T Cell Lines Characterize Events in the Pathogenesis of the Wiskott-Aldrich Syndrome

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Summary

The Wiskott-Aldrich syndrome (WAS) is a severe immunodeficiency and platelet deficiency disease arising from an X-linked defect. The disease is correctable by transplantation of hematopoietic stem cells, but the product of the defective gene is unidentified and the number of defects in patient blood cells is large. The current hurdle is the need to identify the early pathogenic event(s) that are the cause of other defects. As a step toward this goal, we have generated and examined a panel of interleukin 2-dependent allospecific T cell lines from peripheral lymphocytes of seven WAS patients and five normal individuals. WAS cell lines, like normal lines, undergo vigorous proliferation when challenged with specific allostimulant or with phorbol myristate acetate and ionomycin. Both normal and WAS T cell lines express cell surface molecules CD2, CD3, T cell receptor-α/β, human histocompatibility leukocyte antigen class I, CD45 and CD11a, and varying ratios of CD4 and CD8, and are negative for natural killer cell and monocyte surface molecules. WAS T cell lines express CD43 (sialophorin/leukosialin) with molecular weight and in an amount comparable with normal T cell lines. WAS T cell lines thus do not express defects in CD43 (decreased amount, abnormal molecular weight), previously documented in WAS circulating lymphocytes. On the other hand, as detected by scanning electron microscopy, WAS cell lines exhibit severe morphological abnormalities, including decreased size and density of the microvillus surface projections. The morphological abnormalities of WAS T cell lines are similar to, or more extensive than, those previously reported for WAS peripheral lymphocytes, indicating that the generation of morphological (cytoarchitectural) defects is an early pathogenic event in this disease. The findings suggest that the gene that is defective in the WAS encodes a protein that normally functions to maintain or regulate the cytoskeletal structure of blood cells.

The Wiskott-Aldrich syndrome (WAS)[1] is an X-linked disorder involving severe deficiency of platelet and immunologic function (1-3). The number of documented abnormalities in the disease is very large, and, since the product of the defective X-chromosome gene is unidentified, the current challenge is to establish chronology of the pathological events that result from the primary genetic defect.

Among the most dramatic defects associated with the WAS are the severely decreased size and number of circulating platelets thought to be caused by destructive processes (4). Defects of T lymphocytes, the most definitively involved leukocytes, include abnormal cell surface morphology (cytoarchitecture), documented by scanning electron microscopy and characterized by the paucity of the microvillus surface projections found on normal blood lymphocytes (5). At the mo-

lecular level, defects of WAS T lymphocytes include the reduction in quantity, and in some cases, abnormal molecular weight of the cell surface glycoprotein molecule CD43 (formerly sialophorin or leukosialin) (6-10). CD43 was excluded as the possible primary defect because it is encoded by an autosomal gene (11, 12). Other molecular defects of WAS blood cells include abnormalities of the T lymphocyte enzymes that glycosylate CD43 (9, 10). Further molecular defects include abnormalities of Ca^{2+} regulated neutral protease (calpain) of WAS platelets (Kenney, D. M., F. S. Rosen, R. Reid, and E. Remold O'Donnell, manuscript in preparation).

In an effort to identify early pathological events in WAS, we have established and characterized long-term T cell lines from affected males. The patient T cell lines should, in theory, be an excellent experimental system for studying early pathological events in the disease. The cell lines are expected to express the inherited defective gene and the directly resultant defective product(s), but on the other hand, should be free

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[1] Abbreviation used in this paper: WAS, Wiskott-Aldrich syndrome.

of some of the complicating defects that arise in the circulation. This prediction is demonstrated here by the finding that the long-term T cell lines from WAS patients express one defect previously associated with WAS blood lymphocytes, but lack another defect, which therefore likely results from secondary interactions of circulating blood cells.

Materials and Methods

Patients. The diagnosis of WAS was based on male sex, severe thrombocytopenia with platelets of reduced size and, in some patients, a positive family history. The patients had eczema and/or immunodeficiency. None was splenectomized. The clinical profile of the patients is summarized in Table 1. Platelet size was measured as effective mean diameter using an electronic particle analyzer (El-zone Model 112; Particle Data Inc., Elmhurst, IL) calibrated with 2.02 μ latex beads (Coulter Electronics, Inc., Hialeah, FL).

