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Diversity in the Acute CD8 T Cell Response to Vaccinia Virus in Humans\textsuperscript{1,2}

Lichen Jing,* Tiana M. Chong,† Christopher L. McClurkan,‡ Jay Huang,‡ Brian T. Story,‡ and David M. Koelle\textsuperscript{3}\textsuperscript{*†‡§}

Orthopoxviruses have complex proteomes. Infection provokes a brisk CD8 response, which is required in some systems for recovery from primary infection. Little is known concerning the Ags and epitopes recognized by CD8 T cells. We examined the fine specificity of cloned and bulk human vaccinia-specific CD8 CTL by expressing polypeptide fragments from a library of vaccinia genomic DNA. This epitope discovery method emphasizes virus-specific biological activity, as the responder cells are all reactive with whole vaccinia virus. Sixteen novel epitopes, restricted by several HLA A and B alleles, were defined to the nonamer peptide level in diverse vaccinia open reading frames. An additional seven epitopes were mapped to short regions of vaccinia proteins. Targets of the CD8 response included proteins assigned to structural, enzymatic, transcription factor, and immune evasion functions, and included members of all viral kinetic classes. Most epitopes were conserved in other orthopoxviruses. Responses to at least 18 epitopes were detected within a single blood sample, revealing a surprising degree of diversity. These epitopes will be useful in natural history studies of CD8 responses to vaccinia, a nonpersisting virus with long-term memory, and in the design and evaluation of attenuated and replication-incompetent vaccinia strains being tested for variola and monkeypox prevention and for the delivery of heterologous Ags. The Journal of Immunology, 2005, 175: 7550–7559.

Infection with the orthopoxvirus vaccinia protects against smallpox, a deadly disease caused by variola. Primary vaccination by intradermal scarification with replication-competent vaccinia strains is marked by several weeks of productive viral replication at the site of inoculation. Complete elimination of the pathogen occurs, without latency, persistence, or genomic integration. Follow-up vaccinations usually provoke briefer, milder infection, due to pre-existing immunity. Attenuated vaccinia strains such as New York vaccinia (NYVAC)\textsuperscript{1} and modified vaccinia Ankara (MVA) that are replication-incompetent in human cells are under study for smallpox prevention, and are also in clinical trials as vector backbones for delivery of heterologous vaccine Ags. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\textsuperscript{2}This work was supported by National Institutes of Health Grant AI061636.

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Abbreviations used in this paper: NYVAC, New York vaccinia; MVA, modified vaccinia Ankara; ORF, open reading frame; TCM, T cell medium; MOI, multiplicity of infection; LCL, lymphocyte continuous line; ICC, intracellular cytokine cytometry; eGFP, enhanced GFP; SOR, shortest overlapping region; int, intermediate.
that a theoretical ORF does encode immunogenic protein, the identification of candidate immunodominant ORFs containing several epitopes, and an estimate of the diversity of the acute response to primary vaccination. These epitopes may be useful in the design and evaluation of candidate vaccines for the prevention of variola and monkeypox in humans, and of candidate vectors for the delivery of heterologous Ags.

Materials and Methods

Subjects and specimens

Eight adult subjects (Table I) receiving scarification with Dryvax smallpox vaccine for occupational health were consented after approval by the Institutional Review Board. Five had received one previous vaccination, ranging from 32 to 43 years before recent immunization, while one had received two previous vaccinations 28 and 52 years prior to reimmunization and two younger individuals were primary vaccinees. PBMC from peripheral blood obtained by phlebotomy into sodium heparin-anticoagulated syringes at weeks 2, 4, and 6 after vaccination were enriched by Ficoll centrifugation from peripheral blood and cryopreserved. No relationship between time after vaccination, or between primary vs revaccination status, and the yield of mononuclear cells per volume of blood was noted. HLA typing was done at the Puget Sound Blood Center (Seattle, WA).

Cell and viral culture

PBMC were seeded at 10^6/ml in 2 ml of T cell medium (TCM) in 24-well plates (14). Live vaccinia at a multiplicity of infection (MOI) of 10 was added to stimulate lymphocytes (15). IL-2 (Hemagen) was begun on day 5 (32 U/ml). Cultures were split as needed, fed periodically with IL-2, TCM, and CTL assays done on days 12–14. CD8 magnetic-positive selection (Miltenyi Biotec or StemCell Technologies), typically yielding >95% CD8 cells, was followed by functional assays (below), cloning with PHA as mitogen, or bulk T cell expansion with anti-CD3 as mitogen (14). Clones were screened (day 14) by CTL assay. Positive clones were expanded (14) to >10^6 cells and used, or frozen, at the end of an expansion cycle. EBV-transformed B-lymphocyte continuous lines (LCL) were derived from PBMC (16). Vaccinia strain New York City Board of Health (NYCBH; National Institutes of Health AIDS Research and Reference Program, Atlanta, GA) was used at 0.1 µg/ml × 10^6 cells in 75 µl of TCM, 60 min, room temperature, followed by anti-CD8-PE-Cy5 for 30 min, 4°C. Clones were stained with anti-TCRβ and anti-CD8. HLA expression by 48-h transfected Cos-7 was measured by staining HLA-specific mAb (One Lambda; unlabeled, or biotin- or FITC-conjugated) and goat anti-mouse PE or streptavidin-PE (BD Biosciences). ICC data are reported as the percentage of CD8+ lymphocytes that stain positively for IFN-γ (see Results). Data collected on FACScan (BD Biosciences) were analyzed with WinMDI 2.8 (http://facs.scripps.edu/software.html).

