1 of 1

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Rearrangement of RAG-1 Recombinase Gene in Radiation-Sensitive "Wasted" Mice

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Running Title: RAG-1 rearrangement in wasted mice

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Abstract—Mice recessive for the autosomal gene "wasted" (wst) display a disease pattern which includes increased sensitivity to the killing effects of ionizing radiation, immunodeficiency, and neurologic dysfunction. The recent cloning and characterization of recombinase genes (RAG-1/RAG-2) expressed in lymphoid and possibly central nervous system tissues prompted us to examine expression of these genes in DNA repair-deficient/immunodeficient wasted mice. Our results revealed expression of RAG-1 mRNA in spinal cord (but not brain) of control mice; no expression of RAG-1 mRNA was detected in spinal cord or brain from wst/wst mice or their normal littermates (wst/• mice). In thymus tissue, a small RAG-1 transcript (1.0 kb) was detected in wst/wst mice that was not evident in thymus from control mice. In wst/• mice, a two-fold increase in RAG-1 mRNA was evident in thymus tissue. RAG-2 mRNA could only be detected in thymus tissue from wst/• and not from wst/wst or parental control BCF₁ mice. Southern blots revealed a rearrangement/deletion within the RAG-1 gene of affected wasted mice, not evident in known strain-specific parental or littermate controls. These results support the idea that the RAG-1 gene may map at or near the locus for the wasted mutation. In addition, they suggest the importance of recombinase function in normal immune and central nervous system development as well as the potential contribution of this gene family to the normal repair of radiation-induced DNA damage.

INTRODUCTION

Initial reports describing the wasted mouse mutation defined an autosomal recessive defect that resulted in premature death (by 28 days of age), neurologic dysfunction, and increased sensitivity of lymphocytes to damage by ionizing radiation as measured by chromosome breaks (Schultz *et al.* 1982). This strain was considered a model for the human disease ataxia telangiectasia, although later reports documented that not all features of the mouse disorder were similar to those found in the human disease (Goldwitz *et al.* 1985; Inoue *et al.* 1986b; Woloschak *et al.* 1987). The neurologic dysfunction was found to involve a degenerative loss of anterior motor neurons of the cerebellar cortex and spinal cord (Woloschak *et al.* 1987; Lutsep and Rodriguez 1989). The DNA repair abnormality involved both an increased spontaneous incidence of chromosomal damage in bone marrow cells (Schultz *et al.* 1982) and increased sensitivity of lymphocytes to the killing effects of ionizing (but not ultraviolet) radiation (Padilla *et al.* 1990; Inoue *et al.* 1986a; Nordeen *et al.* 1984; Tezuka *et al.* 1986). Numerous reports have documented immunodeficiency in wasted mice, particularly deficiency in IgA expression and immune responses at mucosal sites (Woloschak *et al.* 1987, 1988; Kaiserlian *et al.* 1985, 1986). The mutation has been mapped to chromosome 2 of the mouse through breeding experiments (Beechey and Searle 1982), but the affected genes have not been identified.

In recent work, Oettinger *et al.* (1990) identified RAG-1 and RAG-2 recombinases that synergistically activate V(D)J recombination in both B- and T-lymphocytes. Chun *et al.* (1991) determined that the RAG-1 gene is expressed in both lymphoid and central nervous

system (CNS) tissue. The precise functions of these genes are still undetermined, although their roles both in gene recombination and in gene conversion have been suggested (Oettinger *et al.* 1990; Chun *et al.* 1991; Carlson *et al.* 1991). In previous work, we hypothesized that the entire disease syndrome of wasted mice could be explained by the absence of a recombinase function required for (a) double-stranded DNA repair, (b) lymphocyte immunoglobulin and T-cell receptor rearrangement, and (c) rearrangement of an as-yet-unidentified gene in the CNS (Woloschak *et al.* 1987; Padilla *et al.* 1990). The identification of RAG-1 as a lymphoid- and CNS-specific recombinase suggested that this gene might be involved in the disease pattern of wasted mice.

