

## Minor Histocompatibility Antigens

### *Project Aspects*

#### **A. Specific Aims**

The goal of the Minor Histocompatibility Antigen Component of the 13th IHWG is to provide investigators with the opportunity to map genetic loci that encode human minor histocompatibility antigens (mHA) recognized by cytotoxic T lymphocyte (CTL) clones. Specific Aims are as follows: 1) To distribute a panel of well characterized Epstein-Barr virus-transformed B lymphoblastoid cell lines (EBV-LCL) from 12 extended families (obtained from the Centre D'Etude du Polymorphisme Humain) to investigators who have generated CTL clones that recognize human minor histocompatibility antigens; 2) To assemble and distribute recombinant vaccinia vector constructs encoding HLA-A\*0101, HLA-A\*0201, HLA-A\*0301, HLA-B\*0702 and HLA-B\*4402 for transient expression in EBV-LCL used as targets in CTL assays with clones that recognize mHA; and 3) To provide data analysis support for genomic mapping. Results from this component will represent a first step toward developing a unified nomenclature for human mHA and will help to determine whether the CTL used in different laboratories define similar or possibly identical mHA. Most importantly, results of genomic mapping will create an impetus to identify of peptide components of human mHA recognized by CTL.

#### **B. Background and Significance**

**B.1. Background.** Minor histocompatibility antigens (mHA) provoke T cell immune responses that cause allograft rejection and graft-versus-host disease (GVHD) (reviewed in 1-3). These antigens are encoded by genes outside the major histocompatibility complex (MHC) and are formed by the binding of short polymorphic peptides in the groove between the  $\alpha$ -helices of MHC class I and class II molecules. T cell responses are initiated when the T cell receptor binds to complexes formed by peptides and MHC molecules on antigen presenting cells.

Minor histocompatibility antigens were originally defined in rodents by skin graft rejection and in rodents and humans by cytotoxic assays with T cell clones. Progress was greatly advanced by the development of methods that allowed biochemical definition of the peptide component. This was accomplished by elution, purification and sequencing of peptides from MHC molecules or by expression cloning and peptide screening. With the use of these methods, peptides have been defined for at least nine rodent mHA and four human mHA.

Results of recent studies have demonstrated the potential clinical significance of mHA. Recipient disparity for a mHA termed HA-1 was associated with an increased risk of acute GVHD after HLA-identical allogeneic marrow transplantation (4). This antigen was originally defined by a cytotoxic T lymphocyte (CTL) clone which was recovered from a marrow transplant recipient (5). The HA-1 peptide was identified through elution from HLA-A2 molecules, biochemical purification and sequencing (6). The gene that encodes HA-1 was identified after screening published genome sequences corresponding to the peptide sequence. These advances have now made it possible to develop easily applicable genetic assays for testing HA-1 compatibility in marrow transplantation (7, 8) and to develop methods for generating CTL that could be used for adoptive immunotherapy (9). In the future, it may be possible to use peptide-based treatment to induce tolerance to mHA.

**B.2. Significance.** The impetus for this project emerged from discussions during the First International Symposium on Minor Histocompatibility Antigens, a meeting made possible with support from the NHLBI and NIAID (10). Participants at this meeting recognized that further progress in defining mHA would require a genomic approach that could be greatly facilitated through an international collaboration involving the use of shared resources. Toward this end, participants proposed that a project in the 13th IHWG should be designed to provide investigators with the opportunity to map genetic loci that encode human mHA recognized by CTL (11, 12). It was anticipated that mapping of these loci will represent a first step toward developing a unified nomenclature and will help determine whether the CTL used in different laboratories define similar or possibly identical mHA.

EBV-transformed human lymphoblastoid cell lines (EBV-LCL) can be readily obtained for genetic analyses, but these cells can be tested as targets in CTL assays only when they express the appropriate MHC molecules for peptide presentation. Progress has been hampered by the paucity of material with appropriate MHC molecules and by the difficulty of transfecting EBV-LCL. Laboratories that participate in this component of the 13th IHWG will gain access to recombinant vaccinia vector constructs that encode MHC molecules for transient expression in EBV-LCL, thereby facilitating the use of these cells as targets in CTL assays.

The strategy we propose has been used successfully to map loci encoding human mHA within regions encompassing 21 cM on chromosome 22 and 58 cM on chromosome 11 (11, 12). With the availability of rapidly emerging genome sequence data, we expect that results from genomic mapping of loci encoding mHA will enable investigators to use strategies for immunoselection with deletion mapping (13, 14) and expression mapping (15) to identify the peptide components of mHA.