Vaccinia genomic library

BSC-40 cells at 90% confluent were infected 48 h with vaccinia NYCBH, MOI 10. Nuclear DNA was reduced by lysing cells (450 cm^2) with 1% Nonidet P-40 (17), centrifugation (400 g, 15 min), and retention of the supernatant. The cytoplastic fraction was extracted with chloroform and DNA precipitated with ethanol (17). Vaccinia DNA was digested with DNase I (New England Biolabs) with optimized MnCl2 concentration, temperature, and enzyme/substrate ratio to generate DNA fragments in the 0.1–2 kb range. DNA was purified from the excised agarose gel zone corresponding to 300–500 bp (Quiaquick). Termini were blunt-ended with T4 DNA polymerase and dNTPs. The gel-purified blunt-ended fragments were ligated to a DNA adaptor with a 5′ GA overhang: GAGGGTCCGACAGC (single-stranded overhangs are underlined). Unincorporated oligos were removed by gel purification. The library vector backbone (pEGFP-C1; BD Clontech) was XhoI-digested, partially filled in with dTTP and dCTP, and gel-purified to give TC overhangs complementary to the vaccinia fragments. After ligation and purification of DNA by organic extraction/ethanol precipitation, libraries were created by electrotransformation of DH10B (Invitrogen Life Technologies). Libraries were plated on 150-µm diameter kanamycin-LB plates. Bacteria rinsed from the primary growth plates with 1 ml of broth were frozen in aliquots for glycerol stocks, which were titrated on kanamycin plates. 96-well deep-plate dishes (n = 5) were seeded at 40 colonies/well. Resultant plasmid DNA for transfection was prepared (14) with an average yield of 100–150 µg/well. T. H. Fisher allelic vaccinia DNA was isolated from DNA fragments at a complexity of 40/well. Pools were diluted to an average of 50 ng/µl DNA for screening. Forty single colonies derived from retransformation of selected pools were sequenced to check library insert identity and heterogeneity.

The purity of the vaccinia genomic DNA used for library construction was estimated by restriction endonuclease digestion/agarose electrophoresis. Vaccinia DNA was concentrated by ultracentrifugation and shown to contain reduced cellular DNA. The primary library was estimated, from counting primary growth plates, to contain 3.0 × 10^6 unique kanamycin-resistant colonies. Sequencing of 40 random colonies showed that 90% contained single independent vaccinia DNA inserts, averaging 300-bp long (not shown). High diversity was also observed. The quality of the library 96-well miniprep DNA (14), derived from either pools or single bacterial clones, was verified by transfecting Cos-7 cells and observing enhanced GFP (eGFP) live-cell fluorescence in >50% of cells for most DNA preparations.

HLA cDNA expression plasmids

HLA A*0101, A*0201, and B*4403 cDNAs in pcDNA3.0 (Invitrogen Life Technologies) have been described (19, 20). HLA B*0801 cDNA in pcDNA 3.0 was obtained from Dr. J. Fei (Fred Hutchinson Cancer Research Center, Seattle, WA). Other alleles, including those from subjects’ LCL (RNAeasy; Qiagen) and first strand cDNA synthesis primed with oligo(dT) (Superscript II; Invitrogen Life Technologies). cDNA template was PCR-amplified ( pfu; Invitrogen Life Technologies). HLA T cell activation was detected by IFN-γ ELISA of culture supernatants (17). Exponential standard curves were used to convert OD_{490} values to cytokine concentrations and the level of IFN-γ secreted by nonstimulated T cells subtracted to give specific secretion. For intracellular cytokine cytometry (ICC) (18), peptides (1 µM) were added to 3–5 × 10^6 bulk-cultured T cells in 500 µl of TCM for 15 h. A total of 1 × 10^6 autologous LCL were added as APC. Anti-CD28 and anti-CD49d, and brefeldin A, were added 3 h and 1 h, respectively (18). Each specimen was stained with anti-CD8-PE-Cyanin 5 (Cy5) or -FITC, permeabilized, and then split for staining with control mAb-PE or anti-IFN-γ-PE. Controls were DMSO (1%) and PMA/ionomycin (18).

Flow cytometry

Bulk cultures were stained with anti-TCRβ-FITC (BD Biosciences), anti-CD4-PE, and anti-CD8-PE-Cy5 (Caltag Laboratories). PE-labeled tetrameric complexes of HLA B*0801 and peptide A50R 395–403 (WLKKIRDKYL) supplied by the National Institutes of Health Tetramer Program (Atlanta, GA) was used at 0.1 µg/ml × 10^6 cells in 75 µl of TCM, 60 min, room temperature, followed by anti-CD8-PE-Cy5 for 30 min, 4°C. Clones were stained with anti-TCRβ and anti-CD8. HLA expression by 48-h transfected Cos-7 was measured by staining HLA-specific mAb (One Lambda; unlabeled, or biotin- or FITC-conjugated) and goat anti-mouse PE or streptavidin-PE (BD Biosciences). ICC data are reported as the percentage of CD8+ lymphocytes that stain positively for IFN-γ (see Results). Data collected on FACScan (BD Biosciences) were analyzed with WinMDI 2.8 (http://facs.scripps.edu/software.html).

