FEB 12 1991

**Evidence of T-cell Abnormalities
in Immunodeficient Wasted Mice¹**

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Running Title: CD4⁺/CD8⁺ T-cell Abnormalities in Wasted Mice

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¹Work supported in part by the U. S. Department of Energy, Office of Health and Environmental Research, under contract No. W-31-109-ENG-38.

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ABBREVIATIONS

wst/wst - mice bearing the autosomal recessive mutation wasted

wst/• - unaffected littermates of wasted mice

PFC - plaque-forming cells

MLN - mesenteric lymph nodes

SRBC - sheep erythrocytes

ABSTRACT

Wasted mice bear an autosomal recessive mutation (wst/wst) that manifests itself in neurologic abnormalities, immunologic deficiency, and faulty DNA repair evident by 21 days of age. The immunodeficiency is characterized by a reduction in the thymus-to-body weight ratio, low levels of IgA plasma cells at secretory sites, and abnormal repair of radiation-induced damage in T-cells. Experiments were designed to examine measures of T-cell activity in wasted mice. Initial experiments established that wst/wst mice have percentages of thymic and splenic Thyl⁺ cells equivalent to those of control littermates. Further studies of T-cell subpopulations revealed high percentages of CD4⁺ cells (but normal percentages of CD8⁺ cells) in wst/wst spleen cells (55% CD4⁺ cells) relative to controls (31% CD4⁺ cells). Similar studies with thymocytes have shown normal percentages of CD4⁺ and CD8⁺ cells in wst/wst mice; however, double-labeling experiments showed that CD8⁺ cells were predominantly CD4⁻ in wst/wst mice, while in controls most CD8⁺ cells also expressed CD4⁺. Mesenteric lymph node (MLN) T-cell subpopulations were similar in wasted and control mice. Cell volume studies revealed different volume distribution patterns for T-cell subpopulations for wasted mice relative to controls. Further experiments examining DNA content per cell demonstrated fewer cells in S/G₂+M stages of the cell cycle in wasted mice relative to controls. Studies of plaque-forming-cell (PFC) responses to the T-dependent antigen sheep erythrocytes (SRBC) revealed low direct but normal indirect PFC numbers in wasted mice relative to controls, suggesting that some T-cell abnormality that may underlie many reported B-cell defects in wasted mice.

INTRODUCTION

"Wasted" mice spontaneously develop a disease that resembles ataxia telangiectasia in humans, with features that include faulty DNA repair following exposure to ionizing radiation, neurologic abnormalities, and immunodeficiency (1-3). The autosomal recessive mutation wst is evident in these mice by the presence of neurologic abnormalities at 3 weeks of age, although heterozygotes (wst/+) and wild-type (+/+) littermates are phenotypically normal (1). Wasted (wst/wst) mice have been reported to display thymic and splenic hypoplasia (4) and increased sensitivity to chromosomal breakage and DNA damage following exposure of bone marrow cells to ionizing radiation (1-3). Numerous immunologic abnormalities including deficient secretory immunity (5-7), low IgA synthesis (7-10), and altered mitogen responsiveness (4) have been reported in these mice. The mechanism(s) underlying these abnormalities are unclear, though we and others have noted increased viral expression in affected tissues (11-12), while other experiments have implicated a role for microenvironmental influences (7). Past work has shown that the wst defect maps to a single gene locus or several tightly linked loci on chromosome 2 (13).

The majority of the above abnormalities found in wasted mice have been identified in the B-cell compartment. Previous work has shown normal percentages of Thyl⁺ cells in lymphoid tissues of wst/wst mice (5-6), but studies of T-cell parameters such as subpopulations and function have not yet been done. We report here that T-cells from wasted mice are able to support normal indirect plaque-forming-cell (PFC) responses to SRBC, a T-dependent antigen. However, disproportionate percentages of specific T-cell subpopulations were observed in both spleen and thymus of wst/wst mice relative to

controls. Splenic lymphocytes from wasted mice expressed increased percentages of CD4⁺ cells, while thymocytes expressed lower percentages of CD4⁺ CD8⁺ (double-positive) cells than controls. Volume studies revealed that T-cells from all lymphoid tissues of wasted mice have different cell volume distribution patterns than T-cells from controls; these differences were attributed to cell cycle differences (as determined by DNA content experiments) in the lymphoid cell populations. The results reported here suggest a role for altered T-cell function in the pathogenesis of the immunodeficiency of wasted mice.