Generation of Allospecific T Cell Lines. Patient and normal whole blood anticoagulated with National Institutes of Health (NIH) formula A acid-citrate-dextrose, either freshly drawn or after overnight shipment at ambient temperature, was centrifuged at 150 g, and the platelet-rich plasma was removed. All blood cell isolation was performed at room temperature in plastic or siliconized glass. The packed cells were diluted with an equal volume of Ca^{2+}/Mg^{2+} free HBSS (Whittaker Bioproducts, Walkersville, MD), layered onto Histopaque 1077 (Sigma Chemical Co., St. Louis, MO), and centrifuged at 800 g for 40 min. The interface mononuclear cells were washed three times in PBS and were cocultured at 0.5:1 stimulator to responder ratio with the class II$^+$ B-lymphoblastoid cell line Raji, which had been pretreated with mitomycin C (13). The culture medium was RPMI 1640 (Mediatech, Inc., Washington, DC) with 10% FCS (HyClone Laboratories, Inc., Logan, UT), 10% conditioned media from normal blood mononuclear cells cultured at 10^6/ml with 2 μg/ml of PHA (Sigma Chemical Co.) for 48 h (14), and 40 U/ml of rIL-2 (prepared by Hoffman-LaRoche, Nutley, NJ, and obtained through the Biological Response Modifiers Program of the National Cancer Institute) as the source of lymphokines and growth factors. The cells were stimulated as above every 7 d. They were expanded, and fresh supplement was added as needed (usually every 3 d). The cell lines have been maintained in continuous culture for a minimum of 3 mo and are considered established since they have stable phenotype and growth properties.

Proliferative Assays. T cells were harvested 10 d after allospecific stimulation. The cells were washed and plated at 2 × 10^5/well in 96-well plates, and cocultured at 1:1, 0.5:1, and 0.25:1 stimulator to responder ratio with mitomycin C pretreated class II$^+$ stimulator lines U937 (monocytic leukemia) (15) or SPB-10 cells (EBV-transformed normal B cells provided by Jerrold Schwaber, Hahnemann University, Philadelphia, PA). Alternatively, the cell lines were stimulated with 5 ng/ml of PMA (Sigma Chemical Co.), with or without 0.1, 0.2, or 0.5 μg/ml of ionomycin (Calbiochem Corp., La Jolla, CA). After 48 h, 1 μCi/well of [3H]thymidine (New England Nuclear, Boston, MA) was added, and the cells were cultured for an additional 16 h, harvested onto glass fiber paper (Cambridge Technologies, Waltham, MA), and [3H]thymidine incorporation was quantified by liquid scintillation counting.

Antibodies. mAbs OKT3 (anti-CD3), OKT4 (anti-CD4), OKT8 (anti-CD8), OKM-1 (anti-CD11b) (16), OKT11 (anti-CD2), GAP8.3 (anti-CD45) (17), and W6/32 (anti-HLA-class I) (18) were obtained from the American Type Culture Collection (Rockville, MD). Anti-CD43 mAbs L10 (7) and L11 (4) were generated in this laboratory. The mAb T305 (19) provided by Dr. R. I. Fox

(Scripps Clinic, La Jolla, CA) is an IgG1 that recognizes a mol wt 125,000 isoform of CD43 found on activated lymphocytes (19–22). The anti-CD11a TA-1 mAb (23) was provided by Dr. T. LeBien (University of Minnesota, Minneapolis). The anti-CD16 (24) 3G8 mAb was provided by Dr. J. Unkeless (Rockefeller University, New York). The anti-CD43 mAb DF-T1 (25), and the anti-CD25 (IL-2R) Act-1 mAb were from Dakopatts (Glostrup, Denmark). The anti-α/β TCR WT31 mAb (26) was from Becton Dickinson (Mountain View, CA). FITC-labeled F(ab')$_2$ goat anti-mouse IgG was from Tago, Inc. (Burlingame, CA).

Sialidase Treatment. T cell lines were washed and incubated in RPMI 1640 at room temperature with 0.01 U/ml/10^6 cells of sialidase from *Vibrio cholera* (Calbiochem Corp.) for 30 min and then washed. The cells were stained with mAb before and immediately after sialidase treatment, and after additional 18 h culture in serum-containing media.

Flow Cytometric Analysis. Cells (5 × 10^6) were incubated with mAb for 30 min on ice, washed, and incubated with FITC labeled F(ab')$_2$ goat anti-mouse IgG (4). The cells were washed, fixed, and analyzed on an Epics V cytofluorograph (provided through the courtesy of Wallace H. Coulter, Coulter Electronics, Inc., Hialeah FL).