## C. Preliminary Results

**C.1. Organizational preparation.** Organization of this project began after the First International Symposium on Minor Histocompatibility Antigens in September, 1997. An advisory committee was established to oversee the project. The following tables identify the members of this committee and document their expertise.

**Table 1. Advisory Committee Members**

<u>Member</u>	<u>Affiliation</u>
Paul Martin, MD, (Project Leader)	Fred Hutchinson Cancer Research Center
Patrick Beatty, MD	University of Utah
Els Goulmy, PhD	University Hospital, Leiden
Mark Leppert, PhD	University of Utah
Stan Riddell, MD	Fred Hutchinson Cancer Research Center

**Table 2. Documentation of Expertise**

<u>Member</u>	<u>Area of Expertise</u>	<u>Representative Publications</u>
Martin	mHA in marrow transplantation	(16-20)
Beatty	genomic mapping of mHA	(11-12)
Goulmy	CTL clones specific for mHA	(2, 4-7, 9-10)
Leppert	human genetics	(11-12)
Riddell	vaccinia vectors	(21-23)

As documented in the Experimental Plan, individuals involved in research with CTL clones that recognize mHA were contacted to solicit their interest and participation. With this information, members of the Advisory Committee selected the restriction elements to be included in the project. The Advisory Committee also determined the appropriate number of pedigreed families to include in the project and selected the families most likely to provide informative results based on the numbers of family members and the numbers of catalogued markers. Finally, committee members concluded that methods for stable expression after electroporation (11, 12) or retroviral transfection would not be feasible with a panel of 181 cell lines and 5 restriction elements and recommended that vaccinia vectors be used for transient expression of HLA molecules as restriction elements for presentation of mHA (24). Additional studies were carried out in order to demonstrate the feasibility of using vaccinia vectors for this project.

## C.2. Results of Preliminary Experiments.

*Use of vaccinia HLA-A\*0201 recombinant virus to express HLA-A2 on EBV-LCL target cells.* A vaccinia HLA-A\*0201 (vac-A2) recombinant virus was kindly provided by Dr. Jonathan Yewdell (NIH). HLA A2 negative EBV-LCL were infected at a multiplicity of infection (MOI) of 5 for 15 hours with vaccinia wild type (vac-wt), vac-A2 or were left uninfected and then examined for expression of HLA-A2 by staining with an HLA-A2-specific monoclonal antibody. After infection with vac-A2, a substantial fraction of the cells expressed HLA-A2 at levels equivalent to LCL derived from an HLA-A2-positive individual (Figure 1).

*Vac-A2 infected LCL present HLA-A2-restricted antigens to CD8<sup>+</sup> CTL.* LCL from 8 individuals who lacked HLA-A2 were infected with vac-A2 at an MOI of 5 for 15 hours and assayed as targets for a CD8<sup>+</sup> CTL clone termed PAM-13 previously isolated in our laboratory and shown to be specific for a mHa presented by HLA-A2 (25). At a 10:1 E:T ratio, PAM-13 cells produced 0-3.7% lysis of vac-wild type infected HLA-A2-negative targets (Figure 2). After infection with vac-A2, 3 of the 8 targets had 3.4-7.0% lysis, suggesting that these cells did not express the mHA recognized by the PAM-13 clone. The other 5 targets had 13.9-36.9% lysis, indicating that these cells expressed the mHA recognized by the PAM-13 clone. These results demonstrate that mHA can be presented by target cells that are induced to express an appropriate MHC restriction element by transfection with a vaccinia vector.

## D. Experimental Plan

### D.1. Approach.

*Overview.* Genetic mapping of minor histocompatibility loci-encoding antigens recognized by CTL will be carried out according to the method described by Gubarev et al. (11, 12) using a panel of 181 cell lines from the Centre D'Etude du Polymorphisme Humain (CEPH). The cell line panel represents 12 families each with 3 generations including 2 parental probands, 2-4 grandparents, and 8-15 offspring from the parents. These cell lines have been allele typed previously for thousands of genomically localized polymorphic markers.

