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Identification of immediate early gene products of bovine herpes virus 1 (BHV-1) as dominant antigens recognized by CD8 T cells in immune cattle

Jane Hart, Niall D. MacHugh, Tara Sheldrake, Morten Nielsen and W. Ivan Morrison

Abstract

In common with other herpes viruses, bovine herpes virus 1 (BHV-1) induces strong virus-specific CD8 T-cell responses. However, there is a paucity of information on the antigenic specificity of the responding T-cells. The development of a system to generate virus-specific CD8 T-cell lines from BHV-1-immune cattle, employing Theileria-transformed cell lines for antigen presentation, has enabled us to address this issue. Use of this system allowed the study to screen for CD8 T-cell antigens that are efficiently presented on the surface of virus-infected cells. Screening of a panel of 16 candidate viral gene products with CD8 T-cell lines from 3 BHV-1-immune cattle of defined MHC genotypes identified 4 antigens, including 3 immediate early (IE) gene products (ICP4, ICP22 and Circ) and a tegument protein (UL49). Identification of the MHC restriction specificities revealed that the antigens were presented by two or three class I MHC alleles in each animal. Six CD8 T-cell epitopes were identified in the three IE proteins by screening of synthetic peptides. Use of an algorithm (NetMHCpan) that predicts the peptide-binding characteristics of restricting MHC alleles confirmed and, in some cases refined, the identity of the epitopes. Analyses of the epitope specificity of the CD8 T-cell lines showed that a large component of the response is directed against these IE epitopes. The results indicate that these IE gene products are dominant targets of the CD8 T-cell response in BHV-1-immune cattle and hence are prime-candidate antigens for the generation of a subunit vaccine.

INTRODUCTION

Infection with herpes viruses typically results in an acute lytic phase of virus replication, which may be associated with clinical symptoms, followed by establishment of latent infection that can persist for many years. Latently infected cells express only a few genes, but reactivation can occur, often triggered by stress or immunosuppression, whereby expression of the full complement of viral genes is restored, resulting in the production of infectious virus. CD8 T-cell responses have been shown to be important mediators of immunity to a number of herpesvirus infections. There is compelling evidence that they are required to prevent reactivation of latent infection and that they also contribute to resolution of the acute phase of infection [1, 2]. The prominent role of CD8 T cells in immunity is also consistent with the capacity of herpesviruses, including bovine herpes virus 1 (BHV-1), to inhibit a number of the steps involved in processing and presentation of antigen to CD8 T cells [3, 4], thus reducing the efficiency of the CD8 T-cell response.

The capacity of CD8 T cells to act as effectors against viral infections relies on their ability to recognize and respond to virus-infected cells before they produce large quantities of virus. Herpesviruses are antigenically complex, encoding between 70 and 165 proteins, synthesis of which is regulated in a cascade fashion, with immediate early (IE) genes being expressed first, followed by early (E) and late (L) genes. The IE and E proteins are involved in regulating expression of other viral genes or modulation of host cell function, whereas products encoded by the L genes are generally structural components of the virion. Knowledge of which categories of viral protein are recognized by the CD8 T-cell response and the ability of the specific CD8 T cells to recognize virus-infected cells can inform the choice of antigens for inclusion in new vaccines.
Among human herpes viruses, the antigenic specificity of CD8 T-cell responses has been studied most extensively for cytomegalovirus (CMV), Epstein–Barr virus (EBV) and herpes simplex viruses 1 and 2 (HSV-1 and HSV-2). Initial studies suggested focusing of the CD8 T-cell responses on a few dominant viral antigens, often including IE viral proteins or (in the case of EBV) latent gene products [5, 6]. Subsequent use of more comprehensive antigen screening methods [7, 8] has revealed CD8 T-cell reactivity with over half the viral proteins, including proteins with diverse expression dynamics [7, 8]. However, the relative capacities of the different CD8 T-cell specificities to recognize virus-infected cells and act as immune effectors remain unclear.