Western Blots. Cells were lysed in buffer containing 0.5% NP-40 and clarified by centrifugation as described (4). The proteins were separated by SDS-PAGE (7.5% polyacrylamide) and electroblotted onto nitrocellulose (4) (Schleicher & Schuell, Inc., Keene, NH). The blots were stained as described (4) with mAb followed by biotinylated second antibody and a preincubated mixture of avidin and biotinylated horseradish peroxidase reagents (Vector Laboratories, Inc., Burlingame, CA).

Scanning Electron Microscopy. Cells (2–5 × 10^6) from proliferating or resting cultures (3 and 10 d after allospecific stimulation, respectively) were washed in Ca^{2+}/Mg^{2+} free HBSS (Whittaker Bioproducts), and equilibrated at 37°C for 30 min. The cells were fixed as described (5) in 1.25% glutaraldehyde for 1 h at 37°C and 14 h at 4°C, collected on polycarbonate membranes (0.4 μ) (Nucleopore Corp., Pleasanton, CA), and postfixed in 1% osmium tetroxide for 1 h. Subsequently, cells were dehydrated with graded ethanols, subjected to critical point drying from carbon dioxide, coated with gold/palladium (60:40), and examined on a scanning electron microscope (Model DS130; International Scientific Instruments, Santa Clara, CA). For each sample, cells in randomly selected fields were photographed at a magnification of 2,500–6,700.

Results

Establishment of T Cell Lines. T cell lines were established from peripheral lymphocytes of seven WAS patients (Table 1) and five normal individuals by stimulation with Raji cells. No subfractionation of peripheral mononuclear cells was performed before stimulation so as to obtain lines representative of circulating cells. The T cell lines are dependent for long-term growth on weekly stimulation and addition of growth factors. No cases of spontaneous transformation have been observed.
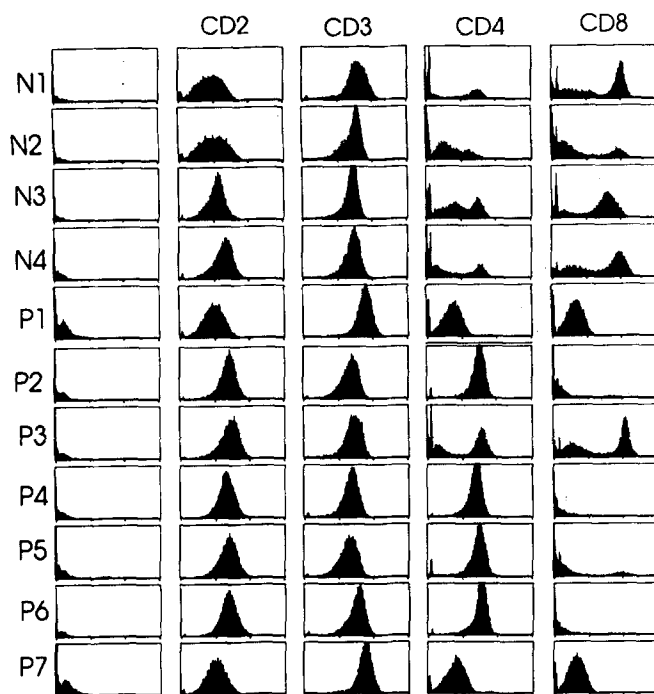
Phenotypic Characterization of T Cell Lines. The patient and normal cell lines are >95% positive for T cell molecules CD2 and CD3, as assessed by flow cytometry (Fig. 1) as well as the TCR-α/β (Table 2). Both patient and normal cell lines exhibit a heterogeneous distribution of CD4 and CD8 molecules (Fig. 1), and are negative for the monocyte and NK

Table 1. *Description of WAS Patients*

Patient	Age	Platelet count/μl whole blood	Mean platelet diameter	Intravenous gammaglobulin therapy	Family history
			μ		
P1	1.6 yr	32,500	1.87	+	−
P2	31 yr	26,600	1.95	−	+
P3*	9 mo	141,000	1.90	+	−
P4	27 yr	33,200	1.92	−	+
P5	29 yr	29,800	1.91	−	+
P6	14 yr	40,900	1.89	+	+
P7	12 yr	13,200	1.90	+	+
N‡		290,000	2.29		
		(± 65,000)	(± 0.12)		

* Whole blood platelet count on patient P3 subsequently decreased to 20,000.
‡ Mean whole blood platelet count and mean platelet diameter ± SEM obtained for 26 normal donors using the equipment and procedures applied to the patient samples.