**Table 3 CEPH Families Selected for Study**

Family Identification Number	Number of Samples Available			Number of Catalogued Markers*
	Grandparents	Parents	Offspring	
1331	3	2	11	10280
1332	3	2	10	10236
1333	4	2	9	3849
1341	4	2	8	3905
1344	3	2	9	3865
1346	4	2	8	3822
1347	4	2	9	9656
1349	3	2	8	3776
1362	4	2	11	10255
1408	4	2	8	3426
1413	2	2	15	9679
1416	4	2	9	9776

\*data provided by Dr. Mark Leppert (Univ. of Utah)

Cell lines of the 24 parental probands will be distributed to the participating laboratories to be tested as targets in assays with CTL clones previously generated and characterized within each participating laboratory. Participating laboratories will receive recombinant vaccinia vectors and protocols for transient expression of HLA-A\*0101, HLA-A\*0201, HLA-A\*0301, HLA-B\*0702 and HLA-B\*4402 as restriction molecules for presentation of minor antigen peptides recognized by CTL clones. Monoclonal antibodies will also be distributed so that expression of MHC restriction molecules can be verified when transfected cell lines are tested as targets in CTL assays. Hybridomas producing monoclonal antibodies specific for HLA-A2, A3 and B7 will be obtained from ATCC. Antibodies specific for HLA-A1 and B44 will be provided by Dr. Paul Teraskai (One Lambda, Los Angeles).

For each CTL clone, the screening phase of testing will serve to identify a set of at least 3 "informative" families in which one parental proband is positive and the other parental proband is negative in the CTL assay. After the screening is completed, participating laboratories will select 3-5 families for further testing with each CTL clone. Results of testing with cell panels will be analyzed as described by Gubarev et al. (11, 12)

*Construction of vaccinia recombinant viruses encoding HLA-A\*0101, HLA-A\*0301, HLA-B\*0702 and HLA-B\*4402.* A plasmid encoding the cDNA for HLA-A\*0101 has been obtained from Dr. Thomas Spies, FHCRC, and the cDNA was subcloned into pcDNA3 (Invitrogen). cDNAs encoding HLA-A\*0301 and HLA-B\*0702 have been obtained in our laboratory by amplification of cDNA from an A3<sup>+</sup> B7<sup>+</sup> EBV-LCL line with primers HLA-A3-5P 5' AAGGTACCATGGCCGTCATGGCGCCCCGA3' and HLA-A3-3P 5' GGTCTAGATCACACTTTACAAGCTGTGAG3' and with HLA-B7-5P 5' AAGGTACCATGCTGGTCATGGCGCCCCGAA3' and HLA-B7-3P 5' GGTCTAGATCAAGCTGTGAGAGACACAT3', respectively. Amplified segments were then cloned into pcDNA3. The cDNA for HLA-B\*4402 will be obtained from Core C or by amplification as described previously (26) and similarly cloned into pcDNA3.

Vaccinia recombinant viruses encoding individual HLA alleles will be constructed using standard homologous recombination methods (27-30). These methods have been used previously in our laboratory to construct vaccinia/CMV recombinant viruses encoding pp150, pp65, IE-1 and IE-2. The pSC11 plasmid (provided by Bernard Moss) encodes flanking sequences for vaccinia virus TK, the lacZ gene driven by the p11-promoter for identification of recombinants, and the p7.5 promoter to drive expression of the insert. pSC11 has been modified in our laboratory by insertion of the multiple cloning site from pBluescript SK (Stratagene) to form pSC11-T7. This facilitates directed cloning of inserts downstream from p7.5 (Figure 3).

The individual HLA cDNA inserts will be excised from the respective pcDNA-3 plasmids using KpnI and Xba and ligated to pSC11-T7 using the same restriction sites. DNA from a positive pSC11 subclone will be transfected (Lipofectin, Gibco) into BSC 40 cells previously infected for 2 hours with wild type vaccinia virus. Cells will be harvested after 2 days, and then frozen and thawed three times to make a stock virus. Recombinant viruses will be isolated from the stock virus by plating dilutions onto HuTK-143B cells, and positive plaques will be selected by BrdU and Xgal staining. Plaques will be purified 3 times, amplified in BSC-40 cells and titred according to standard procedures (28). Aliquots of the titred vaccinia virus will be cryopreserved in 0.5 ml volumes at -70°C.

Vaccinia recombinants will be tested for expression of the encoded HLA allele by infecting EBV-LCL at MOI's of 1, 5, 10 and 20 pfu/cell for 8, 12 and 16 hours. Cells will then be stained by indirect immunofluorescence with a murine monoclonal antibody specific for the respective vac-encoded HLA allele and analyzed by flow cytometry. To demonstrate that the allele introduced by the vaccinia recombinant can present class I-restricted mHA, experiments will be carried out with well characterized T cell clones generated at the FHCRC (30) or in Leiden (2). Briefly, EBV-LCL from the CEPH family panel will be infected with the vaccinia recombinant virus at the optimal MOI and time, labeled with <sup>51</sup>Cr and plated in triplicates in 96 ml round bottom plates with medium alone, NP-40, or CTL clones at effector to target ratios of 5, 10 and 20:1. Supernatants will be harvested after 4 hours, and specific lysis will be evaluated according to the standard formula (23). Variations in the vaccinia infection, effector to target ratio and assay duration will be assessed to define the optimal conditions for use with each vaccinia-HLA recombinant virus. A standard protocol procedure will be developed for dissemination to participating laboratories.