Bovine herpesvirus type 1 (BHV-1) is the most widely distributed pathogenic herpesvirus of cattle. BHV-1 infects cells of the respiratory and reproductive tracts and causes clinical diseases, namely infectious bovine rhino-tracheitis and infectious pustular vulvo-vaginitis [14], which result in significant production losses in affected herds, including the death of some animals. BHV-1-encoding proteins have a number of immunomodulatory activities, including down-regulation of type I interferon responses [15] and expression of class I MHC proteins [16], and interference with chemokine activities [17], which collectively are associated with suppressed cellular immune responses during infection. This immunosuppression is implicated in predisposition to secondary infections with other respiratory pathogens, both viral and bacterial, which cause severe disease and mortality in intensive cattle-rearing units (reviewed in [18]). Vaccination is widely deployed to control disease caused by BHV-1. A number of inactivated and live attenuated vaccines are commercially available, but the latter are more commonly used because of the requirement for only one vaccine dose (reviewed in [18, 19]). A vaccine using a virus containing a deletion of the gene encoding gE is widely used at present; in addition to being highly attenuated, this virus is incapable of reactivation and its use allows differentiation of infected from vaccinated animals based on detection of serological responses to gE [20]. Although live vaccines have proved to be relatively effective, concerns remain about their immunosuppressive activities and some of the most attenuated viruses have been reported to have reduced immunogenicity [20, 21]. Hence, there is continued interest in the possibility of using subunit antigens for vaccination in some animal production sectors. However, this is constrained by a paucity of information on potentially protective T-cell responses elicited by BHV-1.

Although MHC class I-restricted CD8 T-cell responses have been identified in animals immune to BHV-1 [22–24], little is known about the specificity of these responses. This appears to have been partly due to the poor sensitivity of assays using ex vivo-derived T cells and the lack of reliable in vitro systems for the maintenance and enrichment of virus-specific CD8 T cells. Recently, we developed a system to derive virus-specific CD8 T-cell lines from BHV-1-immune cattle using *Theileria annulata*-transformed cell lines infected with the virus as stimulator cells [25]. Herein we describe the use of CD8 T-cell lines generated using this system to identify CD8 T cell target antigens in BHV1. The results demonstrate dominant responses to three IE BHV-1 early gene products.

**RESULTS**

**Selection of viral antigens for T-cell screening**

A panel of 16 BHV-1 genes, including four IE, four E and eight L genes, was selected for antigen screening. The genes and the PCR primers used for their amplification are listed in Table S1 (available in the online Supplementary Material). They include orthologues of genes known to encode CD8 T-cell antigens in HSV-1 and genes encoding the glycoproteins gC and gD, which previous studies suggested may be targets for the CD8 T-cell response to BHV-1 [24].

Cloned DNAs encoding each of these genes, inserted into the pcDNA3.1V5 His TOPO eukaryotic expression vector, were used for screening. The ICP4 gene-coding region was cloned as two fragments overlapping by five codons (nucleotides 1–2077 and 2061–4105), referred to herein as ICP4-1 and ICP4-2, respectively.

**Detection of antigens recognized by CD8 T cells**

Unlike humans and mice, which generally express a fixed complement of three class I genes, the number of bovine class I genes expressed by different MHC haplotypes varies between one and four [26]. The class I genes expressed by a series of haplotypes in Holstein cattle have been fully characterized, and full-length cDNAs in eukaryotic expression vectors were available for the present study. Three BHV-1-immune animals were used for antigen screening, two of which expressed the A10 and A15 class I MHC haplotypes, while one expressed the A11 and A14 haplotypes. All four haplotypes are present at relatively high frequencies (~10%) in most populations in Holstein cattle [27]. The MHC class I alleles expressed by the animals are shown in Table 1. To detect which BHV-1 proteins were recognized by these animals, a CD8 T-cell line from each animal was screened for IFNγ release following incubation with COS-7 cells co-transfected with each BHV-1 gene and a mixture of the class I MHC heavy-chain alleles expressed by the respective CD8 T-cell line from each animal was screened for IFNγ release following incubation with COS-7 cells co-transfected with each BHV-1 gene and a mixture of the class I MHC heavy-chain alleles expressed by the respective CD8 T-cell line. The results obtained by screening with CD8 T cells from one of the A10/A15 animals (3430) are illustrated in Fig. 1, which demonstrates recognition of the expressed products of two IE genes, ICP4 and Circ. Identical results were obtained with the other A10/A15 animal (3109). The A11/A14 animal (1475) also recognized ICP4 and Circ and another IE gene product.
ICP22, as well as an L gene (UL49) that encodes a tegument protein (data not shown). The two A10/A15 animals recognized both expressed fragments of the ICP4 gene (ICP4.1 and ICP4.2), indicating the presence of more than one epitope specificity, whereas the A11/A14 animal only recognized one fragment (ICP4.1).