Table 2. *Flow Cytometric Analysis of Patient and Normal T Cell Lines*

	Percent of cells positive for indicated antigen*						
Cell line	HLA class I	CD11a	TCR-α/β	CD54	CD56	CD16	CD11b
N3	96	92	96	33	3	3	3
P4	92	97	96	41	3	3	3
P5	94	98	97	49	5	5	4

* Patient and normal cell lines were harvested 10 d after allospecific stimulation and stained with mAbs as indicated in Materials and Methods. The mean intensity of fluorescence for each antigen was similar in normal and patient cell lines.

Figure 1. Flow cytometric characterization of T cell lines from normal individuals and WAS patients. Panels from left to right show T cell lines stained with an isotype-matched irrelevant control mAb, and mAbs defining CD2, CD3, CD4, and CD8. Graphs represent cell number versus logarithm of fluorescence intensity. (*N1-N4*) T cell lines derived from lymphocytes of four normal individuals; (*P1-P7*) T cell lines derived from blood lymphocytes of the correspondingly numbered WAS patients described in Table 1. N5 cells analyzed in other experiments and not shown are very similar to lines P1 and P7.

surface molecules CD11b, CD16, and CD56 (Table 2). Patient and normal cell lines are >95% positive for class I MHC molecules and CD11a (LFA-1) (Table 2).

Proliferative Responses of T Cell Lines. Both patient and normal cell lines proliferate vigorously in response to the allostimulant Raji cells, whereas only minimal proliferation was detected when nonallospecific class II positive U937 or SPB-10 cells were used (Table 3). Significant proliferation is also induced by PMA plus ionomycin (Table 3), but not by PMA alone (data not shown). Both normal and patient cell lines proliferate moderately in the presence of rIL-2 (Table 3), and proliferative responses to various stimuli are enhanced in the presence of rIL-2 (not shown).

CD43 Expression on T Cell Lines. Patient and normal cell lines exhibit comparable high expression of CD43 molecules, as assessed by flow cytometry with the mAb L10, which recognizes all CD43 isoforms (Fig. 2). No significant differences were found in the expression of the high molecular weight CD43 isoform recognized by T305 mAb (19, 20) (Fig. 2). In addition, PHA-stimulated lines initiated from two WAS patients and maintained in culture for 6 wk also had normal surface density of CD43, as assessed by flow cytometry (not shown).

When examined by Western blots stained with L10 mAb, CD43 was detected as a broad band of apparent mol wt of approximately 135,000 in lysates of both normal cell lines (Fig. 3, two left lanes) and WAS cell lines (three right lanes). No obvious difference in CD43 quantity was detected between the patient and the normal cell lines. No CD43 degradation products were observed in the patient cell lines, including the 95,000 mol wt L10 mAb positive degradation product previously found on lymphocytes isolated from stored WAS blood (8). CD43 was also detected in Western blots stained with the T305 mAb, which recognizes higher molecular weight CD43 isoforms, and its apparent molecular weight (approximately 135,000) was the same in WAS and normal cell lines (not shown).

CD43 was also examined after sialidase treatment of the T cell lines by the use of several anti-CD43 mAbs recognizing

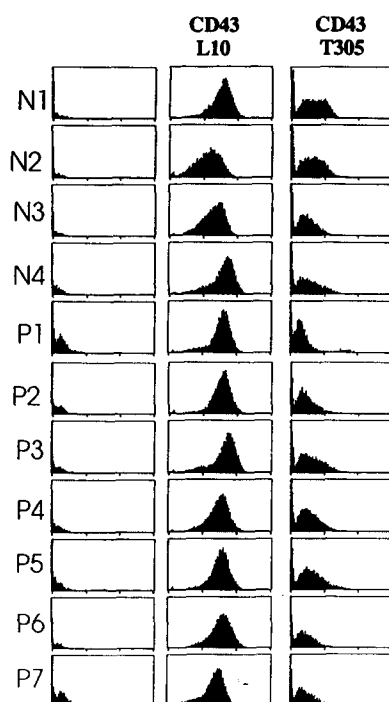**Table 3.** *Proliferative Responses of Normal and WAS T Cell Lines*

Cell line	[³H]Thymidine incorporation in response to indicated stimulating agent					
	None	Raji	U937	SPB-10	PMA + ionomycin	rIL-2
				cpm		
N1	540*	164,400*	ND	ND	60,500§	61,100*
N2	1,100*	178,800*	4,500‖	ND	ND	46,600*
N3	1,000‡	86,300‡	2,200‖	8,500¶	ND	17,900‡
N5	2,200‡	46,600‡	ND	ND	ND	48,300‡
P1	3,400§	41,400§	ND	ND	51,900§	23,100§
P2	800*	106,800*	2,100‖	3,600¶	63,800§	34,200*
P3	600*	31,900‡	ND	ND	ND	20,100*
P4	1,300*	139,600*	ND	4,000¶	ND	40,000*
P5	500*	108,200*	ND	4,100¶	ND	14,500*
P6	1,600*	114,100*	2,200‖	5,000¶	ND	32,100*
P7	1,100**	110,000**	ND	ND	79,000**	71,200**

