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WI-1, a Novel 120-Kilodalton Surface Protein on *Blastomyces dermatitidis* Yeast Cells, Is a Target Antigen of Cell-Mediated Immunity in Human Blastomycosis

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A large body of experimental data has demonstrated the central role of T cells in acquired resistance to the dimorphic fungus *Blastomyces dermatitidis*. We examined the human T-cell response to WI-1, a 120-kDa *B. dermatitidis* yeast cell surface protein recently shown to be an immunodominant antigen of the B-cell response in infected humans. Peripheral blood lymphocytes from 10 blastomycosis patients studied proliferated in response to WI-1 (mean, 19,431 cpm) and to the standard, crude cell wall antigen, *Blastomyces* alkali- and water-soluble antigen (B-ASWS) (mean, 19,131 cpm); lymphocytes from 10 histoplasmosis patients and 10 normal control subjects did not respond to WI-1. WI-1 stimulation of patient lymphocytes and rechallenge with WI-1 or B-ASWS showed that the antigens share immunodominant epitopes. Of 100 WI-1-responsive T-cell clones derived from peripheral blood, 10 were studied in detail to assess the phenotype, function, and ligands recognized. The clones exhibit the $CD3^+$ $CD4^+$ phenotype of helper T cells; 2 of 10 clones (and 21% of antigen-stimulated peripheral blood lymphocytes) use the Vβ8 T-cell receptor gene element to respond to WI-1. All the clones proliferate in response to both WI-1 and B-ASWS but not other fungal antigens, and some mediate potent cytolytic effects on WI-1- and B-ASWS-labeled targets. WI-1 recognition requires antigen processing and presentation of epitopes in association with HLA-DR (to noncytolytic clones) and HLA-DP (to cytolytic clones). From these findings, we conclude that $CD4^+$ T cells with regulatory and cytolytic properties are involved in the development of acquired resistance to *B. dermatitidis*, that the cells are directed against WI-1, and that the manner of display of WI-1 peptide epitopes in conjunction with major histocompatibility complex class II may influence the profile of the immune response.

Blastomycosis, one of the principal endemic systemic mycoses, results from an infection with the thermal dimorphic fungus *Blastomyces dermatitidis*. Humans and lower animals, such as dogs, are primarily infected by inhaling aerosolized conidia from soil, in which the organism dwells as a saprophyte (19). At body temperature, the conidia are converted to the parasitic or invasive yeast forms that cause disease. The acute primary pulmonary infection can be asymptomatic or can produce an influenza or atypical pneumonia syndrome (47). Although acute blastomycotic pneumonia may resolve spontaneously, progressive forms of disease involving the lungs, the extrapulmonary organs (usually the skin, bones, joints, or prostate gland), or both develop in many patients.

The central role of cell-mediated immunity, especially thymus-derived lymphocytes (T cells), in the immunologic control of granulomatous fungal disease is highlighted by the emergence of disseminated histoplasmosis (53), coccidioidomycosis (8) and, most recently, blastomycosis (43) as opportunistic infections in AIDS patients. A large body of experimental data has demonstrated that specific cellular immunity is a principal component of acquired resistance to *B. dermatitidis* (9, 17, 48, 52). In a murine model of blastomycosis, protective immunity can be adoptively transferred to naive mice with T cells (9). In humans, nearly all individuals infected with *B. dermatitidis* develop specific cellular immunity, as determined by in vitro lymphocyte proliferation in response to yeast cell wall determinants in the crude antigen preparation, *Blastomyces* alkali- and water-soluble antigen (B-ASWS) (4, 5, 30, 32). The response correlates with enhanced in vitro phagocytosis and inhibition of the growth of *B. dermatitidis* by peripheral blood monocyte-derived (7) and alveolar (6) macrophages, and the supernatant from antigen-stimulated T cells endows macrophages of nonimmune donors with comparable microbicidal function (6). Murine studies have extended these data and examined the influence of gamma interferon on macrophage killing of *B. dermatitidis*. Peritoneal macrophages treated with a dose of 10,000 U/ml show significantly enhanced killing of yeast cells (10). The results of these studies have led to the conclusion that activated macrophages are the principal effector cells of resistance; they acquire their microbicidal activity after stimulation with lymphokines released from antigen-specific T cells. However, the phenotype and function of the cells and the ligands recognized have not been fully defined.

The development of B-ASWS by Cox and Larsh (15) has permitted experimental (18, 23), clinical (4, 5), and epidemiological (30, 32) studies of cellular immune responses in blastomycosis. The antigen is extracted from yeast cell walls with 1 N NaOH, which cleaves α-1,3-glucan linkages in the outermost layer and yields a complex mixture of lipid, polysaccharide, and protein with a molecular weight of 30,000 to 50,000 (16). Preparative polyacrylamide gel electrophoresis and isoelectric focusing have shown that

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B-ASWS possibly contains three or four fractions (33). One fraction confers the high sensitivity and specificity, whereas the others appear to account for cross-reactivity with heterologous fungi, particularly *Histoplasma capsulatum*. Indeed, lymphocytes from histoplasmin-reactive subjects proliferate in response to B-ASWS (13), albeit to a significantly lower extent than lymphocytes from blastomycosis patients (5). Despite the considerable value of B-ASWS, the technique that isolates the antigen may thwart further attempts to purify and characterize immunodominant T-cell epitopes of *B. dermatitidis*. Alkali extraction denatures (15) and markedly fragments (34) the protein components.

In view of this difficulty in further defining determinants of B-ASWS and, in turn, T cells directed against them, we studied whether *Blastomyces*-reactive lymphocytes from infected persons were directed against a recently described 120-kDa surface protein antigen on *B. dermatitidis* yeast cells, designated WI-1. The results from the study demonstrate that WI-1 is a specific target of the T-cell response in human blastomycosis, that WI-1 and B-ASWS share immunodominant epitopes, and that the manner of display of the epitopes in conjunction with major histocompatibility (MHC) class II may influence the profile of the immune response.

