen, and kidney tissues from EMCV-infected mice contained seven- to eightfold more 2-5A than control tissues did. The nanomolar levels of 2-5A in the tissues of EMCV-infected mice provide evidence that 2-5A may play a role in the antiviral response in an intact animal. In both poly(I) · poly(C)- and EMCV-treated mice, the levels of 2-5A recovered from the tissues were not directly proportional to the amount of 2-5A synthetase present. These results indicate that factors other than the level of 2-5A synthetase controlled the accumulation of 2-5A in tissues.

2′,5′-Oligoadenylate (2-5A), $(p)_nA(2'A)_m$, where $n = 0$–3 and $m \geq 2$, has been shown to be an important component of the response of cultured mammalian cells to interferons (for reviews, see references 14, 29, and 33). The 2-5A system is composed of 2-5A synthetase, a latent 2-5A-dependent RNase (RNase L or RNase F), and the effector 2-5A. In the presence of double-stranded RNA (dsRNA), 2-5A synthetase polymerizes ATP into a series of novel 2′,5′-linked oligoadenylates that activate the endoribonuclease and lead to digestion of single-stranded RNA (5, 40) and inhibition of protein synthesis (2, 15, 16). Since an active 2-5A system depresses protein synthesis, accumulation of 2-5A is believed to contribute to the antiviral state established by interferon after infection by at least some viruses (for a review, see reference 14). The 2-5A system has also been implicated in the regulation of differentiation and cell metabolism (19, 24).

2-5A synthetase has been purified from a number of sources (4, 39, 43) and has been shown to vary in activity in response to different metabolic stresses and disease states. Increased levels of 2-5A synthetase have been reported to occur in growth-arrested cells (21), lymphoblastoid cells treated with glucocorticoid (20), and chick oviducts after withdrawal of estrogen (32). 2-5A synthetase activity was also found to decrease in rat liver after partial hepatectomy (31). After infection with Newcastle disease virus or encephalomyocarditis virus (EMCV) or treatment with dsRNA, increased levels of 2-5A synthetase were observed in mouse liver and spleen (9).

Inactivation of 2-5A may occur through phosphodiesterase or phosphatase activity. 2-5As that lack a 5′-terminal di- or triphosphate or that are composed of less than three adenylate residues are not capable of activating the endonuclease (15, 33). Phosphodiesterase levels were found to be unaffected in a variety of interferon-treated cells (1, 30, 36, 37), although the induction of phosphodiesterase was observed in some systems (17, 20, 27).

Intracellular levels of 2-5A have been reported to vary with the metabolic state of cells. Stark and co-workers observed increased levels of 2-5A in monkey cells after infection with simian virus 40 and in human neuroblastoma cells after growth arrest (24). However, results reported previously contain a large discrepancy in the levels of 2-5A in normal cells. Whereas Laurence et al. (22) measured levels as high as 10 to 100 nM, preliminary results from Brown and Kerr (3) suggest that few mice have levels of 2-5A above 1 nM.

Accumulation of 2-5A in vivo has not been compared with the levels of 2-5A synthetase present, although tissue variations in the basal levels of 2-5A synthetase in normal mouse tissues have been reported (28). If the level of enzyme is the only factor that controls the level of 2-5A in control or infected cells, then 2-5A levels would be expected to increase in proportion to the level of 2-5A synthetase. The effect of a natural infection on intracellular 2-5A levels in an intact animal has also not been described. In the studies reported here, pathogen-free mice were injected with saline, the potent interferon inducer poly(I) · poly(C), or EMCV. The levels of 2-5A synthetase, intracellular 2-5A, and circulating interferon were determined to define the basal levels of these components in uninfected mouse tissues, to investigate the extent to which 2-5A accumulates during a virus infection in an intact animal, and to determine if the level of 2-5A synthetase accurately reflects the level of intracellular 2-5A.