MATERIALS AND METHODS

Mice. wst/wst mice were bred in the sterile, hooded animal facility at Argonne National Laboratory from wst/+ breeding pairs obtained from the Jackson Laboratory (Bar Harbor, ME). Because the wasted trait is inherited as an autosomal recessive disorder, approximately 25% of each litter can be diagnosed (by neurologic examination) as having the disease. The remaining 75% (approximately) of the litter appear normal, so that identification of known wst/+ and +/+ littermates depends upon breeding experiments. In this work, normal littermates of wst/wst mice are labeled wst/•. These wst/• mice should be a mixture of 67% wst/+ and 33% +/+; they serve as age-matched controls. All results reported here are for animals 25-28 days of age.

Previous work from our laboratory has suggested that wst/• mice may not be totally normal (5-7,11). Therefore, as an additional control we include B6C3 F₁ mice (C57BL6 x C3H/HeN F₁) bred in the animal facility at Argonne. All B6C3 F₁ mice were age-matched (within 2 days) with wst/wst mice within a single experiment. The B6C3 F₁ mouse was chosen as a control because it is the parental strain from which wst mice are derived (1).

Immunofluorescence. Lymphocytes were purified from tissues by ficoll-hypaque gradient centrifugation (5,7). Direct immunofluorescence was carried out as described previously (5,7). For single-color immunofluorescence, fluorescein-conjugated rat anti-CD4, mouse anti-Thy1.2, and rat anti-CD8 (all purchased from Beckton-Dickinson) were used. For dual-color immunofluorescence, phycoerythrin-conjugated rat anti-CD4 and fluorescein-conjugated rat anti-CD8 or anti-Thy1.2 were used (also from Beckton-Dickinson). All samples were analyzed on a FACS Analyzer. Background fluorescence was considered to be that caused by the binding of a fluorochrome-labeled, irrelevant antibody to the cell population. (This amount never exceeded 2% of the total fluorescence for the positive cells.) In these experiments, cells pooled from multiple mice were used due to the low yield of lymphocytes from wst/wst mice.

Cell size/volume studies. Cells from immunofluorescence (as described above) were analyzed with forward angle light scatter to estimate cell volume/cell size. Cell populations (Thy1⁺, CD8⁺, CD4⁺, CD8⁺ CD4⁺) from mesenteric lymph nodes (MLN), spleen, and thymus of wst/wst mice were compared to those from normal littermates (wst/•) and B6C3 controls.

DNA content measurements. Lymphoid cells were permeabilized by the addition of 0.1% Triton in PBS prior to DNA staining with propidium iodide (1.0%). The DNA content per cell was measured using a FACS analyzer, and percentages of cells in S/G₂+M stages of the cell cycle were determined.

Jerne plaque assay. Mice were immunized i.p. at 15 days of age with a solution of 1% sheep erythrocytes (SRBC) in phosphate buffered saline (PBS) (0.5 ml), similarly boosted at age 21 days, and sacrificed by asphyxiation at age 25 days; the spleens were immediately removed and homogenized in PBS. Mononuclear cells were purified on ficoll

gradients and washed twice in PBS before an aliquot of cells was counted with a hemocytometer. After the cells were combined with a constant amount of a 20% SRBC solution, they were plated out at densities ranging from 10^5 to 10^6 cells/plate on two-layer plates. The bottom layer contained 1.2% agarose in PBS/Hank's Balanced Salt Solution, and the top layer contained 0.75% low-melting-temperature agarose in Delbecco's Modified Eagle Medium with SRBC and lymphocytes added. To prevent cytolysis, the top layer was allowed to equilibrate to 40°C before the cells and SRBC were added.

Direct PFC plates were incubated at 37°C for 2 h, a 10% solution of guinea pig complement (Cedarlane Laboratories) was added to each, and incubation continued for an additional 2 h at 37°C, followed by a 30-min incubation at room temperature. The cells were fixed overnight in solution containing 0.25% glutaraldehyde in PBS. Plates were scored for numbers of direct plaques. For the indirect PFC assay, plates pre-measured for direct-PFC were incubated at 37°C for 45 min, and a 1% solution of goat anti-mouse IgG (10 µg/ml) or goat anti-mouse IgA (10 µg/ml; Southern Biotechnologies) was added to each plate. Incubation continued at 37°C for an additional hour before the antibody was poured off, and the plates were washed twice with PBS. a 10% solution of guinea pig complement was added to each; incubation was carried out at 37°C for 2 h and then at room temperature for 30 min. The plates were fixed overnight in 0.25% glutaraldehyde in PBS at room temperature; the glutaraldehyde was then removed, and the plaques were counted. Calculation of indirect PFC was determined by prior subtraction of direct PFC. For all experiments, numbers of wst/wst spleen cells were limiting because wst/wst spleens are small (5×10^5 lymphocytes) and wasted mice make up only 25% of the litter.