MATERIALS AND METHODS

Lymphocytes. Peripheral blood lymphocytes (PBL) from patients and control subjects were obtained by Ficoll-Hypaque sedimentation of anticoagulated whole blood as previously described (51). Some cells were cryopreserved in 10% dimethyl sulfoxide by controlled-rate freezing and stored in liquid nitrogen for later use (12).

Antigens. The isolation and partial purification and characterization of WI-1 have been described (31). In that study, the antigen was partially purified by electroelution for the principal purpose of radiolabeling with ^{125}I to detect antibody in infected patients. In the present study, WI-1 from *B. dermatitidis* yeast cells (ATCC 60636) was purified to homogeneity by high-performance liquid chromatography (HPLC) (unpublished data). HPLC-purified WI-1 is a glycoprotein that contains only minimal polysaccharide (4.1% estimated carbohydrate content), as assessed by the phenol-sulfuric acid method (dextran standard) (20).

Other antigens used included B-ASWS, prepared as described previously (15) from ATCC 60636; histoplasmin, prepared from *H. capsulatum* G217B and generously provided by Errol Reiss, Centers for Disease Control, Atlanta, Ga.; *Coccidioides* alkali- and water-soluble antigen (C-ASWS), generously provided by Rebecca Cox, San Antonio State Chest Hospital, San Antonio, Tex.; *Candida albicans* yeast cell cytoplasmic extract, prepared from strain B311A (ATCC 32354); and tetanus toxoid (Wyeth Laboratories Inc., Marietta, Pa.).

Antigen-induced lymphocyte proliferation. Standard microtiter methods were used (4, 30). In brief, 10^5 mononuclear cells (generally about 80% lymphocytes) were cultured in triplicate in 0.25-ml round-bottom microtiter plate wells (Costar, Cambridge, Mass.) containing 0.1 ml of HS-RPMI (RPMI 1640 medium supplemented with 25 mM *N*-2-hydroxyethylpiperazine - *N'* - 2 - ethanesulfonic acid [HEPES] buffer, L-glutamine, penicillin, streptomycin [Flow Laboratories, McLean, Va.], and 10 to 15% [by volume] heat-inactivated nontransfused human serum) and 0.1 ml of test antigen. Following incubation for 5 days with the antigen at 37°C in a 5% CO_2 humidified incubator, the cultures were pulsed with 1 μCi of [*methyl*-^{3}H]thymidine ([^{3}H]TdR) (New England Nuclear, Boston, Mass.). Radiolabeled cultures were incubated for 18 h and harvested with a MASH device (Otto Hiller, Madison, Wis.). The amount of [^{3}H]TdR incorporation was quantitated by liquid scintillation counting. Data are expressed as mean ± standard error counts per minute for antigen-stimulated cells minus that for unstimulated cells or as that for antigen-stimulated cells divided by that for unstimulated cells.

Cloning of T cells. Single-cell-derived clones were generated at a limiting dilution (36). In brief, 6-day cultures of antigen-stimulated lymphocytes were expanded for 48 h with 25 to 50 U of recombinant interleukin-2 (IL-2) (Cetus Corp., Emeryville, Calif.) per ml. Activated lymphocytes were then cultured in 0.25-ml round-bottom microtiter plate wells at an average quantity of 0.8 cell per well with 0.2 ml of HS-RPMI containing 2×10^4 gamma-irradiated (3,000 cGy) autologous PBL, 10^4 gamma-irradiated (8,000 cGy) autologous Epstein-Barr virus B-lymphoblastoid (EBV-LCL) cells, IL-2 at 20 U/ml, a 5% final dilution of a supernatant from a 3-day culture of PBL with 1,000 U of IL-2 per ml, and an appropriate concentration of stimulating antigen. Preliminary experiments showed that 20 μg of B-ASWS per ml was optimal for cloning *Blastomyces*-reactive T cells and that 5 μg of histoplasmin per ml was optimal for cloning *Histoplasma*-reactive T cells. Cultures were incubated at 37°C in a 5% CO_2 humidified incubator. Cells were isolated from wells showing growth after 3 to 4 weeks. They were screened for proliferative and cytotoxic responses (described below) to autologous EBV-LCL antigen-presenting cells in the presence and absence of fungal antigen, and antigen-reactive clones were expanded, each clone on a separate 96-well microtiter plate. Initial clonal expansion and restimulation every 10 to 14 days were performed with microtiter plate wells as in the original cloning. Some clones were cryopreserved at −135°C, thawed, and restimulated in the same manner.

Proliferation assays of clones. Minor modifications were made in antigen-induced proliferation assays to study the antigen responsiveness of clones or antigen-primed PBL in secondary assays. In brief, 10^5 responding cells were cultured in triplicate in 0.25-ml flat-bottom microtiter plate wells (Costar) with HS-RPMI containing 5×10^4 gamma-irradiated autologous EBV-LCL cells (8,000 cGy) or autologous PBL (3,000 cGy) as antigen-presenting cells plus test antigen or medium alone as a control. Following incubation for 48 h, the cultures were radiolabeled, harvested, and counted as described above.

Cytotoxicity assays of clones. Cell-mediated lysis or killing was measured in a standard 6-h ^{51}Cr release assay. Cloned effector cells were diluted in HS-RPMI and plated in round-bottom microtiter plate wells to yield the effector/target ratios described in each experiment. Target cells were gamma-irradiated autologous EBV-LCL cells cultured in test antigen or medium alone overnight and labeled with 250 μCi of ^{51}Cr (New England Nuclear) in 0.3 ml of HS-RPMI for 2 h. After being radiolabeled, target cells were washed twice in medium and diluted, and 5×10^3 cells were added to microtiter plate wells containing effector cells. The plates were centrifuged at $500 \times g$ for 10 min and incubated at 37°C in a humidified incubator containing 5% CO_2. After 6 h, supernatants were collected with Skatron (Sterling, Va.) harvesting frames, and ^{51}Cr release was counted in a gamma counter. The percent cytotoxicity was calculated by using the following formula: % cytotoxicity = [(experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)]

× 100. Spontaneous release and maximum release counts per minute (cpm) were determined by incubating target cells in medium alone and in 0.1% cetrimide detergent (Sigma Chemical Co., St. Louis, Mo.), respectively. In all experiments, spontaneous release counts per minute were ≤20% of maximum release counts per minute. Results are expressed as mean ± standard deviation for triplicate determinations.