Results from different experiments are shown. Individual experiments are identified by footnote symbols. Proliferation assays were performed as described in Materials and Methods.
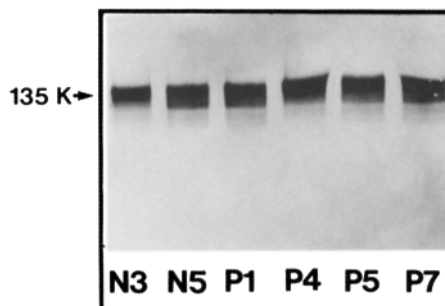
sialylation-dependent or -independent epitopes. Staining with L11 mAb, which is specific for desialylated CD43 (4), is negative in untreated cells (Fig. 4). After sialidase treatment, the cells become L11 positive, and the strong staining of untreated

	CD43 L10	CD43 T305
N1		
N2		
N3		
N4		
P1		
P2		
P3		
P4		
P5		
P6		
P7		

Figure 2. Flow cytometric measurement of CD43 on T cell lines from normal individuals (*N1–N4*) and WAS patients (*P1–P7*). Panels from left to right show cells stained with isotype-matched irrelevant control mAb, L10 mAb, which recognizes all CD43 isoforms, and T305, which recognizes only high molecular weight CD43 isoforms.

cells with the sialylation-dependent anti-CD43 mAbs DF-T1 and T305 is lost. The profiles obtained with L10 mAb are not changed by sialidase treatment, nor are the profiles obtained with GAP8.3 mAb, which recognizes CD45, another heavily glycosylated molecule. After overnight culture, the CD43 epitopes recognized by DF-T1 and T305 mAbs are reexpressed, albeit at lower density, whereas the staining with L11 mAb is significantly diminished. Thus, although the mechanisms leading to restoration of sialylated CD43 have not been clarified, these mechanisms function in an indistinguishable manner in WAS and normal cell lines.

Upregulation of T Cell Activation Molecules during Proliferation. Resting and alloactivated cell lines were analyzed by flow cytometry for expression of activation-associated molecules. The β chain of the IL-2 receptor (CD25) and the
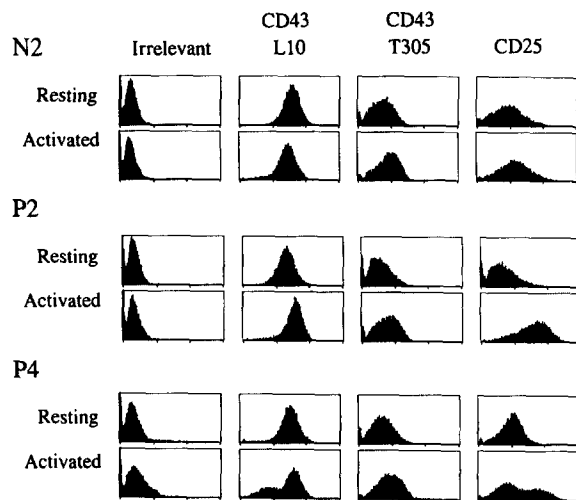

135 K►

N3 N5 P1 P4 P5 P7

Figure 3. Western blot of NP-40 lysates of normal T cell lines N3 and N5 and WAS patient cell lines P1, P4, P5, and P7 stained with L10 mAb. Similar results were obtained for the lines N2, N4, P2, and P6.
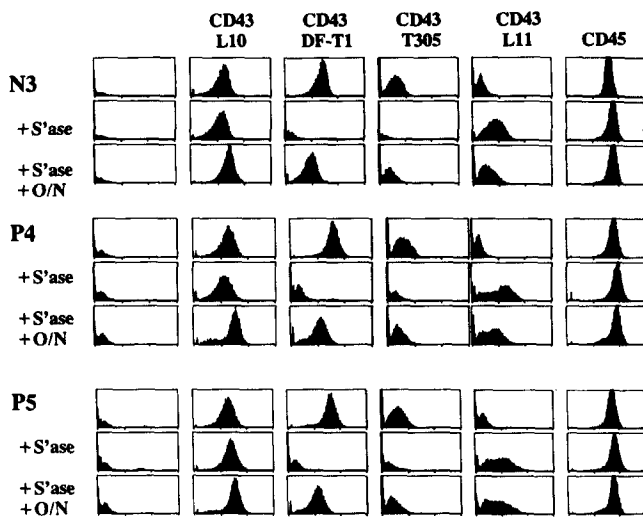
Figure 4. Restoration of sialylation of normal (N3) and WAS (P4 and P5) T cell lines. Cells were stained with mAb and analyzed by flow cytometry before, or immediately after (+ S'ase), treatment of cells with sialidase, or after an 18-h posttreatment culture under normal culture condition (+ S'ase, O/N). L10 mAb recognizes a sialic acid-independent CD43 epitope; DF-T1 and T305 mAb recognize sialylation-dependent CD43 epitopes; and L11 recognizes an epitope of desialylated CD43 (see Materials and Methods). Similar results were obtained with N4 and P6 cells.