Table I. Subjects’ vaccination status and time after vaccination for PBMC specimens

<table>
<thead>
<tr>
<th>Subject</th>
<th>Vaccination</th>
<th>Number of Previous Vaccinations</th>
<th>Time Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Revaccination</td>
<td>1 (43)</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Primary</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Revaccination</td>
<td>1 (34)</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Revaccination</td>
<td>1 (32)</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>Revaccination</td>
<td>2 (52,28)</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>Revaccination</td>
<td>1 (41)</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>Primary</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>Revaccination</td>
<td>1 (36)</td>
<td>4</td>
</tr>
</tbody>
</table>

* The number of previous vaccinations is followed by the number of years, separated by a comma if appropriate, between the previous vaccinations and the recent vaccination.

* The number of weeks between the most recent vaccination and the PBMC collection used to obtain CTL effectors.
puriﬁed for 51Cr CTL assays at an E:T ratio of 20. Allogeneic target cells

2–10). One day later, 5

Life Technologies), followed the next day by vaccinia infection (MOI

cDNA (50 ng/well) using Fugene 6 (Roche) or Lipofectamine (Invitrogen

percent-speciﬁc release from 51Cr CTL assays of candidate clones. Subject 2 is a primary vaccinee and the other subjects are revaccinees. Clones in the

FIGURE 2. Clones with cytotoxic activity toward autologous vaccinia-infected LCL but not mock-infected LCL are readily derived from CD8 cells

purified from PBMC stimulated with live vaccinia. Subject numbers, weeks after vaccination, and the number of clones screened are indicated. Data are

percent-speciﬁc release from 51Cr CTL assays of candidate clones. Subject 2 is a primary vaccinee and the other subjects are revaccinees. Clones in the

upper left quadrants with >20% killing of infected targets and <10% killing of uninfected targets were considered positive.

FIGURE 1. Detection of vaccinia-speciﬁc CD8 lymphocytes in PBMC. Top, Intracellular cytokine cytometry. PBMC from before or 4 wk after

primary intradermal Dryvax were stimulated with live vaccinia for 6 h. The proportion of CD8+ lymphocytes staining positive for IFN-γ is indicated.

Staining with an isotype control is also shown. Bottom, Vaccinia-speciﬁc CD8 CTL activity is present in human PBMC after intradermal vaccination

with Dryvax. After one cycle of restimulation in vitro, CD8+ cells were puriﬁed for 51Cr CTL assays at an E:T ratio of 20. Allogeneic target cells were HLA class I-mismatched.

A*2301 and A*2902 primers were GGCGCTAGCATGGCCGTCATG

g and GGCCTCATGCACTTTTACGCCTGTGAGAGAC (Nhel and XhoI sites underlined). PCR products were digested, gel-puriﬁed, and directionally ligated into similarly digested pcDNA3.1+ (Invitrogen Life Technologies). Low-endotoxin plasmid DNA was prepared (Qiagen) after

sequence veriﬁcation.

Epitope discovery

Details and examples have been published (14, 17). Briefly, functional

HLA expression and restriction were conﬁrmed by transfection of Cos-7

cells. One day later, 5 × 105 cloned CD8 CTL were added in 130 μl of LCL media (16) with 2 U/ml IL-2. As controls, autologous or HLA-mis-

matched LCL were mock- or vaccinia-infected overnight at MOI 10 and cocultured (2.5 × 105 LCL and 5–10 × 104 CD8 CTL) in 96-well U plates for 24 h. Twenty-four hour supernatants were assayed for IFN-γ. If HLA transfection plus infection lead to high IFN-γ release, as described (17), HLA expression was functionally adequate for library screening.

Cos-7 were transfected with 50 ng of HLA cDNA and 150 ng of library

data pool DNA/well. We screened 384 library pools in duplicate, the equivalent of 5.5 × 109 discrete vaccinia genomic fragments. T cells were added

24–48 h later and IFN-γ was measured after an additional day. If multiple

positive pools were detected, up to ﬁve were analyzed. Positive plasmid pools were broken down by retransformation and selection of 96 single
donor bacterial colonies per positive pool, screened as plasmid DNA in a secondary cotransfection assay. Single, biologically active plasmids were sequenced (17).

Candidate peptides were selected by bioinformatics (14). Briefly, if

more than one active plasmid was sequenced, overlapping insert sequences were assembled into a contig (DNASTAR) after trimming. The overlap (or single) region was searched with a basic local alignment search tool

(www.poxvirus.org/); Ref. 21). Typically, the vaccinia insert was within a documented/predicted vaccinia ORF and in-frame with eGFP. Some ex-

ceptions are discussed in Results. Predicted amino acid sequences in the

antigenic fragments were submitted to HLA epitope prediction algorithms

(22, 23) and high-scoring peptides (Synpep) dissolved in DMSO. Or-
thopoxvirus genomes (21, 24) were searched for the presence and sequence of homologous ORFs, antigenic fragments, and peptide epitopes. Alpha-

numeric ORF nomenclature based on vaccinia Copenhagen HindIII di-
gests, and systematic names, are used (21, 25).