MAbs for phenotyping and blocking experiments. Monoclonal antibodies (MAbs) reactive against CD3 (Leu 4), CD4 (Leu 3a), CD8 (Leu 2b), and T-cell receptor (TCR) αβ (WT31) were purchased from Becton Dickinson, Mountain View, Calif. MAbs directed against the Vα and Vβ gene elements of TCR αβ (WT31) and against TCR γδ (TCR δ-1) were purchased from T-cell Sciences, Cambridge, Mass. Flow cytometric analysis was performed with the FACStar or FACScan instrument (Becton Dickinson). Approximately 1×10^5 to 5×10^5 cells were incubated with a fluorescein isothiocyanate (FITC)-conjugated MAb for 30 min on ice, washed, and analyzed. For indirect staining with an unconjugated MAb, cells stained with an MAb as described above were reincubated with goat anti-mouse immunoglobulin G (IgG)–FITC for 30 min on ice, rewashed, and analyzed. Stained cells were analyzed by single-color flow cytometry with 4-decade LOG amplification. Viable cells were gated for stained cultures with forward scatter and propidium iodide (1 μg/ml) (Sigma).

Additional MAbs were used in experiments of antibody blocking of cytotoxicity or proliferation. Anti-HLA class I (monomorphic) (W6/32) and anti-HLA class II (HLA-DR [L243], HLA-DP [B7/21.2], and HLA-DQ [S3/4]) MAbs derived from American Type Culture Collection hybridoma supernatants were generously provided by Fritz Bach, University of Minnesota Medical School, Minneapolis. For assessment of antibody blocking of cytotoxicity, antibody was added to radiolabeled, washed target cells (final dilution, 1:100). Antibody-treated target cells were incubated for 60 min on ice prior to being plated with effector cells. For assessment of blocking of proliferation, antibody was added to plated EBV-LCL antigen-presenting cells (final dilution, 1:100). Antibody-treated EBV-LCL cells were incubated for 60 min at 37°C in a humidified incubator prior to the introduction of responding cells into the wells. In these experiments, isotype and other control antibodies were anti-CD3 (final dilution, 1:200), IgG1 (final dilution, 1:60), and IgG2A (final dilution, 1:20) (Becton Dickinson).

Inhibition of antigen processing. To assess whether intact WI-1 or denatured peptide forms of the antigen are recognized by T cells, we compared viable and paraformaldehyde-fixed EBV-LCL cells for the ability to present antigen to T cells. Light paraformaldehyde fixation, which renders antigen-presenting cells metabolically inert and incapable of processing antigen, was performed as described previously (11). In brief, 5×10^6 to 10×10^6 cells per ml were suspended in a 0.15% paraformaldehyde solution (pH 7.2). The cells were incubated for 5 min at room temperature. Fixation was stopped by the addition of a 2× volume of 0.5 M lysine (Sigma). Fixed cells were centrifuged, washed twice in HS-RPMI, and counted.

RESULTS

Antigen-induced lymphocyte proliferation. Ten blastomycosis patients, 10 control subjects with a prior history of histoplasmosis, and 10 healthy persons with no reported history of systemic mycoses were studied for the response of lymphocytes to WI-1, B-ASWS, and other fungal and control antigens. Six of the blastomycosis patients had localized pulmonary disease, and four had disseminated disease. Lymphocytes from blastomycosis patients responded vigorously to both WI-1 and B-ASWS (Table 1). Lymphocytes from control subjects with histoplasmosis and healthy persons responded to appropriate control antigens but did not cross-react to WI-1. In contrast, lymphocytes from histoplasmosis control subjects responded to B-ASWS, indicating some cross-reactivity to shared *Histoplasma* and *Blastomyces* determinants in this crude preparation.

WI-1 and B-ASWS share T-cell epitopes. We reasoned that WI-1 and B-ASWS might share antigenic determinants, since both antigens are rich in protein derived from either the surface or the outer region of the cell wall. To test this hypothesis, we studied lymphocytes from two representative blastomycosis subjects: patient 5, a 35-year-old Filipino male with disseminated disease of the lungs and skin, and patient 1, a 21-year-old black male with disseminated disease of the lungs and brain. Lymphocytes from the patients were stimulated for 6 days in vitro in separate flasks containing either WI-1 or B-ASWS to stimulate antigen-specific T cells. The cells from each flask were then tested separately in a secondary assay for the ability to recognize and respond to the corresponding stimulating antigen, the reciprocal *Blastomyces* antigen, and other fungal and non-fungal control antigens. Regardless of the *Blastomyces* antigen used initially to stimulate the lymphocytes, the cells responded vigorously to both WI-1 and B-ASWS in the secondary assay but not to other antigens (Table 2).

Cloning of antigen-specific T cells. We cloned T cells from patient 5 that were specific for *Blastomyces* antigenic determinants. The cloning strategy took advantage of the observations that WI-1 contained only (or mostly) *Blastomyces*-unique determinants and that WI-1 and B-ASWS shared immunologically important determinants. Thus, fresh PBL were stimulated in vitro for 6 days to enrich WI-1 antigen-specific T cells, and the cells were expanded with IL-2 (25 U/ml) for 48 h and cloned in the presence of B-ASWS. One hundred twenty-five T-cell clones were obtained from 1,800 wells plated with antigen-activated cells, a cloning efficiency of 6.9%. Of these clones, 80% responded in screening proliferation assays to *Blastomyces* antigens WI-1 and B-ASWS, whereas 20% responded to the antigen-presenting cells alone (autologous EBV-LCL cells) and were therefore directed against Epstein-Barr virus-encoded determinants (24). Twelve representative clones (10 *Blastomyces*-reactive and 2 EBV-LCL-reactive clones) were selected for detailed study, and the remainder were cryopreserved.

