Comparative Medicine
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Vol 52, No 4
August 2002
Pages 359-362

Nucleic Acid Deletions and Copy Number in Rats

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Rats fed either a cereal-based or purified diet of variable folate content (deficient, replete, or supplemented) inadvertently were infected with sialodacryoadenitis virus, which resulted in an increased frequency of hepatic mitochondrial DNA (mtDNA) deletions that persisted for three weeks after the period of acute signs of disease. The amount of the "common deletion" (4.8 kb, bases 8103–12937) in liver was measured by quantitative co-amplification of the mitochondrial D-loop and the mitochondrial deletion, using a real-time quantitative polymerase chain reaction assay. The relative abundance of mtDNA was determined by co-amplifying mitochondrial D-loop versus the rat β-actin gene. Virus-infected rats had more mtDNA deletions ($P < 0.0001$) and higher copy number ($P < 0.0001$) than did uninfected animals. There was no effect of diet on frequency of deletions. Diet affected mtDNA relative abundance in the infected, but not the uninfected rats. Relative abundance was higher ($P = 0.004$) in rats of the high folate group than in rats of the low-folate or folate-replete groups, and was significantly higher in rats of the cereal diet group than that in those of the purified diet group. In conclusion, sialodacryoadenitis virus infection in rats was associated with increased frequency of hepatic mtDNA deletions. Thus, sialodacryoadenitis virus infection mitigated biological processes in the liver of rats, and mtDNA damage was modulated by diet.

Because of reports of possible involvement of mitochondrial alterations associated with carcinogenesis (1-3) and the importance of folate metabolism to the mitochondria (4, 5), we were interested in investigating the relationship of diet, and particularly, nutritional folate status to mitochondrial DNA (mtDNA) damage after cancer chemotherapy in rats (6). During the course of these experiments, some of the rats inadvertently were infected with sialodacryoadenitis virus (SDAV). The SDAV is a member of the coronavirus group and is antigenically related to mouse hepatitis virus (MHV) (7). The lesions typically associated with infection are found in the salivary and lacrimal glands, but abnormalities of the distal portion of the respiratory tract also have been described (8). Viral infection can lead to reproductive disorders in female rats and central nervous system lesions in suckling mice (9, 10). The objective of the study reported here was to evaluate the effect of SDAV infection on rat hepatic mtDNA and whether diet could modulate any damage caused by the infection.

Materials and Methods

Animals. The research protocol was approved by the Institutional Animal Care and Use Committee of the University of Vermont. Animals were cared for and used humanely in accordance with the AALAS "Policy on the Humane Care and Use of Laboratory Animals." Female Fischer 344 rats, weighing approximately 60 g, were obtained from Charles River Canada (St.-Constant, Quebec). At purchase, the animals were virus antibody free. Rats were housed in an AAALAC-approved animal facility, were maintained in groups of three or four for 10 days, and were fed a cereal-based rat chow (Harlan Teklad LM-485; Harlan Teklad, Madison, Wis.). Rats were housed individually in polycarbonate cages, 19 × 10 × 7 in., with wire tops. The contact bedding was sanichips (wood shavings), which was changed weekly throughout the trial. Cages were transported directly to the cagewash area and were immediately disinfected. The photoperiod was 12 h light and 12 h dark. Mean ± SD room temperature was 21° ± 2°C, with 30 to 70% humidity.

Diets. The cereal-based diet consisted of 19.92% protein, 5.67% fat, 4.37% fiber, and 4.05 Kcal of gross energy/g. Its principal ingredients included ground corn, soybean meal, ground oats, wheat middlings, and alfalfa meal, and it did not contain animal protein. The folic acid content was 8.21 mg/kg, and the vitamin B_{12} content was 30 μg/kg. One group continued to consume the cereal-based diet, while the others were fed the AIN-93G diet (Dyets, Inc., Bethlehem, Pa.). This purified diet is based on vitamin-free casein and cornstarch. The folate-replete diet consisted of AIN-93G with a vitamin supplement that provided 2 mg of folic acid/kg and 25 μg of vitamin B_{12}/kg of diet. The folate-deficient diet consisted of AIN-93G with a vitamin mixture containing folic acid. The rats receiving the high folate diet were fed AIN-93G with vitamin supplement, and folic acid (50 mg/kg of body weight, dissolved in 8.4% sodium bicarbonate solution) was administered intraperitoneally daily. Liver specimens were collected for subsequent analyses after anesthesia of the rats with pentobarbital sodium (60 mg/kg, i.p.), followed by cardiac puncture and exsanguination. The liver tissue was stored at -80°C until processing. Total hepatic DNA content was isolated, using the Qiagen DNeasy Tissue kit (Valencia, Calif.).