High-throughput epitope discovery

Peptide epitopes recognized by bulk vaccinia-speciﬁc T cells were also

identiﬁed using a parallel processing variant method. Cos-7 (384 wells) were transfected in duplicate with cDNA encoding one of the subjects’ HLA class I A or B alleles, plus the library. Bulk CD8 CTL (105/well) were substituted for cloned CTL as responders. Single active plasmids were sequenced and contigs assembled and analyzed as above. Candidate pep-
tides were tested by loading (0.01–10 μM) onto autologous LCL (2 × 105 cells, 200 μl of LCL medium, 90 min, 37°C). After washing, stimulators were plated in duplicate or triplicate with 1 × 105 bulk CTL responders in 130 μl of TCM with 2 U/ml IL-2 in 96-well U-bottom plates, and T cell activation detected by IFN-γ ELISA in 24-h supernatants. Speciﬁc responses at 1 μM or lower were considered positive. As an alternative, bulk CTL were tested with synthetic peptides (1 μM) by IFN-γ ICC as detailed above.

Results

Detection and cloning of vaccinia-specific CD8 T cells

Bulk CTL. Vaccinia-speciﬁc CD8 T cells were initially detected by IFN-γ ICC using whole PBMC responders and live vaccinia stimulation. Speciﬁc signals in the range of 1.0% of CD8+ lymphocytes were detected 2–6 wk after Dryvax, but not in vaccinia-naive subjects (Fig. 1, representative subject). To enrich vaccinia-specific
CD8 T cells, PBMC from eight subjects (Table I), obtained 2–6 wk after intradermal vaccination, were restimulated once in vitro. Vaccinia-specific, self-restricted cytotoxicity was detected (not shown), as defined in Materials and Methods, in each subject except subject 1. These cultures were predominantly CD8⁺, CD4⁺, and >95% TCRαβ⁺. CD8⁺ cells were purified from six cultures. For each, strong virus- and self-restricted CTL activity was detected (Fig. 1).

**CTL clones.** Panels of clones (96–144 per subject) were derived from bulk CD8 CTL from one primary and three revaccinees. From 27 to 99% of clones had vaccinia-specific CTL activity (Fig. 2). For each, strong virus- and self-restricted CTL activity was detected (Fig. 1).

**FIGURE 3.** Representative example of cytotoxicity and transfection/infection tests to establish HLA restriction. Top, ⁵¹Cr CTL assays for clone 2.59 from a primary vaccinee vs autologous, fully mismatched, or partially HLA class I-matched (matching alleles indicated) LCL targets with or without vaccinia infection. Bottom, IFN-γ release by clone 2.59 after co-incubation with Cos-7 cells transfected with HLA B*4403 cDNA, infection with vaccinia, or both. Controls at right are co-incubation with autologous LCL. Data are means of triplicate assays.

Vaccinia epitopes recognized by HLA-A*0101, B*0801, B*4403, A*2902, and A*2301 restricted-CD8 CTL clones

We defined peptide epitopes for five CD8 clones. For each, one or more vaccinia plasmids were strongly stimulatory for IFN-γ release, and only when cotransfected with the appropriate HLA cDNA. If multiple library hits were obtained, they were aligned and shortest overlapping regions (SOR) were determined. For example, the HLA B*4403-restricted clone 2.59 from a primary vaccine yielded four independent library hits (Fig. 4). The SOR was the C-terminal 29 aa of the theoretical ORF F3. This 49-aa-long ORF (VACvgp067) is predicted to lie between ORFs F14L and F15L in vaccinia Copenhagen (GenBank NC_001559), but has never been documented at the protein level. Of note, the plasmids RC4 B6 E7, RC1 H11 H8, and RC1 B5 C10 are fusions in which fragments of ORF F3, or the neighboring ORF F15 L, are predicted to be out of frame with eGFP. However, an ATG codon is present at predicted aa 25 of ORF F3. Sequence with features of a vaccinia early promoter (27), 5’ to the predicted initiation codon of F3, occurs in plasmids RC2 B7 A10 (and RC4 B6 E7). Full-length F3, cloned after PCR into pEGFP-C1 as an in-frame fusion, was positive in the IFN-γ Cos-7 cotransfection assay (not shown).

The candidate antigenic region, F3 25–49, was analyzed for peptides with the B*4403-binding motif (22). The peptide F3 41-49 (EEQELLLLY) was positive in CTL assays with an approximate EC₅₀ of 10⁻⁸ molar (Fig. 5). It is likely that internal initiation or transcription from the vaccinia promoter occurred after transfection with the active genomic fragments. We previously documented internal ATG initiation and transcription/translation from viral promoters during similar library-based epitope discovery for HSV type 2 (HSV-2) (17). The presence of specific CD8 CTL in a vaccinia-infected human is the first documentation that F3 encodes a protein. F3 is highly conserved in orthopoxviruses (below), consistent with a role in replication or pathogenesis.

Similar overall strategies were used to discover four additional epitopes recognized by CD8 clones (Fig. 5). For each clone, HLA restriction was documented in CTL and transfection/infection assays (not shown). Each was similarly screened against the vaccinia genomic library, and positive pools were decoded to single active fragments of the proteome. The translational schema for the active fragments of ASOR recognized by a B*0801-restricted clone and fragments of A48R restricted by an A*2301-restricted clone were straightforward, entailing TCRαβ⁺, CD8⁺, and displayed vaccinia-specific lysis in confirmatory assays (data not shown). After expansion, HLA class I or B restriction was unambiguously assigned for most clones using both panels of partially matched APC and by vaccinia infection/HLA transfection assays (example, Fig. 3). Each clone investigated (n = 5) gave identical results with both methods.