We also cloned *Histoplasma*-reactive T cells from a histoplasmin-positive donor, individual 11. Fresh PBL were stimulated with histoplasmin in vitro for 6 days, and the cells were expanded with IL-2 (25 U/ml) for 48 h and cloned in the presence of histoplasmin. Of the 75 T-cell clones obtained, 8 representative clones were selected for limited study, mainly as controls in experiments to assess the specific recognition of WI-1 by *Blastomyces*-reactive T-cell clones.

Phenotype and TCR gene usage of clones. Phenotyping of the cloned cells demonstrated that the 12 *Blastomyces*-reactive clones were 97 to 99% homogeneous in staining with antibodies to CD3, TCR αβ, and CD4 but showed no staining with antibody to TCR γδ or CD8. For examination of the V gene usage of these clones, they were stained with a panel of MAbs directed against the Vα and Vβ gene elements: Vβ5, a Vβ5 subset, Vβ6, Vβ8, Vβ12, Vα2, and a Vα locus not yet defined (recognized by clone LC4 on SUP-T13 cells [34]). Two of the 10 *Blastomyces*-reactive clones were composed

TABLE 1. Lymphocyte proliferation responses to WI-1 and other antigens in blastomycosis patients and in control subjects

Subject tested	Response to the following antigen (cpm)[a]:						
	None (medium)	WI-1	B-ASWS	Histoplasmin	*C. albicans*	Tetanus toxoid	IL-2
Blastomycosis							
1	523	27,051	33,152	13,541	13,183	1,870	39,812
2	424	7,250	15,300	540	23,214	NT	5,069
3	1,471	11,745	21,328	1,002	17,753	NT	10,673
4	304	3,538	3,470	605	4,899	NT	6,966
5	1,166	10,946	11,434	2,610	NT	NT	13,529
6	586	78,162	81,061	2,613	27,733	2,278	15,690
7	818	40,520	7,546	3,473	4,524	1,211	68,608
8	263	2,989	3,443	281	10,128	306	2,424
9	348	2,165	8,463	54,972	34,603	325	11,297
10	378	9,946	6,114	8,717	18,464	447	4,323
Mean ± SE	628 ± 40	19,431 ± 2,390	19,131 ± 2,362	8,835 ± 1,676	17,167 ± 1,019	1,073 ± 142	17,839 ± 2,076
Histoplasmosis							
11	290	311	6,692	31,208	7,230	4,886	46,707
12	1,629	1,494	18,211	6,572	46,387	15,999	21,303
13	1,364	3,187	49,226	31,758	56,513	26,875	36,124
14	1,139	1,320	11,801	28,437	29,342	7,583	33,698
15	773	940	1,401	4,248	844	486	5,266
16	482	563	874	20,359	1,785	257	35,834
17	628	1,275	3,753	18,271	11,222	653	7,449
18	469	731	593	25,513	1,741	444	42,204
19	534	663	2,730	9,276	13,691	555	42,237
20	602	595	1,719	5,251	5,403	630	14,905
Mean ± SE	791 ± 44	1,108 ± 82	9,701 ± 1,504	18,089 ± 1,102	17,443 ± 1,993	5,837 ± 895	28,573 ± 1,516
Normal							
21	630	1,064	543	348	1,189	NT	11,168
22	685	1,693	1,036	504	1,689	NT	14,252
23	455	1,171	878	357	770	NT	8,387
24	1,109	1,400	2,016	1,653	10,081	NT	45,726
25	531	1,375	1,350	938	9,636	NT	41,226
26	548	1,789	1,735	1,350	4,949	NT	18,858
27	617	1,685	1,389	703	3,089	NT	7,245
28	1,672	1,987	1,761	2,279	19,697	NT	6,908
29	435	1,894	802	829	3,891	NT	6,599
30	806	1,893	1,710	1,319	7,710	NT	43,649
Mean ± SE	749 ± 38	1,595 ± 32	1,322 ± 49	1,028 ± 62	6,270 ± 580	NT	20,402 ± 1,643

[a] [^{3}H]TdR uptake by antigen-stimulated and unstimulated lymphocytes was assessed by standard techniques as described in Materials and Methods. Results were recorded as counts per minute for triplicate parallel cultures. A positive lymphocyte response was defined as previously described (15): ≥2,000 cpm over the background response of lymphocytes cultured in medium alone and four times the background counts per minute. Antigens were prepared as described in Materials and Methods. Optimal concentrations were as follows: WI-1, 10 μg/ml; B-ASWS, 100 μg/ml; histoplasmin, 10 μg/ml; and *C. albicans*, 10 μg/ml. Tetanus toxoid was a standard commercial preparation of antigen that contained 340 μg of tetanus toxoid per ml; the final dilution in the assay was 1:10,000. IL-2 was a commercially available preparation used at a concentration of 200 U/ml. NT, not tested.

entirely of Vβ8 T cells; the other 8 *Blastomyces*-reactive clones and the two EBV-LCL-reactive clones showed no staining with the antibodies. In general, only 1 to 5% of resting T cells have been reported to utilize the Vβ8 locus recognized by the MAbs used here [3, 27], suggesting a possible 4- to 20-fold enrichment of Vβ8 T cells from patient 5 in response to WI-1. To exclude the possibility of a selection bias due to cloning or identification of clones for phenotyping, we examined the V gene usage of uncloned fresh lymphocytes from patient 5 in response to stimulation in vitro for 6 days with WI-1 or other stimuli, including B-ASWS, histoplasmin, *C. albicans*, and IL-2. Approximately 21% of cells stimulated with WI-1 utilized Vβ8, whereas only 4 to 8% of fresh PBL or PBL cultured with other stimuli expressed this gene locus on circulating αβ T cells (Table 3). No other preferential usage of V genes in these activated cells was identified by the MAbs.