MATERIALS AND METHODS

Mice

All experiments reported here used wst/wst mice bred from wst/+ breeders obtained from the Jackson Laboratory (Bar Harbor, ME). All wst/wst mice expressed neurologic symptoms (whole body tremors, failure to gain weight, inability to navigate an inclined plane) at 21-22 days of age and were sacrificed at days 25-28 of age. Littermate controls (noted as wst/+ in this manuscript) and BCF₁ parental controls were age- and sex-matched for all experiments reported here. The "wasted" mutation arose in HRS/J mice but has been bred into the BCF₁ background. Known wst/+ mice (which can only be identified by breeding and therefore cannot be age-matched with wst/wst mice) were used for RAG-1 DNA Southern blot experiments.

Hybridizations

Northern blots and Southern blots were done as outlined in the figure legends and as previously described (Woloschak *et al.* 1986, 1990). RNA was separated by using formaldehyde agarose gel electrophoresis as described previously (Woloschak *et al.* 1986). In brief, RNA samples (10 $\mu\text{g}/\text{sample}$) were denatured in 50% formamide, 1.9 M formaldehyde, 0.2 M MOPS, 50 mM sodium acetate, 1 mM Na_2EDTA , pH 7.5, for 15 min at 55 °C, then separated on 1.2% agarose gels in 0.2 MOPS, 50 mM sodium acetate, 1 mM Na_2EDTA , 2.2 M formaldehyde. Northern transfers were performed as described (Woloschak *et al.* 1986). Blots were hybridized to ^{32}P -labeled cDNA probes. Hybridization conditions were 50% deionized formamide, 0.75 M NaCl, 75 mM sodium citrate, 25-50 mM sodium phosphate, pH 6.5, 0.2% SDS, 0.2% bovine serum albumin, 0.2% ficoll, 0.2% polyvinylpyrrolidone, and 50 $\mu\text{g}/\text{mL}$ sonicated denatured herring sperm DNA at 43 °C. Prior to hybridization, all labeled probes were heat-denatured at 90 °C for 5 min. After hybridization, nonspecific binding was reduced by washing the hybridized blots 3 times for 1 h each in 1.5 mM sodium citrate (pH 7.4), 15 mM NaCl, 50 $\mu\text{g}/\text{mL}$ herring sperm DNA (sonicated, denatured), and 0.1% SDS. The blots were then dried and exposed to x-ray film at -20 °C. In some experiments, the same blot was washed and hybridized successively to several different probes. Each probe was eluted by washing for 24 h in distilled deionized water at 43 °C, and blots were checked (for total removal of the labeled probes) by 24-h exposure to x-ray film.

Southern blots were performed as described (Woloschak *et al.* 1986). In brief, DNA was purified from these cells by extraction in Tris-buffered phenol, precipitation from ethanol, and digestion with 10 $\mu\text{g}/\text{ml}$ pancreatic ribonuclease followed by digestion with 50 $\mu\text{g}/\text{ml}$

proteinase K. Liver-derived DNA from wst/wst mice, wst/ mice, and BCF₁, BALB/C and C57BL/6 strain-specific controls was digested with restriction enzyme following manufacturer's specifications and subjected to 1.2% agarose gel electrophoresis in E buffer (40 mM Tris-acetate, 2 mM Na₂EDTA, pH 7.7). In all gels, standard molecular weight markers (HindIII digest of phage λ) were run and DNA was visualized by staining in ethidium bromide. After depurination for 10 min in 0.25 M HCl, DNA was denatured in the gel by incubating for 30 min at room temperature in 0.6 M NaOH, 1.5 M NaCl followed by 30 min in 1.0 M Tris-HCl (pH 8.0), 1.5 M NaCl at room temperature. DNA from the gel was then transferred to an MSI neutral nylon membrane in 10 $\times$ SSC (150 mM sodium citrate, pH 7.4, 1.5 M NaCl) for 18 h at room temperature. Blot-bound DNA was hybridized to the heat-denatured ³²P-labeled RAG-1 DNA probe in Southern hybridization buffer (2.5 μ g/ml poly(A), 50 μ g/ml herring sperm DNA, 0.1% SDS, 0.2% ficoll, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 6 $\times$ SSC) at 65 °C for 18 h. The blot was then washed 3 times at 65 °C for 1 h each in 0.5 $\times$ SSC. Blots were autoradiographed at -70 °C to detect gene-specific sequences.

cDNA clones

We gratefully acknowledge Dr. D. Baltimore's (The Rockefeller University) generous gift of RAG-1 (M6) and RAG-2 (MR2-1) DNA, Dr. David McKean (Mayo Clinic, Rochester, MN) who provided murine IL2 cDNA, Dr. C. Veneziale (Mayo Clinic, Rochester, MN) who provided α -tubulin cDNA.