Vaccinia epitopes recognized by HLA-A*0101, B*0801, B*4403, A*2902, and A*2301 restricted-CD8 CTL clones

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TABLE II. Peptides recognized by vaccinia-specific CD8 T cells

<table>
<thead>
<tr>
<th>ORF</th>
<th>Epitope</th>
<th>Sequence</th>
<th>HLA Allele</th>
<th>Orthopox Conservationb</th>
<th>Molluscum Contagiosum Conservationc</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3L</td>
<td>90–98</td>
<td>DEVASTHDW</td>
<td>B*4403</td>
<td>Primate OP all (+)</td>
<td>DEVASTQDN 8/9</td>
</tr>
<tr>
<td>A3L</td>
<td>264–272</td>
<td>YERFRKVSY</td>
<td>B*4403</td>
<td>Primate OP all (+)</td>
<td>YELKVRPD 4/9</td>
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<tr>
<td>A23R</td>
<td>287–295</td>
<td>HDVGVSNF</td>
<td>B*4403</td>
<td>Primate OP all (+)</td>
<td>AHWYGVHNF 5/9</td>
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<tr>
<td>A24R</td>
<td>278–286</td>
<td>ITDFNIDTY</td>
<td>A*0101</td>
<td>Primate OP all (+)</td>
<td>EDDFIVAEY 3/9</td>
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<tr>
<td>A48R</td>
<td>58–66</td>
<td>TYNHSVNL</td>
<td>A*2301</td>
<td>Primate OP all (+)</td>
<td>No homolog</td>
</tr>
<tr>
<td>A50R</td>
<td>395–403</td>
<td>WILKIRCDY</td>
<td>B*0801</td>
<td>Primate OP all (+)</td>
<td>No homolog</td>
</tr>
<tr>
<td>C12L</td>
<td>326–334</td>
<td>VYINHPFMY</td>
<td>A*2902</td>
<td>Fragmented in MVA (31)</td>
<td>No homolog</td>
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<tr>
<td>D1R</td>
<td>126–134</td>
<td>EERHIFLGY</td>
<td>B*4403</td>
<td>Primate OP all (+)</td>
<td>SEQYFLF 5/9</td>
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<tr>
<td>D5R</td>
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<td>LENGAILRY</td>
<td>B*4403</td>
<td>Primate OP all (+)</td>
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<td>D5R</td>
<td>691–699</td>
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<td>B*4403</td>
<td>Primate OP all (+)</td>
<td>DLIPDFCFQ 5/9</td>
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<tr>
<td>D5R</td>
<td>349–357</td>
<td>VWINWSWRK</td>
<td>A*2301</td>
<td>Primate OP all (+)</td>
<td>VWNHRNCVRF 5/9</td>
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<td>E3L</td>
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<td>B*4403</td>
<td>Primate OP all (+)</td>
<td>No homolog</td>
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<tr>
<td>E3L</td>
<td>41–49</td>
<td>HEQELLLLY</td>
<td>B*4403</td>
<td>Primate OP all (+)</td>
<td>No homolog</td>
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<td>I3L</td>
<td>173–181</td>
<td>IGEQUELGS</td>
<td>B*4403</td>
<td>Primate OP all (+)</td>
<td>MRELLETLA 4/9</td>
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<tr>
<td>IL-18bpd</td>
<td>21–29</td>
<td>DEIKCPNLM</td>
<td>B*4403</td>
<td>Copen (−), MVA, variola, monopeptide (each divergent)</td>
<td>No homolog</td>
</tr>
<tr>
<td>M2L</td>
<td>38–46</td>
<td>AELTIGVNY</td>
<td>B*4403</td>
<td>Deleted in NYVAC</td>
<td>No homolog</td>
</tr>
</tbody>
</table>

- CD8 CTL clones were tested in 3H-crystal assays. Bulk CTL were tested for IFN-γ-release and/or IFN-γ accumulation by ICC and only peptides with two or more positive tests are listed.
- Data from Ref. 21 and J. Tartaglia (unpublished observation). Primate orthopox (OP) analyzed were the primate orthopoxviruses vaccinia Copenhagen, vaccinia Western Reserve, MVA Acambis 3000, monkeypox (MP) Zaire, monkeypox Congo, variola major India, and NYVAC (24). (+), Peptide epitope predicted to be expressed; (−), peptide epitope either altered or deleted and therefore not predicted to be expressed.
- Data from Ref. 21. The sequence of the homologous region of the homologous protein, if any, is shown, followed by the number of identical amino acids at orthologous positions and the total number of amino acids.
- The IL-18 binding protein is named, in vaccinia strain WR, VACWR013 and C12L, (38). It is reported to be absent from Copenhagen (21). The epitope 21–29 is identical between vaccinia NYCBH and vaccinia WR, but is divergent in the homologous proteins in MVA, variola, and monkeypox.
B*2301 binding for both 349–357 and 356–364. These peptides were each tested and only 349–357 lead to detection of IFN-γ/H9253-bright cells at a level above the background seen with DMSO alone.

An additional application of ICC was measurement of the proportion of bulk CD8 CTL responsive to specific epitopes that were defined with CTL clones. For subject 3, 2.95% of bulk CTL recognized A50R 395–403, initially detected with clone 3.94. As 1.4% of cells responded to DMSO, the net response was ~1.55%. Use of an HLA B*0801-A50R 395–403 tetramer to stain the same specimen detected 1.45% Ag-specific CD8 T cells (Fig. 7, right), while a control HSV-2 tetramer (28) was negative.