MATERIALS AND METHODS

Reagents. Poly(I) · poly(C), pppA2′pA2′pA, and A2′pA2′pA were purchased from Pharmacia, Inc., Piscat-

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away, N.J. C_{18} and silica Sep-paks and C_{18} μBondapak columns were obtained from Waters Associates, Inc., Milford, Mass. Microtiter plates (Immunolon I) were purchased from Dynatech Laboratories, Inc., Alexandria, Va. Biotinylated goat anti-rabbit immunoglobulin G, biotinylated rabbit anti-mouse immunoglobulin G, and streptavidin horseradish peroxidase were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Radioactive pppA2'pA2'pA2'pA3'[^{32}P]pCp was generously provided by Robert Silverman and David Krause, Uniformed Services University of the Health Sciences (USUHS).

Mice. Non-pathogen-free BALB/c mice, 8 to 12 weeks old, were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass., and housed in the usual facilities. Pathogen-free, 8-week-old NIH Swiss mice weighing approximately 20 g each were obtained from the National Cancer Institute, Bethesda, Md., and housed in microisolator housing units equipped with polycarbonate filter tops (Lab Products, Inc., Maywood, N.J.). Within 24 h after their arrival, the pathogen-free mice were anesthetized by metofane inhalation and injected intravenously through the tail vein with sterile phosphate-buffered saline (PBS; 0.01 M sodium phosphate [pH 7.4], 0.14 M NaCl), poly(I) · poly(C), or EMCV. These mice were then isolated from other mice for 18 to 24 h before sacrifice. Crude EMCV was the generous gift of Paul F. Torrence, National Institutes of Health, and was purified and assayed as described previously (34). Samples for interferon determinations were obtained from the tail vein immediately before sacrifice.

Extraction of 2-5A from tissue. Mice were sacrificed by cervical dislocation. Each liver, spleen, brain, and kidney was removed and immersed immediately in liquid nitrogen. Tissues from each test group (five mice per group) were pooled and then powdered with a tissue pulverizer under liquid nitrogen. The tissue powder was stored over liquid nitrogen.

Tissue powder was homogenized in 7.5% trichloroacetic acid (TCA; 1:2 [wt/vol]) and extracted as described by Hersch et al. (8). After neutralization of the TCA-soluble supernatant by extraction with trioctylamine-trichlorotrifluoroethane (11:37 [vol/vol]), the extracts were adjusted to 90% acetonitrile and applied to a silica Sep-pak also equilibrated in 90% acetonitrile. The cartridge was washed at a flow rate of about 2 ml/min with 90% acetonitrile. The 2-5A was eluted with 2.0 ml of distilled water and then lyophilized. The dried fraction was dissolved in 0.2 M ammonium acetate (pH 5.0) and applied to a C_{18} Sep-pak prewet with methanol and equilibrated in 0.2 M ammonium acetate (pH 5.0). The cartridge was then washed with 1.0 ml of 0.2 M ammonium acetate containing 2% methanol. The 2-5A-containing fraction was eluted with 100% methanol and evaporated to dryness in a rotary evaporator. All cartridge separations were performed with a Sep-pak cartridge rack (Waters). The sample was subjected to high-performance liquid chromatography (HPLC), and the HPLC fractions were analyzed for 2-5A by an enzyme-linked immunosorbent assay (ELISA) described below.

HPLC separation of 2-5A in tissue extracts. HPLC was performed on a Waters Associates instrument equipped with two model 510 pumps, a model 720 systems controller, a model 730 data module, a model 721 WISP autoinjection system, and a Schoeffel variable-wavelength UV detector set at 258 nm. The dried extract obtained by TCA extraction and Sep-pak fractionation was dissolved in HPLC buffer A (50 mM ammonium acetate [pH 5.0]) and applied to a C_{18} μBondapak column (3.9 mm by 30 cm; 5-μm particle size). The elution gradient was composed of an initial isocratic phase of 0% buffer B (50 mM ammonium acetate [pH 5.0], 25% acetonitrile) for 5 min, followed by linear gradients to 25% B (15 min) and then to 100% B (20 min) at a flow rate of 1 ml/min. By this procedure, trimer triphosphate (pppA2'pA2'pA) and trimer core (A2'pA2'pA) eluted at 24 and 32 min, respectively. A radioactive 2-5A marker, pppA2'pA2'pA2'pA3'[^{32}P]pCp, was included as an internal standard in each run and eluted consistently at 28 to 29 min. Fractions (1.0 ml) were collected, lyophilized, and reconstituted in 125 μl of 0.1 M Tris hydrochloride (pH 7.8).