It should be noted the percentages of Ig⁺ cells in spleens of wasted mice are the same as those from control mice (5).

For all experiments, controls with complement alone, antibody solution alone, or cells without complement produced no detectable plaques.

RESULTS

Immunofluorescence. T-cell subpopulations in lymphoid tissues of wst/wst mice were examined relative to controls by using both one-color and two-color immunofluorescence (Table 1). One-color analyses were used to determine the total percentages of Thyl⁺, CD4⁺ and CD8⁺ cells present in thymus, MLN, and spleen lymphoid populations. In all experiments, age-matched B6C3 control mice showed percentages similar to those of wst/⁺ mice, and results from dual- and single-color fluorescence studies demonstrated similar percentages of total CD4⁺, CD8⁺ or Thyl⁺ cells. The MLN of wst/wst mice consistently contained normal percentages of each cell subpopulation relative to age-matched wst littermate controls (wst/⁺). In splenic populations, however, we observed an increased percentage of total CD4⁺ cells in wst/wst mice relative to controls, which caused an increase in the CD4:8 ratio. Percentages of total CD4⁺, CD8⁺, and CD4⁻ CD8⁺ were similar for wst/wst mice compared with wst/⁺ and other control mice (such as B6C3; data not shown).

While percentages of Thyl-bearing cells in the thymus were normal, the actual plots of fluorescence intensity against cell number in thymus (Fig. 1) revealed a profile of Thyl⁺ cells that was quite different for wasted mice relative to their normal littermates. The profile from wasted mice contained a disproportionately large number of Thyl-bright cells relative to Thyl⁺ thymocytes. Using dual-color fluorescence, we determined that the majority of CD8⁺ thymocytes from wst/⁺ and B6C3 mice also express surface CD4 (Table 1, Fig. 2), as has

been reported by others (14,15). In wst/wst thymocyte populations, however, most cells are either $CD8^+ CD4^-$, or $CD8^+ CD4^+$, with a minor population of cells that bear both surface markers simultaneously. These results demonstrate low percentages of $CD4^+ CD8^+$ double-positive cells in wst/wst thymus, while a high percentage of cells bears either one marker or the other.

Cell volume/DNA content determinations. In the course of the FACS analyses for immunofluorescence, we detected potential volume differences between wst/wst and control T-cells. Figure 3 documents results of experiments comparing cell volumes of T-cell populations ($CD4^+$, $CD8^+ CD4^+$) in wasted mice to cell volumes of T-cells from littermate controls and B6C3 controls. $Thy1^+$ and $CD8^+$ cells gave similar results (not shown). The results demonstrate that all populations of T-cells examined from wst/wst mice have a different volume distribution pattern than that obtained from T-cells derived from control animals. For thymus, the average cell size is larger in wasted mice than in controls. For MLN and spleen, the majority of cells show a subtle shift into the smaller volume fractions.

We considered that these volume distribution shifts may be attributed to differences in cell cycle between wasted and control mice. Figure 4 documents results of experiments examining DNA content per cell in lymphocytes derived from wst/wst mice relative to controls. Percentages reported in the figure are the fraction of cells in S/G_2+M stages of the cell cycle. In resting lymphocytes derived from either thymus or spleen, wst/wst mice have fewer cells in S/G_2+M stages of the cell cycle than control mice.

Responses to T-dependent antigens. Wasted mice were tested for the ability of their B-cells to mount an antibody response (PFC) to SRBC, a T-dependent antigen. Mice were immunized and then boosted with SRBC three days prior to spleen cell harvest. Table 2 documents

results of experiments monitoring both direct and indirect PFC responses of splenic lymphocytes from wasted and control mice. These results demonstrate that splenic lymphocytes of wst/wst mice are able to mount an indirect PFC response equivalent to that detected in control mice, although the direct response is reduced.

Wasted mice are first distinguished from control littermates at 21 days of age, but they die from their disease by 28 days (1). In the experiments reported here, we could detect no differences in immunofluorescence or volume patterns, percentages of T-cell subpopulations for thymus, spleen, and MLN, or response to antigens when we compared known wst/wst mice aged 22 days, 25 days, or 28 days (data not shown).