Microbiological tests. The routine health monitoring pro-

Received: 3/20/02. Revision requested: 4/17/02. Accepted: 5/17/02.
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Supported by grants from the Department of Defense (DAMD17-98-1-8345) and the National Cancer Institute, Bethesda, Maryland (CA 41843). The content of the information does not necessarily reflect the position or policy of the United States government, and no official endorsement should be inferred. Automated sequencing was performed in the Vermont Cancer Center DNA Analysis Facility and was supported in part by NCI P30 CA 22435.
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gram included serologic testing of colony or sentinal animals in all rooms twice a year. Using an ELISA, blood was tested for the following agents: Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus/rat coronavirus (SDAV/RCV), Kilham rat virus, Toolan's H-1 parvovirus, reovirus 3, *Mycoplasma pulmonis*, and rat parvovirus NS1. Additionally, examination for endo- and ectoparasites was done monthly. Necropsy and tissue collection/histologic examination were done on random animals throughout the year, but at a minimum, on the sentinel animals twice a year.

Viral infection with SDAV in the rat colony was diagnosed by use of an RCV ELISA, and abnormalities and results were confirmed by use of an indirect fluorescent antibody SDAV/RCV test (Charles River Laboratories, Wilmington, Mass.). Follow-up testing of the rats was performed, using a solid-phase immunoassay for mouse/rat antibodies to *Mycoplasma pulmonis*, rodent coronaviruses, and Sendai virus (Murine ImmunoComb, Biogal Galed Labs, Galed, Israel). Testing of liver specimens three weeks after clinical infection by use of reverse transcriptase-polymerase chain reaction (RT-PCR; Charles River Laboratories, Wilmington, Mass.) yielded negative results for MHV (coronavirus) nucleic acid.

Oligonucleotide primers and TaqMan probe design. Primers and probes for the rat D-loop, rat mitochondrial deletion, and rat β-actin were designed, using Primer Express software (PE, Foster City, Calif.) with a rat mitochondrial genome from GenBank (accession No. X14848). Primers and probes were synthesized and high-performance liquid chromatography purified by the Oligo Factory (Foster City, Calif.), then primer-limiting experiments were performed to determine the proper primer concentrations. Sequence for the primers and probes has been reported (6).

Real-time PCR for mitochondrial deletion. These methods have been described (6). Mitochondrial deletion expression was quantified by use of a 5'VIC reporter and a 3'TAMRA quencher dye, and D-loop expression was determined, using a 5'6-FAM reporter and a 3'TAMRA-labeled quencher dye. Polymerase chain reaction amplification was carried out in a 50-μl reaction consisting of 1X TaqMan Universal Master Mix (Perkin-Elmer Applied Biosystems, Foster City, Calif.), 200 n*M* each mitochondrial deletion forward and reverse primers, 100 n*M* each D-loop forward and reverse primers, and 100 n*M* each mitochondrial deletion and D-loop probe. Cycling conditions included an initial phase of 2 min at 50°C, followed by 10 min at 95°C, 40 cycles of 15 sec at 95°C, and 1 min at 60°C. Each sample was assayed in duplicate, and fluorescence spectra were continuously monitored by use of the 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, Calif.) with sequence detection software version 1.6.3.