\textit{IFN-γ secretion}

The second IFN-γ test format for high-throughput epitope discovery involved coincubation of bulk CTL with peptide-loaded autologous APC, and measurement of cytokine release into the media (Fig. 8). Most peptides checked were positive in both ICC and IFN-γ secretion tests (example, A3L 264–272, Figs. 7 and 8), but...
IFN-γ secretion was generally more sensitive (not shown). For subject 2, eight additional epitopes (Fig. 8) were documented by IFN-γ release to lie within genomic fragments that were active upon cotransfection with HLA B*4403 (Fig. 6). Responses to the epitope in ORF F3 detected at the clonal level (Figs. 4 and 5) were again detectable among bulk CTL. Of note, three discrete B*4403-restricted epitopes were detected in ORF A3L and two in ORF D5R. Overall, 16 epitopes have been defined by combining clonal reactivity and interrogation of bulk CTL with the IFN-γ ICC and secretion assays (Table II).

Seven additional vaccinia antigenic regions have been identified by cotransfection; definition of their internal peptide epitopes is still underway (Table III). These fragments have been repeatedly positive, contain regions of known ORFs, and are mostly straightforward, in-frame fusions with eGFP. Testing of candidate internal peptides is in progress. Of note, these data are consistent with the presence of additional epitopes in ORF A3L.

The most detailed CD8 epitope data are available for subject 2, a primary vaccinee. The minimal estimate of the overall diversity of the CD8 response in this specimen is 18 epitopes. Specifically, for B*4403, 10 peptides stimulate bulk CTL (Figs. 7 and 8), including one that stimulates a CD8 clone (Figs. 5 and 8). Two additional nonredundant antigenic DNA regions, for which peptide identification is pending, also stimulate B*4403-restricted responses (Table III) for a total of at least 13 B*4403-restricted epitopes. Three antigenic DNA fragments contain epitopes restricted by A*2301 (Table III) that are nonredundant with clone 2.105 (Fig. 5) for a total of at least 4 A*2301-restricted epitopes.

Table III. Regions of vaccinia ORFs that contain putative epitopes stimulating human HLA class I-restricted CD8+ T cells*

<table>
<thead>
<tr>
<th>ORF</th>
<th>HLA cDNA</th>
<th>Predicted Amino Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3L</td>
<td>B*4403</td>
<td>487–567</td>
</tr>
<tr>
<td>A3L</td>
<td>B*4403</td>
<td>393–474</td>
</tr>
<tr>
<td>A24R</td>
<td>A*0101</td>
<td>747–897</td>
</tr>
<tr>
<td>A57R</td>
<td>A*2301</td>
<td>1–62</td>
</tr>
<tr>
<td>F12L</td>
<td>A*2301</td>
<td>147–280</td>
</tr>
<tr>
<td>F12L</td>
<td>A*0101</td>
<td>392–486</td>
</tr>
<tr>
<td>IL-18bp</td>
<td>A*0101</td>
<td>59–126</td>
</tr>
</tbody>
</table>

* Each fragment was repeatedly positive after cotransfection with indicated HLA cDNA. Recognition of an internal peptide has not yet been demonstrated.

We also derived A*2902-restricted clones from this individual (data not shown), increasing the diversity to at least 18.

HLA A*0201-restricted responses are of interest due to the high population prevalence of this allele. Subject 3, a revaccinee, had brisk HLA A*0201-restricted IFN-γ release by bulk CD8 CTL exposed to Cos-7 artificially transfected with A*0201 cDNA and infected with vaccinia. CD8+ clones with HLA A*0201-restricted CTL activity and IFN-γ release were also derived from this subject (data not shown). For unknown reasons, discussed below, screening of the vaccinia genomic library for A*0201 epitopes was negative for both clonal and bulk CTL responders. We used the ICC assay to probe bulk CTL from this subject with five previously reported (11–13) A*0201-restricted epitopes (Fig. 7, bottom). Three peptides, B22R 60-68, C7L 74-82, and D6R 498-506, gave responses above background, while A26L 6-14 and H3L 184-192 did not (data not shown).

**Discussion**

In the present study, we have identified vaccinia virus Ags and epitopes recognized by CD8 T cells in humans recently vaccinated with Dryvax. These results should be useful in comparing this replication-competent vaccine with other candidate products currently under evaluation for smallpox prevention. We have also made a preliminary identification of candidate immunodominant Ags containing a high density of epitopes and gained an insight into the diversity of the response during acute infection. The contribution of responses to these epitopes to protection from orthopoxvirus challenge are unknown but could be addressed in challenge studies using HLA-transgenic animals after epitope-based vaccination.

This report describes 16 novel discrete epitopes within 15 vaccinia ORFs that are recognized in the context of four HLA class I alleles (HLA A*0101, A*2301, A*2902, and B*4403). HLA A*2301 belongs to the A24 supertype, while B*4403 belongs to the B44 supertype. A*0101 and related A*0101 supertype members are also prevalent in the population (29, 30). Although reactivity with other members of these supertypes will have to be studied empirically, the epitopes described in this report greatly extend published reports, limited to 5 epitopes restricted by A*0201 (11–13), and should allow monitoring of expanded patient cohorts. As almost all of the epitopes described in this report are conserved in MVA and NYVAC (Table IV), these epitopes should also be useful in monitoring the immune response to these replication-incompetent candidate vaccine strains.