Immunoassay for 2-5A in HPLC fractions. Reconstituted HPLC fractions were assayed in a competition ELISA essentially as described elsewhere (12, 13). Briefly, monoclonal antibody (Hy21-3AC9) specific for $(p)_mA2'p(A)_n$ (m = 1–3; n = 2–4) was used to assay fractions obtained from the region of the chromatograph where 5'-phosphorylated forms of 2-5A eluted. A rabbit antiserum, 80-019b, specific for $A2'p(A)_n$ ($n \geq 2$) was used to assay fractions containing non-5'-phosphorylated core forms of 2-5A. Microtiter plates were coated with 0.02 μg of pA2'pA2'pA per ml covalently attached to modified Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.) (13). After blocking with 0.1% ovalbumin in PBS, antibody and sample (or standard or water) were added to each well. After incubation at 30°C for 90 min, the plate was washed with PBS with 0.05% Tween 20 and probed first with 1 μg of biotinylated anti-mouse or anti-rabbit immunoglobulin G per ml and then with a 1/1,500 dilution of streptavidin horseradish peroxidase. Plates were developed with ABTS substrate (Kirkegaard and Perry, Gaithersburg, Md.) and read in a Multiscan MCC microtiter plate reader (Flow Laboratories, Inc., McLean, Va.) at 414 nm. 2-5A levels were determined by comparing the degree of antibody inhibition obtained with each HPLC fraction with a standard inhibition curve obtained with pA2'pA2'pA or A2'pA2'pA. Fractions that tested positive for 2-5A were pooled and tested for authenticity by assuring that the antibody-reactive material was sensitive to snake venom phosphodiesterase and bacterial alkaline phosphatase (for pppA2'pA2'pA) and resistant to RNase T_2. The interassay standard deviation for ELISA determinations was less than 10% in all cases.

Assay of 2-5A synthetase and interferon. 2-5A synthetase levels were determined by using the immunossay method described by Johnston et al. (13). Briefly, tissue powder was homogenized in DBG-50 buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid] [pH 7.4], 50 mM KCl, 7 mM 2-mercaptoethanol, 20% glycerol, and 1.5 mM magnesium acetate) (1:2 [wt/vol]) with a polytron probe and centrifuged at 10,000 × *g* for 10 min. The supernatant was bound to poly(I) · poly(C)-cellulose (39), washed, and incubated for 18 h at 32°C in DBG-50 buffer containing 5 mM ATP. The supernatants were removed and assayed for 2-5A in a competition ELISA with the monoclonal antibody Hy21-3AC9. The standard deviation in synthetase assays was usually 10 to 20% and was calculated from two to three determinations. Serum interferon titers were determined by Stephanie Vogel, USUHS (38).

RESULTS

2-5A and 2-5A synthetase levels in control mice. The ability to recover and accurately quantify the level of 2-5A is critical in elucidating the role of 2-5A in normal and virus-infected cells and tissues. To that end, an isolation scheme to recover 2-5A in high yield from tissues was established. TCA-soluble

tissue extracts were fractionated on silica and C_{18} cartridges and then by HPLC before detection in an ELISA. The efficiency of the two-step cartridge chromatography for the recovery of 2-5A was examined by determining the percent recovery of known amounts of 2-5A in the methanol elution from the second (C_{18}) cartridge. Although adenine did not bind to the silica cartridge and ATP bound poorly to the C_{18} cartridge, A2'pA and pppA2'pA2'pA were fully retained on both cartridges and recovered under the elution conditions described (results not shown). The C_{18} cartridge bound core 2-5A strongly, but a low pH was necessary to attain >85% retention of 5'-phosphorylated 2-5A; at pH 7.0, trimer triphosphate (pppA2'pA2'pA) was retained with an efficiency of <50% (data not shown).

When >1 pmol of endogenous 2-5A per g was added to tissues before homogenization, over 85% was recovered. Over 55% was recovered when 0.5 pmol of sample was added (data not shown). 5'-Phosphorylated 2-5A (pppA2'pA2'pA) added before extraction was recovered with over 90% yield in the phosphorylated region of the HPLC chromatograph, indicating that the extraction process did not result in detectable cleavage of the phosphodiester backbone or the 5'-terminal phosphates (data not shown). This does not, however, eliminate the possibility that degradative activities occurred before the homogenization process, although the tissues were immersed in liquid nitrogen within 1 to 2 min of sacrifice.