Real-time PCR for mitochondrial D-loop. The D-loop expression was quantified by use of the aforementioned probe, and b-actin expression was quantified, using a 5'VIC reporter and a 3'TAMRA-labeled quencher dye. Polymerase chain reaction amplification was carried out in a 50-μl reaction consisting of 1X TaqMan Universal Master Mix (Perkin-Elmer Applied Biosystems), 200 n*M* each β-actin forward and reverse primers, 50 n*M* each D-loop forward and reverse primers, and 100 n*M* each β-actin and D-loop probe. Cycling conditions included an initial phase of 2 min at 50°C, followed by 10 min at 95°C, 40 cycles of 15 sec at 95°C, and 1 min at 60°C.

Determination of the white blood count and serum biochemical analytes. White blood count was measured, using a Coulter Counter (Model ZBI) according to the manufacturer's instructions. Analyses of blood urea nitrogen (BUN) concentration and lactic dehydrogenase (LDH), serum alanine transaminase (SALT), and creatine kinase (CK) activities were performed, using Sigma Diagnostics (St. Louis, Mo.) procedure Nos. 66-UV, 500, 505, and 661, respectively.

Statistical analyses. In this system, a smaller ΔC_T (mitochondrial deletion [C_{Tdel}] - mitochondrial D-loop [$C_{T\ D\text{-}loop}$]) value indicated more deletions, whereas a smaller ΔC_T (mitochondrial D-loop [$C_{T\ D\text{-}loop}$] - β-actin [$C_{T\ ,\text{-}âctin}$]) value indicated less total mtDNA. Relative expression was calculated, using the equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T$ equals $\Delta C_{TCopyNumber} - \Delta C_{TCalibrator}$, where the calibrator was the ΔC_T of no drug in the folate-replete purified diet. Relative expression indicated the fold difference in deletions or copy number, compared with that of the folate-replete purified diet.

Analysis of variance was used to test the significance of differences in mtDNA deletions and copy number. Student's *t* test was used to test the significance of differences between white blood count and biochemical values in virus-infected and uninfected rats.

Results and Discussion

Rats were fed a cereal-based diet or on a purified diet of variable folate content. The SDAV infection was diagnosed clinically by one of the authors (SAM). A caretaker reported unusual red-tinged tears and bulging eyes. On examination, conjunctivitis and enlarged salivary glands were observed. The room was "quarantined" on first clinical identification of the SDAV. Previously, neither SDAV nor any other virus had ever been identified in rats used in our experiments during routine health monitoring. After identification of the virus, other animals were not allowed to enter or leave the room. Signage was placed on the doors of infected rooms, and personnel were required to wear caps, masks, disposable gowns, gloves, and booties when in the room, and all apparel was disposed of in the room. Despite these quarantine measures, rats in all rooms became infected. Every animal fed every diet involved in this experiment became infected at five weeks, and antibody testing results were positive for SDAV. There were no deaths among the infected animals.

The liver of all animals was collected three weeks later, when all had clinically recovered from the infection. Liver specimens from uninfected rats were obtained from those fed the same diets for the same duration in a previous experiment (6). Since the liver tissues used as controls for this experiment were collected approximately three months prior to this SDAV outbreak, it is highly unlikely that the "control" animals were similarly infected. At the time the liver was collected, measurements were performed—hematocrit, white blood cell count, BUN concentration, and LDH, SALT, and CK activities. There were no significant differences between virus-infected and uninfected rats of any of the dietary groups for hematocrit, LDH, or SALT values (data not shown). The BUN concentration of virus-infected rats was consistently and significantly ($P < 0.05$) higher than that of uninfected rats of all dietary groups, but the difference was small (1.16 ± 0.04 mg/dl versus 0.97 ± 0.05 mg/dl [mean ± SEM]). There were no significant differences in white blood count except in the cereal diet-fed animals (5,199 ± 1,582