Identification of the class I MHC restriction elements

To determine which class I alleles are involved in presentation of the viral antigens, further screens were conducted in which COS-7 cells were co-transfected with the viral genes that gave positive reactions in the antigen screens, along with individual class I heavy-chain cDNAs. The results are shown in Figs 2 and 3 and the overall findings are summarized in Table 1. Again, identical results were obtained with the two A10/A15 animals; ICP4 was recognized in combination with both the 3*00102 (A10) and 1*00901 (A15) alleles, and an additional positive reaction was detected with the Circ gene product presented by 3*00102 (A10). No positive reactions were detected against cells transfected with the remaining class I alleles expressed by these animals (2*01201, 4*02401 and 2*02501 – data not shown). In the A11/A14 animal (1475), both ICP4 and UL49 were presented by the 3*01701 (A11) allele, Circ was presented by the 2*01801 (A11) allele and ICP22 was presented by the 4*02401 (A14) allele. No positive reactions were detected against cells transfected with the remaining class I alleles expressed by this animal (1*02301, 2*02501 and 6*04001 – data not shown). Thus in each animal individual antigens are presented by one or two of the available class I alleles.

Identification of the CD8 T-cell epitopes in ICP4, Circ and ICP22

Two parallel approaches were pursued to identify the epitopes in the three IE gene products recognized by CD8 T cells, namely screening of truncated genes and synthetic peptides for recognition by the T-cell lines, and in silico use of

<table>
<thead>
<tr>
<th>Table 1. MHC class I genotypes of animals used for antigen screening and BHV1 antigens detected</th>
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<tr>
<td><strong>MHC allele</strong></td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>2*01201</td>
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<tr>
<td>3*00201</td>
</tr>
<tr>
<td>1*00901</td>
</tr>
<tr>
<td>2*02501</td>
</tr>
<tr>
<td>4*02401</td>
</tr>
<tr>
<td>2*01801</td>
</tr>
<tr>
<td>3*01701</td>
</tr>
<tr>
<td>1*02301</td>
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<tr>
<td>6*04001</td>
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</tbody>
</table>

*Detected in animal 1475 but not in animals 3109 or 3430.

Fig. 1. Results of antigen screening using a CD8 T-cell line from a BHV-1-immune animal (3430 –MHC A10/A15). T cells were incubated with COS-7 cells that had been co-transfected with the individual BHV-1 genes and a mixture of the MHCI cDNAs expressed by the donor animal. Release of IFNγ into the culture supernatant was measured after 48 h using a specific ELISA. T cells incubated with autologous and allogeneic T. annulata-transformed cells infected with BHV-1 were included as positive and negative controls, respectively.

Fig. 2. MHC restriction of T-cell responses to BHV-1 proteins recognized by a CD8 T-cell line from animal 1475 (MHC A11/A14). T-cell recognition was examined by incubation of T cells with COS-7 cells co-transfected with individual BHV-1 genes and MHCI alleles and measurement of IFNγ release into the culture supernatant after 48 h using a specific ELISA. Panels (a), (b) and (c) show the results obtained with cells transfected with the 3*01701, 2*01801 and 4*02401 MHCI alleles, respectively. None = COS-7 cells transfected with MHCI cDNA only; BHV = autologous T. annulata-transformed cells infected with BHV-1.
antigen specificities, two contiguous overlapping peptides were found to stimulate T-cell reactivity, suggesting the presence of a single epitope.