DISCUSSION

Our experiments defined several T-cell abnormalities evident in wasted mice, including a high ratio of CD4: CD8⁺ T-cells in both spleen and thymus, decreased percentages of CD4⁺ CD8⁺ (double-positive) cells in thymus, differences in volume distribution patterns of T-cells from all subpopulations derived from all tissues and cell cycle abnormalities in thymic and splenic lymphocyte populations. Spleen cells from wasted mice were able to generate a PFC response to the T-dependent antigen SRBC, although numbers of direct PFC were reduced relative to those of controls. These results suggest the presence of a T-cell imbalance in wasted mice that may underlie many of the reported B-cell-derived immune deficiencies.

The wasted mouse has been proposed as a model for AIDS because of its wasted appearance (16) and abnormal virus expression (11, 12). The abnormal CD4:CD8⁺ ratio observed in splenic and thymic lymphocyte populations of wasted mice is interesting in light of current work being done with AIDS patients. However, while AIDS patients have low Th:Ts/c in peripheral blood lymphocyte populations (which we could not

examine in wasted mice because of low yields of lymphocytes from the peripheral blood), few studies have documented similar low Th:Ts/c in lymphoid tissues such as spleen or thymus from these patients. High Th:Ts/c ratios have been documented in several autoimmune diseases, especially the mouse models for systemic lupus erythematosus (NZB, MRL, bxs mice; 17,18). We have found no evidence for an autoimmune component to the wasted mouse disease. Investigators who have previously examined the pathologic sections of brain, spinal cord, spleen, lymph nodes, gut, and other tissues reported no evidence of immune complex deposition in immune-mediated cell damage (5). In addition, we have found no evidence for high numbers of spontaneous PFC in any of the mice (see Table 2), a feature common to several autoimmune diseases (19). On the basis of the presence of an IgA deficiency, low IL2 production in response to Con A, a premature thymic involution, and other thymic abnormalities (5-10), we hypothesize that the high CD4:CD8⁺ cell ratio in wasted mice may be a compensatory mechanism for the absence of a lymphokine or the functional absence of a T-cell subset.

T-dependent B-cell responses in wasted mice were somewhat reduced for direct PFC, but similar controls for indirect PFC; this suggests the ability of T-cells to support a B-cell response in wasted mice. In general, though, the high fraction of T-cells bearing the CD4 marker (as an indicator of Th-cell numbers) in wst/wst mice relative to controls should support a high PFC response in wasted mice. It is possible that a lymphokine deficiency may be responsible for the low numbers of direct PFC specific for SRBC. Experiments are currently underway to determine levels of functional IL4 and IL5 produced by wst/wst mice. Recent evidence suggests that IL2 production by thymus of wst/wst may be high relative to controls (Padilla and Woloschak, unpublished observations).

Our current findings do not enable us to determine whether any of the immune parameters examined are causally related to the onset of the wasted disease or whether they are the consequence of the disease. Wasted mice cannot be identified from their normal littermates until 21 days of age when neurologic symptoms, (tremulousness, inability to negotiate an incline, etc.) first become evident (1,5). Others have reported a general decline in immune function with age in these mice, with the lowest mitogen responses being evident at day 28 (the average lifespan of wasted mice; 4). In examining the percentages of CD4⁺ CD8⁺ cells in the thymus, we found no differences in wasted mice aged 22 days, 25 days, or 28 days. This finding suggests that the thymic abnormalities may be evident prior to the onset of neurologic symptoms and may not be related to the deterioration of the health of the wasted mice with age. Studies of this sort would be greatly facilitated by the presence of some marker for disease development that could be detected prior to 21 days of age.

The increased average volume (based on forward angle light scatter) in thymocytes of wst/wst mice relative to controls suggests an overall abnormality in T-cells that is not confined to any one subset since all T-cell populations examined expressed this difference. In fact, histograms suggest the virtual absence of a "very small" thymus T-cell population and a shift to the right of all other T-cells (see Fig. 3). Because patterns of DNA content suggest an absence of cycling cells in lymphoid tissues of wasted mice relative to controls, it is not likely that cell volume differences can be directly attributed to high cell turnover.

ACKNOWLEDGMENTS

The authors wish to thank Mr. Louis Sandoval and Mrs. Chin-Mei Chang-Liu for excellent technical assistance and Ms. Jane Perrin for assistance with animal care. Dr. Thomas Ellis kindly aided this work

with his interpretation of FACS data. We also thank Drs. Frank Collart, Carol Giometti, Fred Stevens, and Andrew Kligerman for critical review of the manuscript.