Figure 5. Upregulation on activation of the IL-2R (CD25) and the T305 epitope of CD43. The normal cell line N2 and the patient cell lines P2 and P4, were stained with the indicated mAbs 10 d (resting cells) or 3 d after allospecific stimulation (activated cells). Similar results were obtained with P5.

activation-associated epitope of CD43 recognized by the T305 mAb (22) are upregulated in WAS patient and normal T cell lines when examined on the third day of allostimulation, the time of peak proliferation, with no detectable differences in patient and normal cells (Fig. 5).

Surface Morphology of T Cell Lines. Because scanning electron microscopy served to document substantive cytoarchitectural defects in the case of WAS blood lymphocytes, this approach was applied to the analysis of the T cell lines. Cells in normal T cell lines were found to be relatively homoge-

neous in size and morphology and, like normal blood lymphocytes, virtually all cells exhibit a dense surface display of microvillus projections (Fig. 6). WAS T cell lines, on the other hand, are heterogeneous in size and morphology (Fig. 7 A) and are abnormal in two respects (Fig. 7, A–D). Although some cells exhibit normal lymphocyte surface morphology, a large number of cells in each sample show the paucity of microvillus surface projections previously documented for WAS PBLs. In addition, some WAS cells have qualitatively abnormal surface projections that are bulbous or lamellar; other cells have elongated or otherwise irregular shapes. These cells with qualitatively abnormal cytoarchitecture have not been detected in WAS peripheral blood cells, and were found
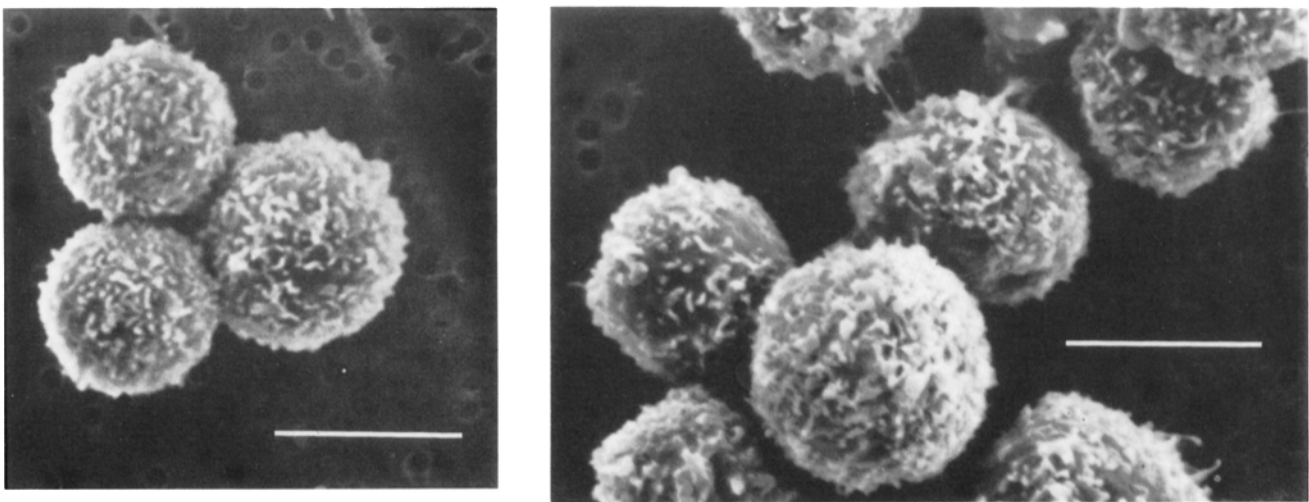
Figure 6. Scanning electron micrographs of a T cell line from a normal individual. Cells are homogeneous in size and morphology with the surfaces of most cells showing a dense display of microvillus projections. The micrographs shown are representative of four normal cell lines examined (N1, N2, N3, and N5). The normal cell lines are indistinguishable from normal blood lymphocytes (Reference 5, comparison not shown). Bars = 5 μm.
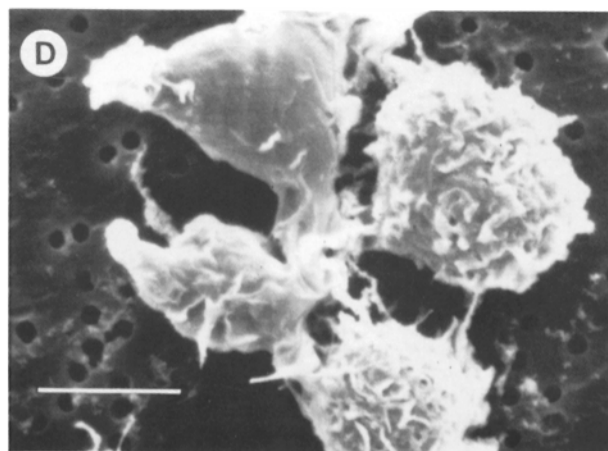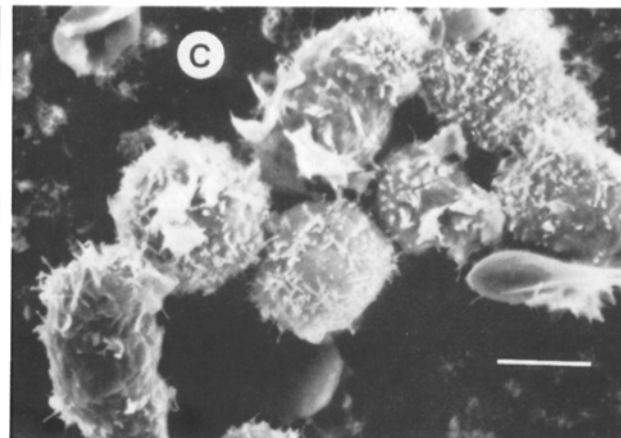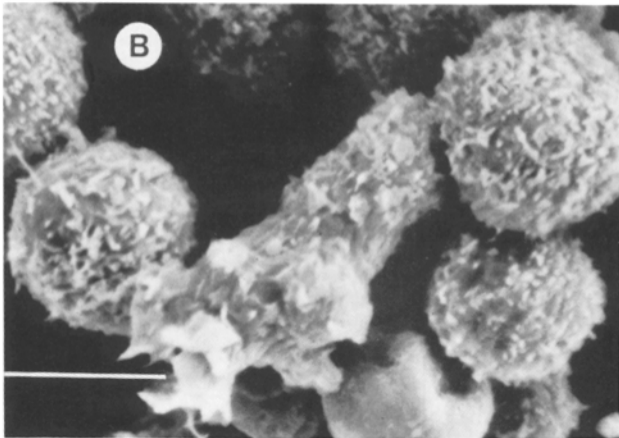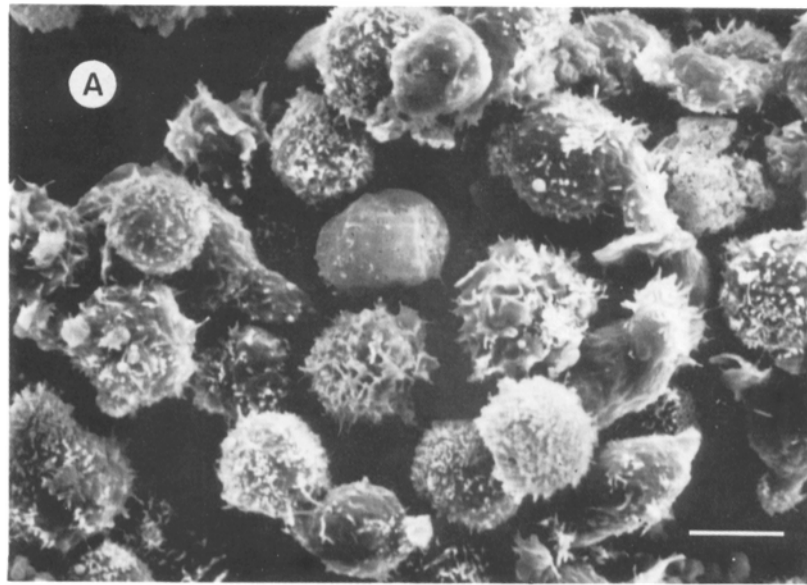
Figure 7. Scanning electron micrographs of WAS patient T cell lines. The patient cells show heterogeneous morphology (*A*). Some cells are spherical with a display of surface microvillus projections (*A–C*). Other cells have relatively smooth surfaces depleted of microvilli (*A–C*) or show qualitatively abnormal projections that are blunted, bulbous, or lamellar (*A–C*). Still other cells in the patient population have elongated shapes (*B*, *C*, and *D*). The micrographs shown are representative of three WAS cell lines examined (P1, P2, and P5). Bars, 5 μm.