Further fine mapping to identify the epitopes, defined as the minimal length peptide that retained near-maximal T-cell recognition, was conducted using truncated peptides covering the 17-mer peptides recognized by CD8 T cells. Similar results were obtained with both A10/A15 animals. For the ICP4-1 restricted ICP4 epitope recognized by these animals, 12-mer, 11-mer, 10-mer, 9-mer and 8-mer peptides were used for screening. The results obtained with the CD8 T-cell line from animal 3019 (A10/A15) using the 12-mer and 9-mer peptides are illustrated in Fig. 4, and they identify a 9-mer (ICP4 1159–1167) as the likely epitope. Synthetic peptides representing step-wise truncation at either end of the remaining positive regions were used to map a further five epitopes; the results of these screens are shown in Table 2. In each case, identification of the C-terminal amino acid residue was relatively straightforward, as truncation at this residue resulted in an abrupt loss of T-cell reactivity. By contrast, identification of the N-terminal residues was in some cases more ambiguous. For example, 10-mer and 9-mer peptides for the ICP4-3*00201 and ICP22-4*02401 epitopes (shown in Table 2) showed strong T-cell recognition, but shorter 9-mer and 8-mer peptides truncated by one residue at the N-terminus also retained substantial T-cell recognition.

**In silico validation**

The identified epitope regions and the corresponding BoLA restriction elements were analysed using the NetMHCpan (version 2.9) prediction method [28]. Earlier studies have demonstrated the ability of this method to accurately predict peptides that bind to MHC I, including BoLA-1 molecules [29–31], and the method has proved useful as a guide to confirm whether identified epitope sequences are indeed minimal epitopes [32]. Therefore, these analyses set out to: (i) quantify the degree to which *in silico* predictions are capable of identifying the validated epitopes; and (ii) identify possible alternative peptide variants (extensions or fragments) of the validated epitopes predicted to have improved binding affinity to the BoLA-I restriction element. The results of the analyses, shown in Table 3, reveal several important points. First and foremost, they confirm the high predictive capacity of NetMHCpan for most BoLA molecules. Four of the identified epitopes were confirmed, including the ICP4-3*00201 AGPDQLQRAL epitope discussed above, which is predicted to have much stronger MHC binding than the shorter peptide GPDLQLARL that also showed T-cell recognition. In one case (ICP22-4*02401), *in silico* screening suggested an alternative truncated version (PGAFCPEDW) of the epitope identified by peptide screening (PGAFCPEDW); although the former showed slightly weaker T-cell recognition, its superior predicted MHC binding indicates that it is likely to represent the minimal epitope. Based on these findings, in four out of six cases the F-rank value for the (alternatively) validated epitopes is below 2%. The two molecules with the poorest performance (BoLA-1*00901 and BoLA-3*01701) both have protein sequences that are very
distinct from the sequences of the BoLA molecules included in the training of the NetMHCpan method, which probably accounts for the overall low prediction accuracy for these molecules (see Table 3).

**Abundance of epitope-specific CD8 T cells**

Although the IFNγ ELISA allowed detection of the viral proteins and the location of the epitopes recognized by CD8 T cells, it did not provide information on the relative abundance of T cells specific for each of the epitopes in the CD8 T-cell lines. Therefore, further experiments were undertaken to provide an estimate of the proportions of cells that are specific for the different epitopes. CD8 T-cell lines from one of the A10/A15 animals (3430) and the A11/A14 animal (1475) were analysed both by intracellular staining for IFNγ and using an IFNγ ELISpot assay. The responses of T cells against autologous *T. annulata*-infected cells pulsed with peptides containing each of the identified epitopes were compared with the responses of the same cells infected with BHV-1. Controls included T cells incubated with autologous *T. annulata*-infected cells alone or pulsed with an irrelevant peptide and MHC-unrelated *T. annulata*-infected cells infected with BHV1. The results obtained with both CD8 T cell lines are illustrated in Fig. 5.

Intracellular IFNγ staining of CD8 T cells from the A10/A15 animal (3430) detected 6.5 and 17.2 % positive cells (following subtraction of background levels) for the ICP4 epitopes presented by 2*00901 (A10) and 2*00901 (A15), respectively, compared to 54 % positive cells reactive with whole BHV1 (Fig. 5b). The lack of a detectable response to the Circ epitope recognized by this animal is thought to reflect a lower frequency of CD8 T cells specific for this epitope and the relative inability of this assay to detect cells present at a frequency of <1 %. Staining of responding CD8 T cells from the A11/A14 animal (1475) detected 6 and 14 % positive cells for the ICP4 and Circ epitopes presented by 3*01701 and 2*01801 (both A11), respectively, compared to 21 % for whole BHV1 (Fig. 5d).