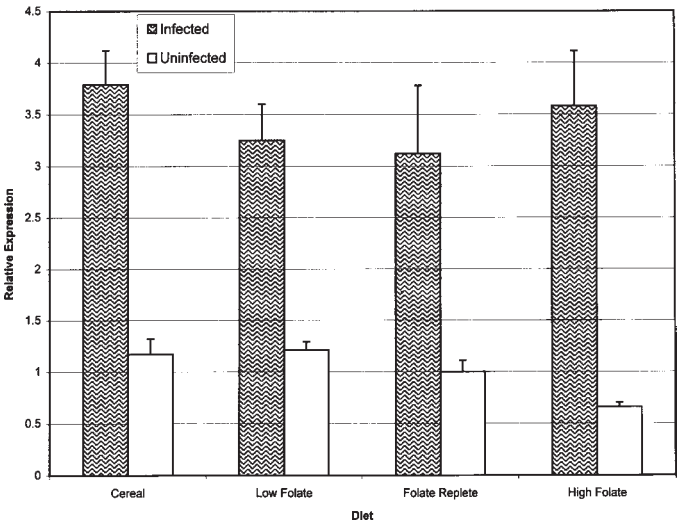

Figure 1. Mitochondrial DNA deletions in the liver from uninfected rats and rats infected with sialodacryoadenitis virus and fed a cereal-based diet or a purified diet of variable folate content. The number of deletions is expressed relative to that of uninfected rats fed the folate-replete diet. Virus-infected rats had more deletions ($P < 0.0001$), but there was no dietary effect. Error bars represent SEM; 6 rats/group.

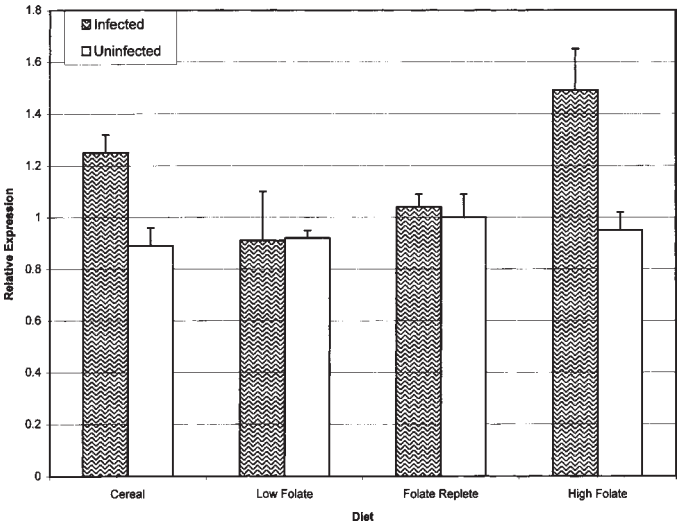

Figure 2. Mitochondrial DNA copy number in the liver from uninfected rats and rats infected with sialodacryoadenitis virus and fed a cereal-based diet or a purified diet of variable folate content. The copy number is expressed relative to that of uninfected rats fed the folate-replete diet. Infected rats had a higher relative copy number than did uninfected rats of the cereal and high-folate diet groups ($P < 0.0004$), but there was no significant difference in rats fed the low-folate and folate-replete diets. *See* Fig. 1 for key.

cells/mm^3 for infected versus 3,267 ± 1423 cells/mm^3 for infected; $P = 0.05$). The CK values were slightly but significantly ($P = 0.02$) higher in the uninfected (0.22 ± 0.01 U/ml) than in the virus-infected (0.20 ± 0.01 U/ml) rats of the folate-replete group. There was no other significant difference in CK values for rats of the other dietary groups.

The amount of the "common deletion" (4.8 kb, bases 8103–12937) was measured by quantitative co-amplification of the mitochondrial D-loop and the mitochondrial deletion, using a real-time RT-PCR assay with the specific primers reported previously (6). The amount of mtDNA (abundance) relative to genomic DNA was determined by co-amplifying mitochondrial D-loop versus rat β-actin gene. This method allowed not only determination of changes in the amount of mitochondrial deletion relative to total mtDNA after viral infection in rats fed different diets, but also measurement of changes in total mtDNA relative to genomic DNA.