Two precautions were taken to prevent the artifactual identification of 2-5A in tissue extracts. First, the HPLC column used for biological samples was never exposed to optical density levels of 2-5A. Such levels led to carry-over of small but antibody-detectable levels of 2-5A for several succeeding blank runs (results not shown). Instead, each column was calibrated with NADP and pppA2'pA2'pA2'pA3'[^{32}P]pCp, which did not interfere with antibody assays. Second, fractions that contained immunoreactive material were subjected to various digestive enzymes (i.e., snake venom phosphodiesterase, RNase T_2, and bacterial alkaline phosphatase) and retested for immunoreactivity.

Mice (BALB/c, non-pathogen-free), each injected with 20 μg of poly(I) · poly(C), were sacrificed at various times to determine the optimal time for tissue collection. Kidney samples were collected at 0, 6, 12, 24, and 36 h after injection and examined for 2-5A and 2-5A synthetase. Circulating interferon levels were measured and found to peak at 12 h (Fig. 1). Synthetase levels reached a maximum at approximately 24 h, whereas 2-5A levels may have been increasing slightly at 36 h. The time courses for circulating interferon and 2-5A synthetase induction were in agreement with previous reports (6, 9). We chose to compare tissues collected at 24 h postinjection to gain perspective on the state of the 2-5A system at a time when synthetase levels determined by an in vitro enzyme assay were at maximum.

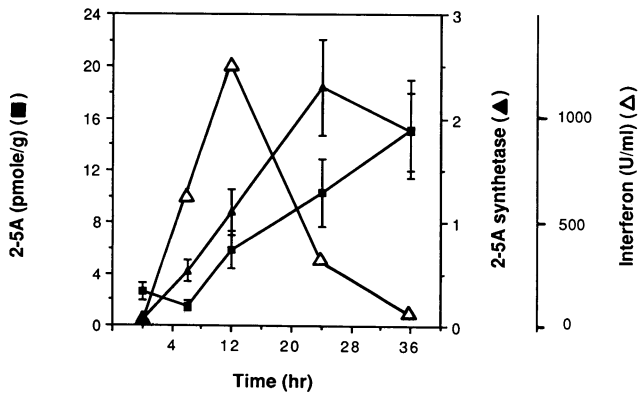

FIG. 1. Levels of 2-5A, 2-5A synthetase, and circulating interferon as a function of time. Non-pathogen-free BALB/c mice were injected intravenously with 20 μg of poly(I) · poly(C), and kidneys were collected at various times. Serum interferon levels were also determined (Δ). Kidneys were examined for 2-5A (■) and 2-5A synthetase (▲) as described in Materials and Methods. 2-5A synthetase levels are expressed as picomoles per hour per gram. Error bars depict one standard deviation from the mean of two to three determinations.

TABLE 1. Levels of 2-5A in the tissues of pathogen-free mice[a]

Treatment	2-5A recovered (fmol/g of tissue) (% cores)			
	Liver	Spleen	Kidney	Brain
Saline	780 (92)	760 (30)	660 (28)	410 (17)
poly(I) · poly(C) (μg)				
2	460 (53)	840 (57)	920 (37)	740 (8)
20	7,100 (90)	3,000 (82)	8,500 (87)	2,900 (82)
200	8,700 (98)	7,200 (96)	15,800 (98)	4,060 (80)

[a] Pathogen-free NIH Swiss mice were injected intravenously with saline or poly(I) · poly (C), and the tissues were collected after 24 h. Tissue extracts were analyzed for 2-5A in a competition ELISA after separation by HPLC as described in Materials and Methods. The interassay standard deviation was <10%; the intraassay standard deviation was ≤25%.