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FIGURE LEGENDS

- Figure 1. Plots of fluorescence intensity vs. cell number in wst/ $\bullet$ or wst/wst thymocytes stained directly with anti-Thy1.2 (Thy1.2) anti-CD8 (Lyt2), or anti-CD4 (L3T4). Unshaded areas represent the Thy1.2 $^{+}$ cells in all samples.
- Figure 2. Plots representing wst/wst, wst/ $\bullet$, or B6C3 thymocytes; MLN lymphocytes; or splenocytes bearing the following cell surface phenotypes as determined by dual-color immunofluorescence: CD8 $^{-}$ CD4 $^{-}$ (area 1) CD8 $^{-}$ CD4 $^{+}$ (area 2), CD8 $^{+}$ CD4 $^{+}$ (area 3), CD8 $^{-}$ CD4 $^{+}$ (area 4).
- Figure 3. Plots of cell volume vs. cell number in (A) CD4 $^{+}$ and (B) CD8 $^{+}$ CD4 $^{+}$ cells derived from thymus, MLN, and spleen of wst/wst, wst/ $\bullet$ or B6C3 mice. The line is drawn through the 10^1 volume to provide a measure of comparison among panels.
- Figure 4. Plots of DNA content per cell vs. cell number as determined by propidium iodide staining of resting thymic or splenic lymphocytes. Numbers in the right hand corner indicate the percentages of cells to the right of the gate, i.e., those cells in S/G $_2$ +M of the cell cycle.

Table 1. Percentage of Lymphocytes Positive for CD4/CD8^a

Lymphocyte Source	CD8 ⁻ CD4 ⁺ ^b	CD8 ⁺ CD4 ⁻ ^b	CD8 ⁺ CD4 ⁺ ^b	CD4 ⁺ /CD8 ⁺
<u>wst/wst</u> spleen	25.8	8.0	1.5	$\frac{27.3}{9.5} = 2.9$
<u>wst/•</u> spleen	13.6	6.2	1.4	$\frac{15.0}{7.6} = 1.9$
B6C3 F ₁ spleen	16.3	6.8	1.2	$\frac{17.5}{8.0} = 2.2$
<u>wst/wst</u> MLN	39.6	14.9	1.5	$\frac{41.1}{16.4} = 2.5$
<u>wst/•</u> MLN	46.0	14.2	1.8	$\frac{47.8}{16.0} = 2.9$
B6C3F ₁ MLN	39.5	14.9	2.3	$\frac{41.8}{17.2} = 2.4$
<u>wst/wst</u> thymus	57.5	18.8	15.9	$\frac{73.4}{34.7} = 2.1$
<u>wst/•</u> thymus	18.3	9.6	64.8	$\frac{83.1}{74.4} = 1.1$
B6C3F ₁ thymus	13.0	7.3	64.6	$\frac{77.6}{71.9} = 1.1$

^aDetermined by direct immunofluorescence. These results are a representative experiment from three independent experiments. In each case, pooled cells from 3-4 mice were used. (A single wst/wst mouse gives insufficient cells for analyses). Error estimates for each antibody with this technique are < 2%.

^bDetermined by dual-color immunofluorescence.

^cThe same results were consistently obtained if CD4:CD8 was determined by single- or by dual-color immunofluorescence.

Table 2. SRBC-PFC Response in Wasted Mice^a

Mice	Direct PFC/10 ^{6b}		Indirect PFC/10 ^c	
	Unimmunized	Immunized	Unimmunized	Immunized
<u>wst/wst</u>	0	10 ^d	2	202 ^e
<u>wst/•</u>	0-2	191-195 ^f	1	142-195 ^f
B6C3 (controls)	0-1	182-393 ^f	0-2	122-242 ^f

^aMice were immunized intraperitoneally with 0.1% SRBC in PBS and boosted 1 week later. Five days later, splenic lymphocytes were harvested and analyzed.

^bDirect plaque-forming cells/10⁶ total splenic lymphocytes.

^cIndirect plaque-forming cells/10⁶ total splenic lymphocytes.

^dPFC were the same for three wst/wst mice from different litters.

^ePFC were the same for two wst/wst mice from different litters.

^fRange from 10 mice.

Fig. 1

Thymus

Fig. 2

Fig. 3A

Ly12⁺ L3T4⁺

Fig. 3B

DNA Content: Resting Cells

Thymus

Spleen

B6 C3

wst/.

wst/wst

DNA CONTENT

Fig 4