Phenotyping of the eight *Histoplasma*-reactive clones studied demonstrated that they were 95 to 99% homogeneous in staining with antibodies to CD3, TCR αβ, and CD4 but showed no staining with antibody to TCR γδ or CD8.

Proliferative and cytotoxic functions. The *Blastomyces*-reactive clones proliferated vigorously in response to both WI-1 and B-ASWS (presented by autologous EBV-LCL cells) but not at all in response to the heterologous fungal antigens from *H. capsulatum*, *C. immitis*, and *C. albicans* (Table 4). EBV-LCL cells labeled with the non-*Blastomyces* antigens retained the ability to stimulate appropriately re-

TABLE 2. Examination of shared determinants on *B. dermatitidis* antigens WI-1 and B-ASWS

Patient	Stimulating antigen	Response to the following antigen(s) (cpm)[a]:					
		LCL alone	LCL and WI-1	LCL and B-ASWS	LCL and histoplasmin	LCL and *C. albicans*	IL-2
5	B-ASWS	1,100	16,524	16,758	NT	701	7,703
	WI-1	16,270	46,186	64,388	14,965	17,612	105,586
1	B-ASWS	8,986	42,509	33,184	17,059	13,754	115,240
	WI-1	14,882	27,807	25,554	14,024	15,065	98,609

[a] Mononuclear cells from patients 5 and 1 were antigen activated in vitro for 6 days in the presence of 100 μg of B-ASWS per ml or 10 μg of WI-1 per ml. Responder populations of mononuclear cells were then challenged in a secondary assay with the indicated antigen incubated in the absence or presence of autologous antigen-presenting B cells (EBV-LCL cells [LCL]). [^{3}H]TdR uptake by antigen-stimulated and unstimulated lymphocytes was assessed. Results were recorded as counts per minute for triplicate parallel cultures. When the responder populations were incubated with medium alone, the background counts for the proliferation assays for patient 5 were 273 cpm for B-ASWS and 1,067 for WI-1, and those for patient 1 were 450 cpm for B-ASWS and 1,316 cpm for WI-1. For calculation of the specific response to an antigen, the response to LCL must be subtracted from the response to LCL plus antigen. Thus, the specific response for the patient 5–WI-1 combination to WI-1 is 46,186 minus 16,270, or 29,916, and that to B-ASWS is 64,388 minus 16,270, or 48,118. The antigen concentrations used are described in Table 1, footnote *a*. NT, not tested.

sponsive T cells. The *Histoplasma*-reactive clones proliferated in response to histoplasmin but not WI-1. Clones K · 3f and K · 11b, directed against Epstein-Barr virus-encoded determinants, proliferated in response to EBV-LCL cells in medium alone and also in the presence of each fungal antigen. The results of the proliferation assays were similar when autologous PBL rather than EBV-LCL cells were used as the antigen-presenting cells (data not shown).

Two of the *Blastomyces*-reactive clones, K · 15f and K · 21b, demonstrated potent cytotoxic functions. At the low effector/target ratio of 10:1, these clones lysed 50 to 60% of EBV-LCL targets labeled with WI-1 or B-ASWS (Table 5). They did not lyse EBV-LCL targets labeled with other fungal antigens. Nor did *Histoplasma*-reactive cytotoxic clones lyse targets labeled with WI-1. EBV-LCL-reactive clones K · 3f and K · 11b lysed targets vigorously, independently of the nature of antigen labeling. The remainder of the *Blastomyces*-reactive clones tested showed little or no cytotoxic functions, even at the higher effector/target ratio of 30:1. Neither of the two *Blastomyces*-reactive Vβ8 T-cell clones (K · 1e and K · 20b) demonstrated cytotoxicity.

Antigen processing and HLA requirements for WI-1 antigen presentation. Paraformaldehyde fixation of EBV-LCL antigen-presenting cells abrogated the proliferative response of clone K · 24c to either WI-1 or B-ASWS, whereas this clone responded to either antigen after processing by nonfixed cells (Fig. 1). In contrast, in a parallel experiment, paraformaldehyde fixation did not influence the presentation by EBV-LCL cells of mycobacterial peptide antigens to appropriately responsive γδ T-cell clones (data not shown) (21).

Antibody blocking experiments were used to identify the MHC molecules that displayed WI-1 to the T-cell clones. The clones recognized WI-1 in association with HLA class II D loci of the MHC. The cytotoxic clones recognized epitopes together with HLA-DP, whereas the noncytotoxic clones recognize epitopes together with HLA-DR. Results for representative clones are illustrated in Fig. 2. The lysis of targets by cytotoxic clones K · 15f and K · 21b was reduced approximately 75% by an MAb directed against the CD3 polypeptide of the TCR complex, nearly abrogated by an MAb directed against a monomorphic determinant of HLA-DP, and relatively unaffected by an MAb directed against HLA-DR, HLA-DQ, or MHC class I molecules. Similar restriction patterns for these clones were demonstrated in proliferation assays (data not shown). In contrast, the proliferative response of clones K · 1e and K · 20f and other *Blastomyces*-reactive clones that proliferated but did not kill was virtually eliminated by an MAb directed against HLA-DR but relatively uninfluenced by MAbs directed against other class II loci. Isotype controls IgG1 and IgG2A had no influence on proliferation or cytotoxicity (data not shown).