A total of 16 distinct peptide epitopes recognized by human CD8 T cells were newly detected in this study. The conservation of epitopes between vaccine strains and pathogens is of interest for vaccine design. A summary of database (21) searches for epitope...
Table IV. Selected virologic characteristics of novel human CD8 Ags in vaccinia

<table>
<thead>
<tr>
<th>ORE a</th>
<th>Function a</th>
<th>Kinetic Class a</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3L</td>
<td>Major core protein</td>
<td>Early (39)</td>
</tr>
<tr>
<td>A23R</td>
<td>Transcription factor</td>
<td>Early (40)</td>
</tr>
<tr>
<td>A24R</td>
<td>DNA-dependent RNA polymerase</td>
<td>Early-int-late (41)</td>
</tr>
<tr>
<td>A48R</td>
<td>Thymidylate kinase/synthase</td>
<td>Early (42)</td>
</tr>
<tr>
<td>A50R</td>
<td>DNA ligase, virulence protein</td>
<td>Early (43)</td>
</tr>
<tr>
<td>A57R</td>
<td>Guanylate kinase homolog (21)</td>
<td>Unknown</td>
</tr>
<tr>
<td>C12L</td>
<td>Serine protease inhibitor-like (44)</td>
<td>Unknown</td>
</tr>
<tr>
<td>D1R</td>
<td>mRNA capping enzyme subunit (45)</td>
<td>Early (46)</td>
</tr>
<tr>
<td>D5R</td>
<td>Nucleoside triphosphatase, role in DNA replication (47)</td>
<td>Unknown</td>
</tr>
<tr>
<td>E3L</td>
<td>ss RNA binding, immune evasion (48)</td>
<td>Early (49)</td>
</tr>
<tr>
<td>F3</td>
<td>Unknown; previously not documented to encode protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>F12L</td>
<td>Infectious enveloped virus protein; extracellular enveloped virus formation, virulence protein (51)</td>
<td>Early and late (50)</td>
</tr>
<tr>
<td>F3L</td>
<td>ssDNA binding protein (51)</td>
<td>Early (52)</td>
</tr>
<tr>
<td>IL-18bp</td>
<td>Immune evasion (53)</td>
<td>Early (38)</td>
</tr>
<tr>
<td>M2L</td>
<td>Unknown</td>
<td>Early (54)</td>
</tr>
</tbody>
</table>

a Nomenclature from HindIII digest of vaccinia Copenhagen (24)
b Syntheses of data referenced and other reports, and texts (27)

cconservation in primate orthopoxviruses (Table II) indicates that most of the CD8 epitopes are identical in varicella and monkeypox. Vaccinia MVA and NYVAC are replication-competent strains with deletions and disruptions of many ORFs (24). Almost all of the CD8 epitopes are predicted to be present in MVA and NYVAC, with the exception of Copenhagen M2L, which is not present in NYVAC (24), and C12L, which is fragmented in MVA (31). The epitope in the IL-18-binding protein, DEIKCPNLN, is identical in NYVAC and vaccinia WR. The homologous ORF is not present in vaccinia Copenhagen. Although predicted IL-18-binding proteins are present in MVA, varicella, and monkeypox, the epitope region diverges at 2 or 3 aa (21). Specifically, the MVA and varicella sequence is VETKCPNLN, with changes at aa 1 and 3, and the monkeypox sequence is VETKCPNLN, with an additional change at the ninth residue. Most of the epitopes are present and identical in ectromelia, an orthopoxvirus of mice, but are divergent in canarypox, the backbone of the ALVAC vaccine vector (1), and molluscum contagiosum virus, a human pathogen (Table II). It has been speculated (27) that decreased smallpox vaccination may predispose individuals to molluscum contagiosum. The molluscum virus is only distantly related to vaccinia (27), and several of the antigenic vaccinia ORFs identified in this study do not have homologs in the molluscum virus (Table II). One epitope is relatively conserved (A3L 90–98 in vaccinia) at 8 of 9 aa, including anchor residues, but has a nonconservative difference at position 7. The other epitopes are quite divergent. Empiric testing of predicted molluscum contagiosum virus peptides would be required to definitively measure any possible cross-reactivity.

The virologic features of the vaccinia proteins newly identified as CD8 Ags in humans are diverse (Table IV). The known functions include enzymes, transcription factors, immune evasion proteins, and structural virion proteins. Of note, we have not detected epitopes in envelope proteins or in known targets of neutralizing Abs. Vaccinia genes are transcribed in several coordinated waves, designated early, intermediate, and late. Each kinetic class is immunogenic, with early proteins particularly well represented.

The determinants of immunodominance the polypeptide level are largely unknown (32). We showed that several vaccinia ORFs contain multiple CD8 epitopes and are thus candidate immunodominant Ags. Specifically, A3L contains at least four epitopes (each B*4403 restricted), D5R at least three epitopes, and A24R, F12L, and IL-18-binding protein at least two epitopes each. These epitopes were discovered in independent iterations of an unbiased genome-wide screen, reducing the chance that epitope grouping is an artifact. Because the responder cells used in this report were studied after one cycle of expansion in response to live vaccinia virus, it is possible that some bias was introduced during restimulation that favored detection of some epitopes over others. The potential dominance of the vaccinia Ags mentioned above is testable by examination of subjects with diverse HLA type by ELISPOT or related techniques using short peptides from these ORFs.