2-5A and 2-5A synthetase in dsRNA-treated mice. Pathogen-free mice were injected intravenously with sterile saline or increasing doses of poly(I) · poly(C). After 24 h, mice were sacrificed and their tissues were assayed for 2-5A as described in Materials and Methods. Mice injected with saline had very low levels of 2-5A (Table 1). These levels were consistently lower than those observed in BALB/c mice that were not pathogen free (Fig. 1 and results not shown). Mice that received doses of poly(I) · poly(C) greater than 2 μg per mouse possessed significantly higher amounts of 2-5A in all tissues examined compared with amounts in controls. The greatest percent increase occurred in the kidney, where the amount of 2-5A rose approximately 24-fold. Interestingly, with increasing amounts of total 2-5A recovered, there was an accompanying increase in the percentage of 2-5A recovered as non-5'-phosphorylated cores [A2'(pA)$_n$, where $n \geq 2$]. For example, in kidney tissue, the percentages of 2-5A recovered as core were 28% in saline-treated mice and 98% in mice that received 200 μg of poly(I) · poly(C). This was not a response unique to synthetic dsRNA, as similar results were obtained after EMCV infection as discussed below.

Levels of 2-5A synthetase increased in mice that received poly(I) · poly(C) (Fig. 2). Although each tissue showed increasing amounts of 2-5A synthetase with increasing amounts of dsRNA, the increase was significant only at 200 μg of poly(I) · poly(C). Spleen contained the highest absolute level of synthetase, but the percent increase over the control was not significantly different among the different tissues. Thus, both synthetase and 2-5A increased in mice treated with poly(I) · poly(C).

Tissue levels of 2-5A were plotted as a function of the level of 2-5A synthetase (Fig. 3). A positive slope, indicating an increase in the amount of 2-5A recovered per U of enzyme, was observed with doses of 2 to 20 μg of poly(I) · poly(C) per mouse. However, at 200 μg of dsRNA, the mean level of 2-5A accumulated per U of enzyme was less than that at 20 μg of dsRNA. This observation suggests that factors other than the absolute level of 2-5A synthetase may be involved

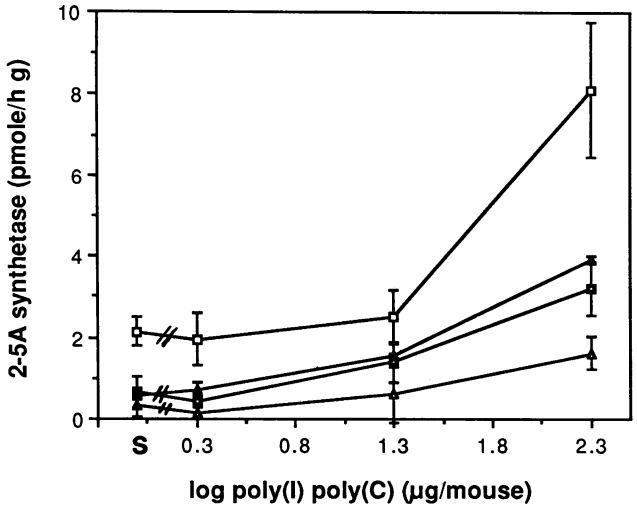

FIG. 2. 2-5A synthetase levels in poly(I) · poly(C)-treated mice. Pathogen-free NIH Swiss Mice received various amounts of poly(I) · poly(C) or saline (S) intravenously. After 24 h, the mice were sacrificed, and liver (△), spleen (□), kidney (■), and brain (▲) were assayed for 2-5A synthetase as described in Materials and Methods. Error bars depict one standard deviation from the mean of two determinations.

in regulating the accumulation of 2-5A in tissues. This finding may be of particular interest to those monitoring 2-5A synthetase in patients receiving interferon or dsRNA therapy (7, 10, 23).

2-5A and 2-5A synthetase in mice infected with EMCV. Pathogen-free mice received intravenous injections of sterile PBS and 5×10^5 or 5×10^6 PFU of murine EMCV to permit analysis of an infection comparable to a previously investigated cell culture system (18, 41). Mice were sacrificed after 24 h and processed for 2-5A as described above. Although 2-5A synthetase was elevated significantly only in the spleens and brains of mice that received the higher dose of EMCV (Fig. 4A), all tissues from EMCV-treated mice contained significantly elevated levels of 2-5A (1.5 to 5.7 pmol/g) (Fig. 4B). The percentage of 2-5A recovered as non-5'-phosphorylated cores ranged from 80 to 98%, and the highest levels of 5'-phosphorylated 2-5A were recovered from the brain. The 2-5A/synthetase ratio yielded results that were similar to those reported above for poly(I) · poly(C)-injected mice (data not shown). The ratio of accumulated 2-5A per U of enzyme varied approximately 20-fold in tissues from EMCV-infected mice.