The relative expressions for mitochondrial deletions and relative abundance in liver from SDAV-infected and uninfected rats are shown in Fig. 1 and 2. Overall, virus-infected rats had significantly more deletions ($P < 0.0001$) and a higher relative mitochondrial copy number ($P < 0.0001$) than did uninfected rats. Two-way analysis of variance indicated significant statistical interaction between group and diet factors ($P = 0.023$). Deletions were increased in virus-infected animals by more than twofold, but there was no dietary effect. There was no effect of diet on mtDNA relative abundance in the uninfected animals. Relative copy number was significantly higher in the infected, compared with uninfected animals, fed the cereal and high-folate diets ($P < 0.0004$), but there was no difference in relative copy number among rats fed the low-folate and folate-replete diets. The paired comparisons indicated that, in rats of the infected group, relative abundance was significantly ($P = 0.004$) higher in those of the high-folate group than in those of the low-folate or folate-replete groups, and relative copy number was significantly higher in rats of the cereal diet group than in those of the folate-replete group. Since the cereal diet and the folate-replete purified diet had approximately the same folic acid content, the latter effect was due to some other constituent of the cereal diet.

These results indicated that clinically mild viral infection with SDAV can cause a marked increase in mtDNA deletions in rats. Mitochondrial defects, particularly deletions, accumulate in senescent tissues and have been implicated in chronic progressive external ophthalmoplegia; Kearns-Sayre syndrome, and its associated condition, Pearson's syndrome; ischemic heart disease; cirrhotic liver; and several types of cancer, including hepatocellular carcinoma (1-3). The "common deletion," which removes the DNA between the *ATPase 8* and *ND5* genes (*ATPase 6* and *ND5* in rats) and is flanked by a 13-bp direct repeat (16 bp in rats) (11, 12) is often used as a specific indicator of general damage (13). Experiments in adult and senescent rats confirmed that the "common deletion" accumulates during aging (14). Our study supports the notion that chronic viral infection may contribute to the accumulation of hepatic mtDNA damage.

This study also indicated that SDAV infection increased mtDNA abundance under certain dietary conditions. In rats fed a purified diet, folic acid supplementation significantly increased mtDNA abundance, compared with that associated with lower folate content diets, whereas a cereal-based diet increased mtDNA abundance, compared with a purified diet with approximately the same folate content. Therefore, more than one dietary constituent can influence mtDNA abundance after viral infection.

The factors that control mtDNA copy number are largely unknown (15). Generally mtDNA values are linked to organelle number, and mammalian cells tend to maintain a constant mass of mtDNA rather than a constant number of mitochondrial genomes (15). Of possible relevance to the study reported here, it has been suggested that control of the total mitochondrial mass

is regulated by the size of the organellar nucleoside pools (16). Folic acid metabolism is critical to maintenance of nucleotide pools (5). In addition, rats fed purified diets are reported to manifest imbalances in intracellular nucleotide pools, compared with animals fed natural ingredient diets (17). Mitochondrial DNA depletion in humans has been found in patients with acquired immune deficiency syndrome and treated long term with nucleoside analogs and in patients with mutations in the thymidine phosphorylase gene (15). Therefore, the effects of diet on mtDNA abundance in the virus-infected rats reported here may be mediated by changes in nucleotide pools.

An alternative mechanism by which folate may modulate mtDNA damage and number caused by viral infections is by decreasing oxidative stress. The tissues of senescent rats have increased oxidative damage and increased mtDNA copy number, compared with their adult counterparts (13). It has been proposed that, during the aging process, cells tend to compensate for decreased functionality of mtDNA transcripts by generating additional copies of mtDNA (18). An increase in mtDNA copy number in lung fibroblasts is an early event in response to oxidative stress (19). Folic acid recently was reported to have free radical scavenging behavior, with a resulting potential to reduce oxidative stress (20). In addition, Lim and colleagues (21) reported positive correlation between blood mtDNA content and serum folate concentration in healthy women. They postulated that increased folate concentration decreases plasma homocysteine concentration, which is a known cause of oxidative stress (21). It seems plausible that the observed increase in mtDNA abundance in rats of our experiments is a compensatory mechanism in the virus-infected animals that manifest frequent mtDNA damage as a result of oxidative stress. These observations suggest that dietary manipulations such as folic acid supplementation may promote this compensation and perhaps offset some of the deleterious effects of mtDNA damage.

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