The vaccinia Ags that were found to stimulate CD8 responses belonged to diverse functional and kinetic classes. Notably, viral regulatory and immune evasion genes and enzymes were well-represented, while we only detected one structural or envelope proteins that was a CD8 Ag (ORF A3L). None of the major neutralizing proteins (9) on infectious intracellular mature virion or extracellular-enveloped virion were targets of CD8 T cells. Viral proteins synthesized at early times after infection were particularly well-represented. If cross-presentation is an important mode of Ag presentation for vaccinia-encoded Ags, as implied by some studies (33, 34), we would predict that abundant structural proteins would be better represented. We did not note any overlap at the ORF level with the ORFs previously reported to contain A*0201-restricted epitope, or with a set of ORFs recognized by CD8 T cells in mouse strain C57BL/6 (35). It is therefore likely that many additional antigenic ORFs remain to be uncovered, and that detailed analyses of many persons and HLA alleles will be required to assess the structural and kinetic correlates of CD8 antigenicity.

Our studies differ in several ways from other approaches to epitope discovery for complex viral pathogens. No knowledge of the viral genome sequence or predicted ORFs was used to generate our initial positive antigenic "hits". The vaccinia genome was probed in an unbiased fashion and Ags were identified by library screening. Expression cloning should therefore be useful for studying T cell reactivity for unsequenced microbial pathogens or for identifying previously unsuspected ORFs. HLA peptide-binding motifs and algorithms were only used to define peptide epitopes within small (~100 aa) antigenic fragments, and were not formally necessary, as the fragment size allows economical molecular truncation analyses and/or screening of internal peptides (19). Although peptide-binding motifs are known for some prevalent HLA alleles, HLA class I loci are extremely diverse, and reliance of these motifs for epitope discovery will exclude some HLA alleles from analysis.

The cells we probed for specificity by expression cloning are reactive with whole vaccinia, because they were studied after one cycle of in vitro expansion stimulated by live vaccinia. Our CD8 clones, in addition, recognize vaccinia-infected cells in CTL assays. Both the clonal and bulk responders in our studies are documented to express CD8α. We used relatively low peptide concentrations in some assays (Figs. 5 and 8). Taken together, these factors are consistent with the detection of vaccinia-specific CTL and decrease the likelihood of detection of cross-reactive T cells. Most likely, both peptide-based and molecular methods such as...
expression cloning will be required to completely analyze the cellular immune response to vaccinia. We initially validated our vaccinia library system using CTL clones (Fig. 5), as previously reported for HSV-2 (19), but adapted the method to bulk-cultured CD8 T cells to speed epitope discovery. This variant offers higher throughput, but without loss of precision. Use of bulk CD8 T allowed rapid identification of antigenic genomic fragments (Fig. 6) and internal epitopes using IFN-γ release (Fig. 8) or ICC (Fig. 7). Overall, the “hit” rate for candidate peptides that we synthesized within antigenic genomic fragments was ~70% for both cloned and bulk responder cells. This is far higher than the ~1% rate obtained from bioinformatic scans of predicted ORFs and analyses of whole PBMC (11). In the ICC format, we noted bright, specific IFN-γ accumulation in CD8⁺ cells when some peptides were used. These cells are unlikely to be NK cells, as the responding bulk cultures are >98% TCRαβ⁺ (not shown). Down-modulation of surface TCRαβ and associated molecules has been reported after activation through TCR (36). It is most likely that the IFN-γ⁺ cells in our ICC assays started as CD8⁺ cells and down-modulated surface CD8α during our long (15 h) stimulation period.

Our results are likely influenced by technical limitations. Any molecular library will have gaps, for example, if epitopes are downstream from viral promoters that are inactive after transfection into uninfected cells, or if epitopes require posttranslational modification by other virus-encoded or -activated functions. As mentioned above, we were unable to score “hits” when screening HLA A*0201-restricted CTL clones, or bulk CTL lines with A*0201-restricted activity, using our genomic library. This was somewhat surprising, as bulk CTL reactivity was detected against known A*0201 epitopes (Fig. 7) in ORFS B22R, C7L, and D6R that do not have posttranslational modification, and should have been included in our library. The A*0201 expression plasmid was checked and protein expression was demonstrable in transfected Cos-7 cells (data not shown). Assessment by PCR with primers spanning these epitopes should allow assessment of whether these epitopes are represented in our library and this type of analysis could be useful for quality control of next-generation libraries. Our analysis of the diversity of vaccinia-specific responses is not exhaustive, as a gradation of IFN-γ responses was detected when bulk CTL were detected against library pools, and not all positive pools have been decoded to single active plasmids (Fig. 6). We cannot yet determine whether we have detected the quantitatively most abundant responses within individuals, but the epitopes disclosed in this report should be useful for designing tetramer and peptide ELISPOT or ICC assays to examine this issue. In summary, the human CD8 T cell response to vaccinia is robust at early times after vaccination. Expression cloning, including a new high-throughput variant, has disclosed that the response can be very diverse within an individual. Several candidate immunodominant Ags, containing multiple epitopes, have been described. These Ags and epitopes should be useful in evaluating candidate smallpox vaccines and modified poxviruses (37) being developed as vectors for heterologous Ags.

Acknowledgments

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Disclosures

L. Jing and D. M. Koelle are inventors listed on a provisional patent application filed by their employer, University of Washington. The patent concerns the use of the vaccinia ORFs discussed in their paper as possible smallpox vaccines.

References