The response of specific tissues to EMCV varied consid-

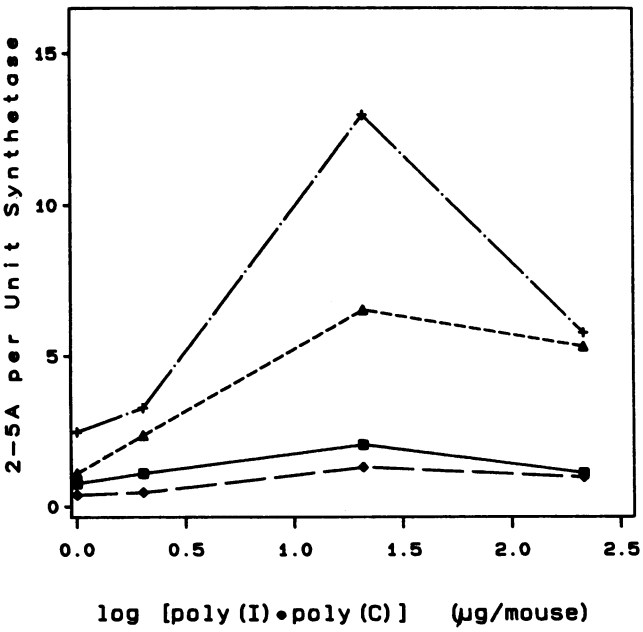

FIG. 3. Comparison of 2-5A concentrations to 2-5A synthetase levels in mice treated with poly(I) · poly(C). The ratio of the mean 2-5A content to the mean 2-5A synthetase level was calculated for liver (+), spleen (◆), kidney (▲), and brain (■) of pathogen-free NIH Swiss mice and is expressed as hour^{-1}.