RESULTS AND DISCUSSION

Experiments were designed to examine RAG-1 and RAG-2 expression in lymphoid and CNS tissues of wasted mice as well as in tissues from littermate and parental controls. Figure 1, Northern blots for RAG-1 and RAG-2 mRNA expression, documents that thymus tissue of wst/wst mice expressed a small RAG-1 transcript (1Kb) while age-matched parental and littermate controls expressed significant amounts of the normal sized mRNA in thymus. In addition, wst/ mice expressed at least two-fold more RAG-1 mRNA than parental controls (BCF₁ mice) or affected wst/wst littermates. Low levels of RAG-1 mRNA were found in spleen of BCF₁ and wst/ mice, but RAG-1 transcripts were undetected in spleen from wst/wst mice (Fig.1). In addition, RAG-1 mRNA was not detected in brain of any mouse strains examined (BCF₁, wst/ or wst/wst), but was evident in high abundance in spinal cord of BCF₁ mice, but not in spinal cord from wst/ or wst/wst mice (Fig. 1). RAG-2 mRNA was not detected in spinal cord or brain of any mouse strains examined (not shown), but was evident in thymus of wst/ mice (Fig. 1). Re-hybridization of these blots to IL2 (for thymus and spleen) and α -tubulin (for brain and spinal cord) revealed equal amounts of intact mRNA on each blot (not shown). Similarly, expression of RAG-2 mRNA was not detected in thymus tissue derived from wst/wst mice, but was expressed at elevated levels in wst/ thymus (Fig. 1D). Thymus from 28-day old wasted mice is involuted and has fewer cells than age-matched controls (Kaiserlian *et al.* 1985, 1986; Woloschak 1988). Northern blots were based on μ g quantities of RNA loaded rather than tissue weight.

Southern blots were performed to determine the status of the RAG-1 gene in wst/wst mice relative to controls. Figure 2 presents the results of such a Southern blot done with the

RAG-1 clone using liver DNA derived from wst/wst mice, wst/· littermates, and BCF₁ controls as well as BALB/c and C57BL/6 strain controls. In each digest of liver DNA, novel or altered bands evident in wst/wst DNA but not in controls are marked with arrows. Bands evident in controls were also detected in DNA from wst/wst mice, suggesting that wasted mice do not have a total deletion of the RAG-1 gene. The sizes of RAG-1 fragments in control mice for all digests were similar to those reported previously (Oettinger 1990). It is also interesting that the wst/· mouse used here expresses some of the abnormal bands evident in the wst/wst affected littermates, suggesting a heterozygosity in this wst/· mouse at this locus. In contrast to RAG-1, hybridization to RAG-2 clone, β-actin clone (generously provided by Dr. Larry Kedes, Stanford Univ.), and chromosome 2-specific c-ets clone (provided by American Type Culture Collection) revealed identical patterns of hybridization among wst/wst, wst/·, and BCF₁ DNA samples (not shown).

The results of a RAG-1 gene alteration in wst/wst mice suggest that the RAG-1 gene may map at or near the wst locus. While we and others have reported low expression of several genes in wasted mice relative to controls (Woloschak 1987; Inoue 1986), no reports have established an associated alteration in any gene at the DNA level. Because the RAG-1 gene shows such an alteration, we suspect that the primary genetic defect of wasted mice may be associated with the RAG-1 deficiency. RAG-2 gene appears to be intact based on Southern-blot analysis; however, we have failed to detect expression which may be the result of rearrangement into a non-expressed area of the genome. Experiments are underway to test this.

Several reports have suggested that scid mice are defective in RAG-1 expression (Oettinger *et al.* 1990; Chun *et al.* 1991; Carlson 1991). The total immunoglobulin/T-cell receptor immunodeficiency of scid mice (which express faulty V-D-J joining) is quite distinct from the abnormalities of wasted mice. The same genes may be involved in both defects, but with a combination of some other contributing genetic lesion resulting in the scid manifestation of altered immune disorders. On the other hand, the genetic lesion in scid mice may not involve the recombinase per se, but rather a protein (working in association with the recombinase) that is required for immunoglobulin and T-cell receptor gene rearrangement but is not involved in CNS function. The similarities between the wasted syndrome and ataxia telangiectasia in humans will provide for interesting future analyses of RAG-1 involvement in the human disorder.