**D.2. Responsibilities of Participants.** This component of the 13th IHWG will entail collaboration between a core laboratory, participating laboratories and a data analysis unit.

*Core Laboratory.* The core laboratory will assemble the panel of cell lines from 12 selected CEPH families, construct recombinant vaccinia vectors for expression of MHC restriction molecules, develop robust and reliable protocols for transfection with recombinant vaccinia vectors, validate expression of MHC molecules by staining with monoclonal antibodies, validate the identity of each cell line with a panel of variable number tandem repeat (VNTR) polymorphisms, and distribute cell lines from the 24 parental probands together with transfection protocols, vectors and monoclonal antibodies for initial screening, provide backup support and trouble-shooting for transfection and CTL assays, and distribute cell panels from selected families for follow-up testing.

*Participating Laboratories.* Each participating laboratory will test the cell panel only with its own CTL clones or genetic assays. Laboratories will register each CTL clone by specifying the MHC restriction element and approximate antigen frequency and by describing how the clone was originally generated. Laboratories will test the 24 parental proband cell lines transfected with the appropriate MHC restriction molecules, select at least 3 informative families for further testing with each CTL clone, test informative families and report results for data analysis. Participating laboratories will also provide results with appropriate positive and negative controls for each CTL clone tested. Each participating laboratory will be expected to use its own resources to support all 13th IHWG-related activities.

**Table 4. Participating Laboratories and CTL Clones Specific for mHA**

## HLA Restriction Allele

Investigator	Location	HLA Restriction Allele				
		<u>A*0101</u>	<u>A*0201</u>	<u>A*0301</u>	<u>B*0702</u>	<u>B*4402</u>
Dolstra	Nijmegen		1			1
Falkenberg	Leiden		1		1	
Goulmy	Leiden	1	2			
Martin	Seattle			1	1	
Roosnek	Geneva		1	1	1	
Warren	Seattle		4	1	6	2
Yeo	Seattle		1			
Total		1	10	3	9	3

*Analysis of Data.* Data will be analyzed by Drs. Patrick Beatty and Mark Leppert at the University of Utah. Results of genomic mapping with each CTL clone will be disclosed before the 13th IHWG meeting to the participating laboratory that reported the data. Global results for all CTL clones will be reported at the 13th IHWG meeting.

**D.3. Expected Results.** With a panel of 24 parental probands from 12 families, there is  $\geq 80\%$  probability of identifying at least 3 informative families (one parent positive and the other negative) if the antigen frequency is between 20% and 80%. Likewise, there is  $\geq 60\%$  probability of identifying at least 3 informative families if the antigen frequency is between 15% and 85%. More extreme antigen frequencies are associated with lower chances of success. If at least 3 informative families are identified, then genomic mapping is almost certain to be successful.

**D.4. Potential Pitfalls.** Genomic mapping will not be successful if samples are misidentified at any stage of the process. Therefore, participating laboratories will be advised that scrupulous precautions must be taken to avoid such errors. Laboratories will be asked to report results with sufficient time before the workshop meeting to allow further testing as needed to resolve discrepancies. Participating laboratories will also be asked to retain DNA samples from each cell line to assist in tracing errors with the use of VNTR markers.

CTL that recognize peptide antigens can crossreact with HLA class I alloantigens. In order to be able to assess this possibility, we plan to type the HLA-A, B and C alleles of the 181 cell lines to be used in this project. This task will be completed in collaboration with Cores C and E.

## D.5. Calendar

### Time Table and Deadlines

<u>Deadline</u>	<u>Core Laboratory</u>	<u>Participating Laboratory</u>	<u>Data Analysis</u>
June, 2000	Distribute recombinant Vaccinia vectors, monoclonal antibodies and cell lines for parental probands.	Register CTL clones.	
August, 2000		Report results of CTL screening.	
September, 2000	Distribute cell lines for selected families.		
December, 2000		Report data.	
January, 2001			Identify discrepancies.
March, 2001		Resolve discrepancies.	
April, 2001			Final analysis.
May, 2001			Write report.
June, 2001			13th IHWG meeting.