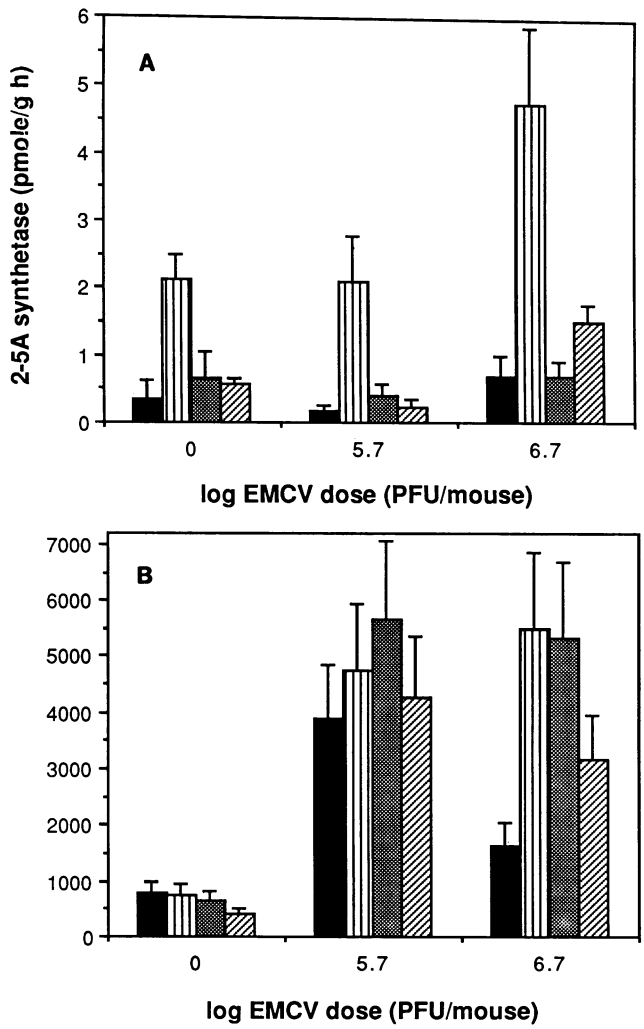

FIG. 4. Levels of 2-5A, 2-5A synthetase, and circulating interferon in mice infected with EMCV. Pathogen-free NIH Swiss mice were injected intravenously with 5×10^5 or 5×10^6 PFU of EMCV. Circulating interferon at 24 h was determined from a serum sample obtained immediately prior to sacrifice and was <10 U/ml for control mice, 250 U/ml for mice that received 5×10^5 PFU, and 650 U/ml for mice that received 5×10^6 PFU. Error bars depict one standard deviation from the mean of two to three determinations. After 24 h, mice were sacrificed, and liver (▬), spleen (▥), kidney (▩), and brain (▨) were examined for 2-5A synthetase (A) and 2-5A (B). Zero depicts the saline control.

erably from the results obtained with poly(I) · poly(C). The most apparent difference was in liver and kidney tissues, in which 2-5A levels increased significantly more with poly(I) · poly(C) treatment than with EMCV infection. With the highest dose of poly(I) · poly(C), the ratio of 2-5A levels was about 4:2:2:1 for kidney-liver-spleen-brain. In contrast, with EMCV (5×10^6 PFU), the ratio of extracted 2-5A was approximately 3:1:3:2. These differences could not be accounted for solely by differences in the levels of induced 2-5A synthetase. For example, with EMCV (5×10^6 PFU), the ratio of enzyme levels in kidney-liver-spleen-brain was about 1:1:7:2. Finally, there was consistently more total 2-5A accumulated per U of enzyme in the kidney tissues of both poly(I) · poly(C)- and EMCV-treated mice than in other tissues.

DISCUSSION

The 2-5A system is believed to contribute to an interferon-mediated antiviral state through degradation of RNA and inhibition of protein synthesis. However, the occurrence and role of 2-5A in uninfected cells remain to be clarified. The results presented here argue for a limited role for 2-5A in uninfected mouse tissue. We detected 400 to 800 fmol of 2-5A per g of a variety of tissues. These values correspond to subnanomolar levels of total 2-5A (approximately 0.4 to 0.8 nM). Further, only a small portion of accumulated 2-5A was recovered as biologically active pppA2′pA(2′pA)$_n$, which was detected at levels far below the concentration required to achieve measurable activation of RNase L in cell extracts (11, 15). Failure to recover higher levels of 5′-phosphorylated 2-5A was not due to a 5′-phosphatase active during the extraction procedure. Although it is possible that as yet undiscovered enzymes may be activated by these low levels of 2-5A, such activities have yet to be described. In separate experiments, higher total levels of 2-5A (100 to 3,000 fmol/g) were detected in mice that were not certified as pathogen free (Fig. 1 and data not shown).

Our results are in contrast to the substantially higher levels of 2-5A reported by Laurence et al. (22). Possible sources of discrepancy include the following. (i) One source may have been differences in the mice used. Laurence et al. used normal and germfree C3H/He mice, while most of the mice used here were pathogen-free NIH Swiss mice. Laurence et al. reported somewhat lower levels of 2-5A in germfree mice, but those levels (50 to 100 nM; 54 to 100% core) were still substantially higher than those reported here. (ii) While Laurence et al. used antibodies raised by immunization with conjugated 2-5A dimer, the antibodies used in our analysis probably did not detect 2-5A molecules with less than three adenylate residues. Cross-reactivity with dimers was 40- to 170-fold lower than that with trimer and longer forms (12, 13). Although 2-5A dimers are not biologically active, they may have been present as degradation products of larger 2-5A molecules. Based on the observations of Laurence et al. (22), we would predict that inclusion of dimers would increase our estimate by less than twofold. The results reported here are more in agreement with preliminary results reported by Brown and Kerr (3), who detected 30 to 100 fmol or more of pppA2′pA(2′pA)$_n$ per g in liver or kidney tissues, or both, of only 5 of 27 mice surveyed. Others had <30 fmol of biologically active 2-5A per g. Because we pooled tissues to provide sufficient material for replicative determinations, animal-to-animal variations were not analyzed. Although it is impossible to rule out the existence of locally high levels of 2-5A, 2-5A does not appear to be a major component of uninfected mouse tissue.