For the wst/⁺ mice used in these experiments, we have demonstrated elevated RAG-1 and RAG-2 expression in the thymus, perhaps the result of over-compensation by the one copy of the normal gene for the production of an aberrant RAG-1 transcript from the altered allele. This is further supported by the faint hint of an altered transcript (1.0 kb) in wst/⁺ thymus (Fig. 1A) and the presence of some altered RAG-1 DNA fragments in wst/⁺ mice on Southern blot (Fig. 2).

The demonstration that mice expressing the wasted phenotype (i.e., sensitivity to ionizing radiation, mucosal immunodeficiency, and neurologic dysfunction) express abnormal RAG-1 transcript suggests an important role for this gene in normal development, since wst/wst mice do not live beyond 32 days of age (1). The radiation sensitivity profiles of wasted mice implicate RAG-1 as a mammalian DNA repair enzyme required for complete

repair of damage induced by ionizing radiation (double-strand breaks?), since failure to express the gene in this mouse system results in an extreme sensitivity to the killing effects of gamma rays. The fact that other investigators have shown variable results in expression of RAG-1 mRNA in CNS tissue (Oettinger *et al.* 1990; Chun *et al.* 1991) could be attributed to the fact that expression is specific for spinal cord; the amount of contamination of brain tissue with spinal cord could vary from one preparation to the other. The importance of RAG-1 expression in spinal cord and for CNS function suggests the presence of some as-yet-unidentified gene recombination event which is essential for maintenance and function of anterior motor neuron cells in the mouse cerebellar cortex and spinal cord. It is interesting that anterior motor neuron cells have been reported to be the most radiation-sensitive cells in mouse CNS tissues (Moss and Lewkowitz 1985).

The immunodeficiency of wasted mice is specifically directed toward defective IgA-mediated mucosal immunity, potentially relating RAG-1 function with IgA responses. Several reports have suggested that IgA responses require multiple rounds of DNA synthesis and especially frequent recombination events in mice (Woloschak and Tomasi 1983; Gearhart and Cebra 1991). It has been hypothesized in the gut that the high antigen concentration is required to drive this IgA expression (Gearhart and Cebra 1982). Defective IgA expression in wasted mice could be associated with a failure of normal recombination in gut-associated mucosal lymphocytes needed to drive an IgA response. This would explain the IgA deficiency at mucosal sites, a prominent feature of the wasted mouse immunodeficiency. On the other hand, a recent report by George and Cebra (1991) has shown that a single B-cell can be driven to IgA expression in the absence of cell division, showing that at least some

IgA-producing cells do not require prior cell division. Studies of the wasted mouse model may further resolve this issue.

Finally, several recent reports (Mombaerts *et al.* 1992; Shinkai *et al.* 1992) have analyzed mice genetically altered at either the RAG-1 (Mombaerts *et al.* 1992) or the RAG-2 (Shinkai *et al.* 1992) locus. These mice share some, but not all, features with wasted mice, especially the CNS abnormality which is not evident in either RAG-1 or RAG-2 mutated mice. We believe these differences could be attributed to several possible explanations: (1) wst/wst mice may produce some functional RAG-1 product, even though the gene is altered. (2) wst/wst mice may express a rearrangement of RAG-1 gene such that another gene (into which it is rearranged) is altered. (3) wst/wst mice show abnormalities in expression of RAG-1 and RAG-2, while the genetically engineering mutant mice have only one or the other gene affected. Further experiments will be required to examine these possibilities.

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FIGURE LEGENDS

Figure 1. Northern blot hybridization of (A) thymus poly(A)+ RNA to RAG-SV1, (B) spleen poly(A)+ RNA to RAG-1, (C) CNA derived poly(A)+ RNA to RAG-1, (D) thymus poly(A)+ RNA to RAG-2.

Figure 2. Southern blot of liver-derived DNA to RAG-1 clone. Liver DNA from wst/wst (w/w), wst/· (w/·), BCF₁, BALB/C and C57BL/6 mice was digested with the indicated enzyme prior to electrophoresis, blotting and hybridization to RAG-1 cDNA clone.

Rag-1
Thymus

Fig 1A

BCF1

wst/•

wst/wst

Rag-1
Spleen

Fig 1 B

Fig 1C

BCF1

wst/•

wst/wst

Rag-2
Thymus

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Fig 1D

Fig 2

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