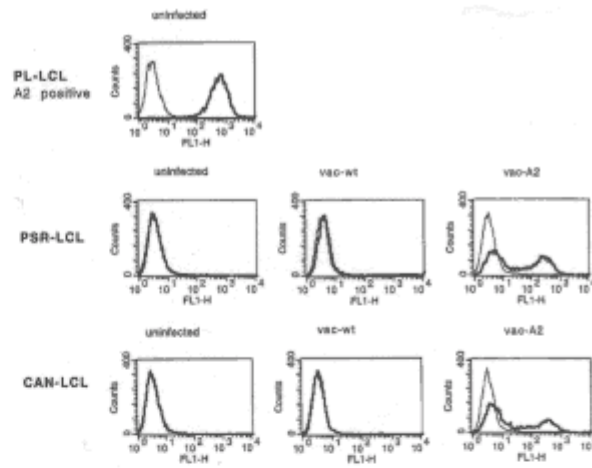
### *Program Aspects*

The focus on peptide alloantigens that bind to HLA molecules represents an extension from the traditional scope of previous Histocompatibility Workshops. This extension is justified by the knowledge that peptide binding is the primary function of HLA molecules. As discussed in the Experimental Plan, the identification of loci that encode mHA can be approached through genomic methods, and this effort will be facilitated through the international collaboration sponsored by the 13th IHWG.

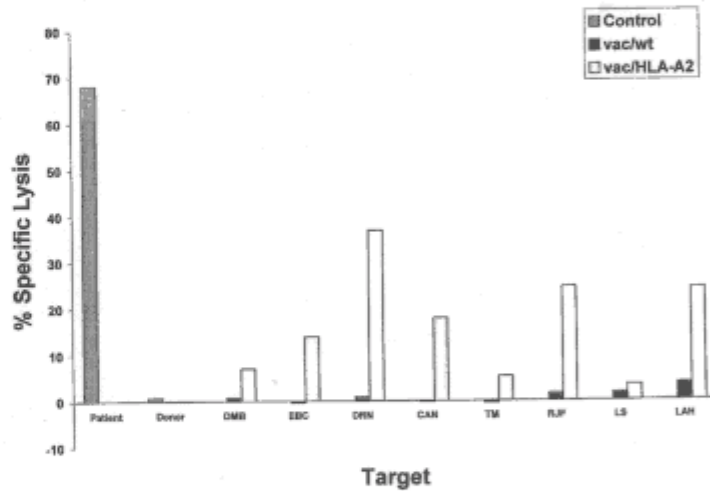
Operational aspects of this project will require support from Cores C and E. Core C will provide cDNA encoding HLA-B\*4402, acquire the panel of 181 CEPH cell lines and manage their distribution. Cores C and E will share responsibility for HLA-class I allele typing of the 181 cell lines.

Although the scientific aspects of this project clearly stand alone, we envision that interactions between Project 11 and Project 9 might emerge in the future. If genomic mapping leads to the identification of a candidate gene that encodes a mHA, interactions with Project 11 could help to define the exact peptide that is recognized by the CTL. After a genomic assay becomes available, interactions with Project 9 could help to determine whether disparity for a mHA affects outcome after transplantation.

**Figure 1.** Expression of HLA-A2 after infection with wild type vaccinia (vac-wt) or with a vaccinia vector containing an HLA-A2 cDNA insert (vac-A2). Thin lines depict control staining with a FITC-conjugated antibody specific for murine Ig. Thick lines depict indirect immunofluorescent staining with an HLA-A2-specific monoclonal antibody. PL-LCL is an HLA-A2 positive control, while PSR-LCL and CAN-LCL were derived from HLA-A2-negative individuals.



**Figure 2.** Lysis of a panel of HLA-A2-negative cell lines by a CTL clone specific for a mHA presented by HLA-A\*0201. The PAM-13 CTL clone was derived from the blood of a patient who had received a marrow transplant from an HLA-identical sibling (25). The donor-derived CTL clone showed strong lytic activity against an EBV-LCL derived from the recipient but had no activity against an EBV-LCL derived from the donor. The PAM-13 clone did not lyse HLA-A2-negative EBV-LCL infected with wild type vaccinia virus (vac-wt). Three of the eight EBV-LCL (DMB, TM, LS) showed < 10% lysis after infection with vac-A2, while the remaining five EBV-LCL (EBC, DRN, CAN, RJF and LAH) showed > 10% lysis after infection with vac-A2.



**Figure 3.** pSC11-T7 vector with multiple cloning sites.



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