only rarely in T cell lines from normal individuals. No obvious differences were noted between WAS resting and proliferating cell lines (not shown).

Discussion

A panel of long-term allospecific T cell lines from WAS patients and normal individuals has been maintained in culture for longer than 3 mo. The rationale for establishing patient cell lines was the anticipation that the cells would (a) express the inherited abnormal gene product and the immediate or early abnormalities resulting from that product, and (b) be free of some secondary abnormalities generated in vivo when defective cells circulate in the blood. Following this rationale, cell lines, particularly nontransformed cell lines, should offer a simplified experimental system in which to study early defects resulting from the inherited defective gene. The normal and patient cell lines exhibit the phenotype characteristic of T lineage cells, being positive for CD2, CD3, TCR-α/β, HLA-class I, CD11a, and CD43, and undergo vigorous proliferation when challenged with allostimulant or with PMA plus ionomycin. The cells are IL-2–dependent for long-term growth, and no spontaneous transformation has been detected.

Flow cytometry and Western blots showed that surface density and molecular weight of CD43 are not detectably different in WAS and normal T cell lines. The WAS cell lines differ in this respect from WAS blood lymphocytes, previously shown to be deficient in CD43 (7–10, 27) and to express abnormal molecular weight CD43 isoforms in some patients (3 of 8 patients [7], and 10 of 12 [9]). The presence of CD43 abnormalities in WAS blood lymphocytes and their absence in WAS T cell lines suggest that these defects are not an early pathological event in the WAS. One possible explanation is that CD43 defects are generated in the circulation (4).

On the other hand, scanning electron microscopic evaluation demonstrated that the cytoarchitecture of WAS T cell lines differs from normal T cell lines. The cytoarchitectural abnormalities found in the WAS T cell lines include those previously documented in WAS blood lymphocytes. In both cases, the dense display of slender surface microvillus projec-

tions that characterize normal blood lymphocytes and normal T cell lines are diminished in number and size. For the case of blood lymphocytes, the mean morphology score, which quantifies microvilli, is 3.62 ± 0.22 for normal individuals and 2.89 ± 0.27 for 18 WAS patients (p <.001, [5]). Indeed, the paucity of blood lymphocyte surface microvilli, since it is consistently found in WAS patients and is specific to the disease, has proved useful as a criterion for diagnosis in difficult cases (5). The cytoarchitectural defects in the T cell lines from WAS patients were found to be more extensive than the defects of WAS blood lymphocytes, since they include qualitative abnormalities in addition to the paucity of microvilli. The findings of cells with qualitatively defective cytoarchitecture in the WAS T cell lines, but not in WAS patient blood, may reflect mechanisms that remove qualitatively defective cells from the circulation.

The documentation in WAS T cell lines of abnormal cytoarchitecture as previously shown in WAS blood lymphocytes verifies that the inherited defective gene is expressed in the T cell lines. It is not known whether the paucity of microvilli detected by scanning electron microscopy has an exact correlate in the living cells, or only a related correlate. It is possible that the absence of microvilli results from the action of the scanning electron microscopy cell fixing procedure in combination with an underlying cellular defect in the WAS patients' cells. Nonetheless, the findings strongly indicate an underlying defect in cell cytoarchitecture or its regulation.

This study thus shows that cytoarchitecture is profoundly defective in T cell lines from WAS patients. On the other hand, expression of a multitude of cell surface molecules including CD43, as well as proliferative responses to specific allostimulant and mitogen, are normal in the WAS T cell lines. The expression of the cytoarchitectural defect in WAS cell lines maintained in an environment free of the pathodegradative events that occur in the circulation strongly suggests that the generation of the cytoarchitectural defects is an early pathogenic event in this disease, and is closely linked to the inherited primary defect. The cumulative findings suggest that the gene that is defective in WAS patients encodes a protein that normally functions to maintain or regulate the cytoskeletal structure of blood cells.

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