TABLE 3. Selective enrichment of Vβ8 T cells in the response of PBL to WI-1[a]

Stimulus	% of PBL that were:		
	T cells	Vβ8 cells	Vβ8 T cells
Medium	72.0	3.0	4.2
WI-1 (10 μg/ml)	77.9	16.1	20.7
B-ASWS (100 μg/ml)	83.6	6.7	8.0
Histoplasmin (10 μg/ml)	82.7	4.9	5.9
C. albicans (10 μg/ml)	91.4	5.9	6.5
IL-2 (100 U/ml)	43.7	2.3	5.3

[a] Fresh PBL from blastomycosis patient 5 were cultured with the stimulus indicated and analyzed on day 1 (medium) or day 7 (other stimuli) by direct or indirect immunofluorescence. For determination of the percentage of T cells, cells were stained directly with anti-CD3–FITC and the corresponding isotype control, IgG1-FITC. Cells were also stained indirectly with a mouse MAb directed against the Vβ8 locus of the TCR and the corresponding isotype control, IgG2B, followed by goat anti-mouse IgG–FITC. Stained cells were analyzed by single-color flow cytometry. The value for Vβ8 T cells was calculated by dividing the value for Vβ8 cells by the value for circulating T cells and multiplying by 100.

DISCUSSION

In this report, we demonstrate that WI-1, a 120-kDa surface protein of *B. dermatitidis*, is a specific target of the cellular immune response in blastomycosis. Lymphocytes from recently and actively infected patients recognized and responded to the antigen in a standard in vitro proliferation assay. This finding should not be surprising, since WI-1 is an immunodominant target of antibody directed against *B. dermatitidis* (31), and most B-cell responses to protein antigens require antigen-specific T-cell help for antibody production (26). In contrast to findings for blastomycosis patients, lymphocytes from histoplasmosis and other control subjects showed no response to WI-1, and T-cell clones reactive with WI-1 showed no response to heterologous fungal antigens. Although there is extensive sharing of cell wall determinants in *B. dermatitidis* and other dimorphic fungi (45), our findings support the view that WI-1 does not contain broadly cross-reactive epitopes. This view is consistent with previous serologic observations that demonstrated

TABLE 4. Antigen-induced proliferation of WI-1-responsive and other human T-cell clones

Clone[a]	Response to the following antigen(s) (cpm)[b]:						
	LCL alone	LCL and WI-1	LCL and B-ASWS	LCL and histoplasmin	LCL and C-ASWS	LCL and *C. albicans*	IL-2
Blastomycosis							
K · 1e	2,259	25,391	24,801	1,545	2,032	1,738	43,440
K · 4d	1,927	34,170	36,944	2,118	2,147	2,102	61,715
K · 13e	2,751	19,091	17,257	1,802	2,144	2,128	54,329
K · 15f	2,228	24,834	25,233	1,702	1,912	1,842	92,238
K · 20b	2,540	13,204	39,349	1,643	2,178	1,679	41,499
K · 20f	2,553	24,258	38,903	2,026	2,067	2,054	25,314
K · 21a	2,138	26,652	28,247	2,592	2,562	2,003	17,454
K · 21b	1,945	13,898	29,531	2,002	2,103	1,804	20,219
K · 24a	2,625	9,850	18,071	1,645	2,128	1,624	10,697
K · 24c	2,093	36,159	43,927	2,045	2,062	1,800	6,827
K · 3f	83,110	75,204	76,227	78,276	79,049	81,463	79,712
K · 11b	31,516	25,481	26,480	26,582	25,105	26,225	6,018
Histoplasmosis							
J · 3B10	223	450	NT	22,425	NT	NT	65,333
J · 7A8	344	560	NT	19,894	NT	NT	45,657
J · 1D3	307	592	NT	21,654	NT	NT	75,241
J · 5E4	1,636	846	NT	19,947	NT	NT	30,723

[a] Clones derived from blastomycosis patient 5 are designated by the prefix "K," and those derived from histoplasmin-positive donor 11 are designated by the prefix "J."

[b] Cells were incubated in the absence or presence of autologous EBV-LCL antigen-presenting cells (LCL) and an appropriate concentration of antigen, as indicated in Table 1, footnote *a*, for 48 h. They were radiolabeled with [^{3}H]TdR, incubated, harvested, and counted as described in Materials and Methods. NT, not tested.

negligible titers of antibody to WI-1 in histoplasmosis, coccidioidomycosis, and sporotrichosis patients with high titers to the respective fungi and suggests a lack of cross-reactive B-cell epitopes on WI-1 (31).

It appears that WI-1 shares important T-cell epitopes with B-ASWS, which has been the standard T-cell antigen used for cellular studies of blastomycosis. We showed that enriching patient lymphocytes for WI-1 (or B-ASWS)-reactive T cells produced an equivalent response to either WI-1 or B-ASWS in a secondary proliferation assay. The T-cell cloning strategy that we used was based on this observation. We hypothesized that lymphocytes enriched for WI-1-reactive T cells would be directed against cell wall determinants uniquely present in *B. dermatitidis* and that cloning of the cells in the presence of B-ASWS would expand T cells that recognize the dominant epitopes of B-ASWS also expressed on the WI-1 molecule. The antigen reactivity and specificity of the clones that we derived support this hypothesis. They all responded to both *B. dermatitidis* antigens but not other antigens.