A high percentage of extracted 2-5A was found as non-5′-phosphorylated cores, particularly in tissues of mice treated with poly(I) · poly(C) or EMCV. This suggests that a 2′,5′-phosphodiesterase may not be the sole mechanism by which 2-5A is inactivated in vivo. Phosphatase may prove to play an important role in regulating the levels of active 2-5A, particularly when 2-5A accumulates to higher levels (>1 pmol/g) (Table 1 and results not shown). Furthermore, since pA2′pA2′pA has been reported to be an antagonist of 2-5A binding to endoribonuclease in some systems (35), phosphatase could conceivably augment or relieve the negative control of the 2-5A system exerted by pA2′pA2′pA. Indeed, 2-5A degradation products inhibitory to RNase L and containing free terminal phosphate(s) have recently been shown to be produced in extracts of interferon-treated HeLa cells incubated with reovirion cores or with poly(I) · poly(C) and in intact interferon-treated cells incubated with poly(I) · poly(C) (41).

Direct comparison of intracellular levels of 2-5A with the levels of 2-5A synthetase has not been reported previously. When either EMCV or poly(I) · poly(C) was injected into mice, tissue levels of 2-5A did not correlate directly with the levels of 2-5A synthetase. Perhaps most interesting was the observation that at the highest dose of poly(I) · poly(C) (200 μg) or EMCV (5×10^6 PFU), the ratio of accumulated 2-5A per U of enzyme activity appeared to be depressed (Fig. 3 and results not shown). Samanta and co-workers (25) demonstrated previously that stimulation of purified 2-5A synthetase activity decreases at dsRNA concentrations above optimal. The results presented here suggest that this observation may be applicable to an in vivo situation. The identity and intracellular concentration of the dsRNA activator of 2-5A synthetase in these studies, particularly in control tissues, remain to be investigated.

Each tissue displayed a unique response to treatment with poly(I) · poly(C) and EMCV infection. In mice injected with poly(I) · poly(C), liver and kidney tissues showed the greatest accumulation and percent increase in 2-5A at 24 h, even though the levels of synthetase in these tissues were lower than those in spleen and brain. This may have resulted from preferential uptake of the injected dsRNA by liver and kidney and subsequently higher levels of 2-5A synthetase activation in those tissues. A similar explanation may also be valid in the case of EMCV-infected mice. Although 2-5A levels were consistently high in spleen and kidney tissues, the greatest percent increase in 2-5A levels occurred in kidney and brain tissues of EMCV-infected mice. Whereas brain tissue had intermediate levels of synthetase and total 2-5A after injection of 20 μg of poly(I) · poly(C), the same tissue after injection of 5×10^5 PFU of EMCV had a higher level of 2-5A despite significantly lower enzyme levels. These differences in accumulated 2-5A per U of enzyme may have resulted from different locations or lower levels of intracellular dsRNA in brain tissues of poly(I) · poly(C)-treated mice compared with those of EMCV-injected mice. Alternatively, differences in 2′,5′-phosphodiesterase levels may have contributed to differences in 2-5A accumulation.

Another factor that may have slightly influenced the levels of 2-5A and 2-5A synthetase in various organs is the amount of blood present. Contamination by peripheral mononuclear cells was probably negligible given their small percentage in whole blood. However, if mouse plasma contains a level of 2-5A that is similar to that reported for human plasma (0.12 nM [26]), plasma contribution to the level of 2-5A in control

tissues would be only about 1 fmol of 2-5A per g for every 1% contamination of the tissue.

The observation that individual tissues respond differently with respect to induction of 2-5A synthetase and 2-5A accumulation clearly delineates the complexity of interpreting synthetase levels as being reflective of 2-5A accumulation. This may be true of circulating cells as well as of tissues. The relative importance of 2-5A synthetase, dsRNA, ATP, and 2-5A degradative enzymes in regulating the intracellular concentration of 2-5A remains to be established.

ACKNOWLEDGMENTS

The authors thank Robert Silverman and David Krause, Department of Pathology, USUHS, for radiolabeled 2-5A; Stephanie Vogel, Department of Microbiology, USUHS, for serum interferon determinations; and William Ruyechan for helpful comments on the manuscript.

This work was supported by a Public Health Service grant from the National Institutes of Health (AI20162) and grants from the National Science Foundation (PCM 8309051) and USUHS (C07146) to M.I.J.

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