TABLE 5. Cytotoxic effects of WI-1-responsive and other human T-cell clones[a]

Clone	% Specific cytotoxicity with the following antigen(s):					
	LCL alone	LCL and WI-1	LCL and B-ASWS	LCL and histoplasmin	LCL and C-ASWS	LCL and *C. albicans*
Blastomycosis						
K · 15f	0	51	51	0	0	0
K · 21b	0	38	49	0	0	0
K · 13e	0	7	2	0	0	0
K · 20b	0	2	13	0	0	0
K · 21a	0	0	1	0	0	0
K · 20f	0	0	2	0	0	0
K · 24c	0	NT	8	NT	NT	NT
K · 4d	0	NT	5	NT	NT	NT
K · 3f	44	46	49	42	50	44
K · 11b	58	58	64	67	72	66
Histoplasmosis						
J · 2G12	0	0	NT	24	NT	NT
J · 3G12	0	1	NT	68	NT	NT
J · 7G12	0	1	NT	44	NT	NT
J · 10C1	0	0	NT	31	NT	NT

[a] The cytotoxic effects of clones derived from blastomycosis patient 5 (designated by the prefix "K") and histoplasmin-positive donor 11 (designated by the prefix "J") were assessed in a standard 6-h chromium release assay. Results reported are for an effector/target ratio of 10:1. Targets were autologous EBV-LCL cells (LCL) that were antigen labeled by overnight incubation with an appropriate concentration of antigen, as indicated in Table 1, footnote *a*. NT, not tested.

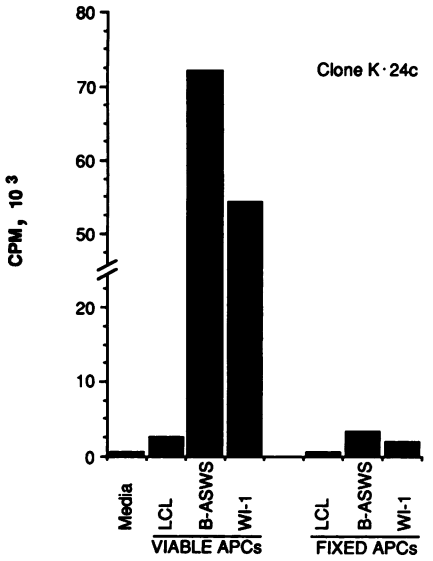

FIG. 1. Influence of paraformaldehyde fixation on the processing and presentation of WI-1 and B-ASWS. Viable and fixed autologous antigen-presenting cells (APCs) (EBV-LCL cells [LCL]) were assessed for the ability to present antigen to clone K · 24C. Fixation was performed as described in Materials and Methods. Antigen concentrations were as described in Table 1, footnote *a*.

All of the clones studied in depth are αβ T cells that express the CD4 antigen, an accessory molecule of the TCR complex that enables interactions with class II MHC molecules. We cannot exclude the possibility that some of the other clones that were not phenotyped and studied in detail express a different phenotype. Alternatively, the initial enrichment of antigen-specific T cells and cloning conditions may have been biased toward the growth and selection of $CD4^+$ T cells. Another study has shown that cloning in the presence of IL-4, rather than IL-2, leads to the proliferative expansion of $CD8^+$ T cells (42). However, the processing of a soluble fungal antigen through the exogenous pathway and the subsequent association of the antigen with class II MHC molecules would likely dictate that a major component of the response would be mediated by class II-restricted $CD4^+$ T cells (38).

Further phenotyping of the clones suggested the preferential usage of a particular V gene element of the TCR, Vβ8, in the immune response to WI-1. Although only 1 to 5% of resting human T cells in peripheral blood express the Vβ8 locus, 2 of the 10 WI-1-reactive T-cell clones expressed this V gene element. The enrichment of Vβ8 T cells in PBL stimulated with WI-1 but not other antigens argues against a cloning artifact and supports a preferential and specific usage of the Vβ8 gene element. The lack of enrichment of Vβ8 T cells in PBL stimulated with B-ASWS may simply have been due to the crude nature of this antigen preparation, leading to the stimulation of other lymphocyte populations by nonshared determinants and a corresponding dilution of Vβ8 T cells. We recognize that our observations of Vβ8 usage are based on clones and PBL from only a single individual. In

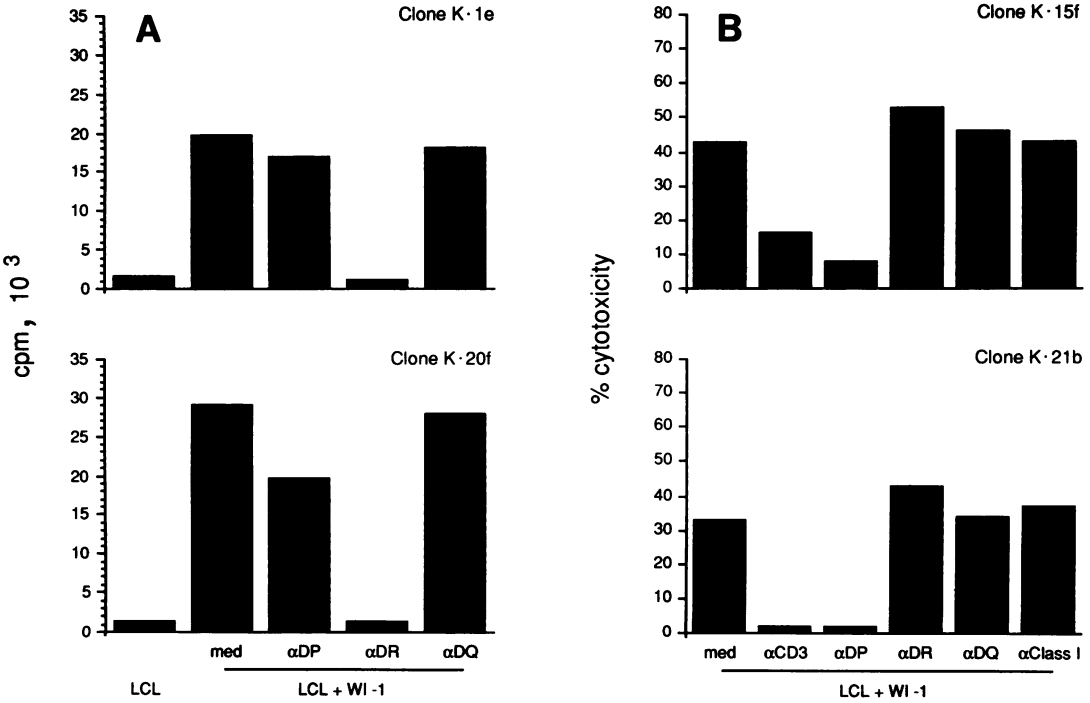

FIG. 2. HLA requirements for WI-1 recognition by proliferative and cytotoxic T-cell clones. MHC class I and II molecules involved in antigen presentation were assessed in antibody blocking experiments. Proliferation (panel A) or cytoxicity (panel B) was assessed in the presence of medium alone (med) or the designated MAb in parallel. The MAbs used were as follows: HLA-DR (αDR; L243); anti-HLA-DP (αDP; B7/21.2); anti-HLA-DQ (αDQ; S3/4); anti-HLA-A, anti-HLA-B, and anti-HLA-C (αClass I; W6/32); and anti-CD3 (αCD3). The experimental conditions and final dilutions of the MAbs used for blocking are described in Materials and Methods. Antigen concentrations were as described in Table 1, footnote *a*. LCL, EBV-LCL cells.

general, however, the preferential usage of V gene elements thus far has been a property that reflects the nature of the antigen rather than the individual (35). We cannot exclude the possibility, however, that subsets of blastomycosis patients exhibit restricted patterns of V gene usage in response to WI-1, as has been shown in subsets of patients with sarcoidosis, whose T cells exhibit the preferential usage of Vβ8 (37).

A subset of the $CD4^+$ T-cell clones that we studied demonstrated strong, specific cytotoxic responses against WI-1 antigen-labeled targets. To our knowledge, antigen-specific T cells with direct cytolytic effects have not been described previously for the immune response to *B. dermatitidis* or other dimorphic fungi. However, recent studies with mice and humans infected with *Mycobacterium* spp. and other intracellular organisms have identified cytolytic T cells (1, 28, 29, 39, 44, 46). In murine models of listeriosis and tuberculosis, $CD8^+$ cytolytic T cells confer protection to naive mice in adoptive transfer experiments (29, 39), suggesting that these cells play a key role in acquired resistance. The precise role of $CD4^+$ cytolytic T cells in the immune response has not been clarified, but recent studies of human tuberculosis have proposed that such cells may be involved in regulating T- and B-cell responses by directly lysing the MHC class II-bearing cells presenting the 65-kDa heat shock protein antigen of mycobacteria (41). These investigators suggested that this type of immunoregulation may explain why tuberculoid leprosy patients are good cytotoxic T-cell responders and have low specific antibody titers, whereas the opposite is true for lepromatous leprosy patients. A similar striking discordance between antibody production and the proliferative T-cell response has been documented in cases of progressive coccidioidomycosis (14), but the involvement of cytolytic T cells has not been explored. The finding of cytolytic T cells in human blastomycosis raises several questions that require future study. (i) Do these effector cells kill or inhibit the growth of conidia or yeasts or produce tissue injury and immunopathology? (ii) Is cytotoxicity mediated directly by effector-target binding and the release of lytic granules (25) or indirectly by secretion of lymphokines, such as tumor necrosis factor β (22)? (iii) Are cytolytic T cells a consistent feature of the immune response in blastomycosis, or might the unusual HLA restriction pattern of the potent killers K · 15f and K · 21b here indicate that this feature is unique to a subset of patients with a particular HLA background?

We examined the requirements for antigen processing and presentation of WI-1 antigen to T-cell clones. Paraformaldehyde fixation of EBV-LCL cells eliminated the metabolic activity of the cells and abrogated the presentation of WI-1. These observations are consistent with the view that WI-1 is a large globular protein that requires uptake and denaturation within antigen-presenting cells and subsequent charging of peptide epitopes with MHC molecules for surface membrane display. We were surprised that fixation also impaired the presentation of B-ASWS to the clones, since that antigen is somewhat denatured and certain other peptide antigens can bind directly to cell surface MHC and be presented to T cells without processing (50). Our findings suggest, however, that the fragmented determinants in B-ASWS require additional processing for proper display of the T-cell epitopes. The small residual T-cell response to either of these antigens presented by fixed EBV-LCL cells could reflect a response to either very low molecular weight breakdown products present in limited quantities in the antigens or very minor epitopes that can be recognized on unprocessed forms of the antigens, as has been described for selected epitopes on the fibrinogen molecule (2).

MHC class II molecules were required for the display of WI-1 and B-ASWS epitopes to the clones. Antibody blocking experiments demonstrated that most of the *Blastomyces*-reactive clones responded to epitopes displayed by class II products of the HLA-DR gene locus. The potent cytotoxic clones, however, responded to epitopes displayed by class II products of the HLA-DP locus. It is not known whether these clones responded differently because they recognized a different antigenic epitope or because a common epitope was displayed in a different manner by an unusual restricting element. Very few soluble antigens whose presentation requires MHC products of the HLA-DP locus have been described (40). Might the allelic expression of genes at this locus influence binding to selected epitopes and, in turn, the profile of the immune response? Using a murine model of leishmaniasis, Scott et al. (49) showed that a single HPLC fraction of soluble leishmania antigen contains a component that may activate either a Th1 subset of helper T cells that confers protection to naive animals or a Th2 subset that exacerbates the disease in these animals. This observation suggests either that different epitopes alter the profile of the immune response or that common epitopes displayed differently alter this profile. This suggestion raises the question of how the HLA background and various allelic forms of class II MHC molecules may influence either the manner of binding and presentation of immunodominant epitopes of WI-1 or the composition of T cells in the host's repertoire generated during thymic selection and, in turn, the profile of the immune response to WI-1.

Clarification of the immunodominant epitopes of WI-1 recognized by the clones described here, the regulatory and effector functions of the clones themselves, and the influence of human HLA loci and alleles on the response of T cells to WI-1 will serve to advance our understanding of the pathogenesis of blastomycosis and of the cellular defense mechanisms that control this and other endemic systemic mycoses.

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