The ELISpot assays yielded much smaller percentages of positive cells (<1 %) in both T-cell lines, but detected substantial responses, relative to those against the whole virus, to the same epitopes detected by intracellular IFNγ staining in each animal (Fig. 5a, c). A weaker response to the Circ epitope recognized by the A10/A15 animal (3430) was detected in this assay. The ICP22 epitope presented by 4*02401 (A14) in the A11/A14 animal (1475) was also included in this assay and yielded a positive result of
similar magnitude to that detected for the ICP4 and Circ epitopes.

The results of these experiments indicate that the epitope-specific T cells detected in these studies represent major components of the virus-specific CD8 T-cell lines.

**DISCUSSION**

The current study has, for the first time, provided information on the fine specificity of CD8 T-cell responses to a natural herpesvirus infection in a non-primate species. The development of a system to generate virus-specific CD8 T-cell lines from BHV-1 immune cattle, employing Theileria-transformed cell lines for antigen presentation, has enabled us to investigate the viral antigens recognized by CD8 T cells in the current study. Screening of a panel of 16 candidate viral gene products with 3 CD8 T-cell lines identified four antigens, including three IE gene products and a tegument protein. The epitope-containing regions of these antigens and the respective presenting MHC class I alleles were identified and semi-quantitative analyses of the epitope-specificity of the CD8 T-cell lines indicated that the IE antigen epitopes represent a major component of the CD8 T-cell response to the virus.

Previous analyses of the specificity of immune responses to BHV-1 have focused largely on a subset of the glycoproteins. The gB, gC, gD and gH glycoproteins were shown to induce readily detectable antibody and/or CD4 T-cell responses [33, 34], and one previous study reported evidence of recognition of the gC and gD glycoproteins by CD8 T cells from immune cattle [24]. Animals vaccinated with a DNA plasmid encoding gB were also shown to generate a gB-specific CD8 T-cell response [35]. However, the CD8 T cell lines used in the current study did not have detectable reactivity with cells transfected with the gB, gC or gD genes, and in a previous study we showed that the same cell lines did not recognize cells infected with recombinant vaccinia viruses expressing these glycoproteins [25]. Previous antigen screening by Denis et al., [24] was based on cytotoxicity assays using virus-stimulated whole PBMC as effectors and was confined to four glycoproteins expressed

| Table 2. Mapping of CD8 T-cell epitopes in BHV-1 IE proteins by screening for recognition of truncated peptides |
|---|---|---|---|---|---|
| Viral protein/MHC allele | T-cell line | Peptides | Response to peptide† |
| ICP4/3*00201 | 43-40 (A10/A15) | | |
| | | 59AGPDILQRAL | 76 | 81 | 75 |
| | | 40GPDLQLRAL | 57 | 26 | 1 |
| | | 40GPDLQLRAL | 63 | 37 | 3 |
| | | 40GPDLQLRAL | 0 | 1 | 0 |
| | | 40PDILQLRL | 3 | 1 | 0 |
| ICP4/3*01701 | 1475 (A11/A14) | 207GRATGARAGYAA | 25 | 1 | 0 |
| | | 297GRATGARAGYAA | 16 | 3 | 0 |
| | | 217GRATGARAGYAA | 25 | 1 | 0 |
| | | 288RATGARAGYAA | 31 | 4 | 0 |
| | | 289ATGARAGYAA | 29 | 2 | 0 |
| | | 290TGAARAGYAA | 45 | 13 | 0 |
| | | 290GARAYGA | 12 | 14 | 3 |
| Circ/3*00201 | 43-40 (A10/A15) | 1475FTTPEIELIE | 35 | 39 | 45 |
| | | 1475FTTPEIELIE | 38 | 26 | 0 |
| | | 1475TPEIELIE | 37 | 47 | 41 |
| | | 1475TPEIELI | 0 | 0 | 0 |
| | | 1475TPEIELI | 3 | 0 | 0 |
| Circ/2*01801 | 1475 (A11/A14) | 235AAPAPSGAL | 54 | 39 | nt |
| | | 235AAPAPSGAL | 51 | 38 | nt |
| | | 235AAPAPSGAN | 0 | 0 | nt |
| | | 235APAPSGAN | 14 | 12 | nt |
| | | 235APAPSGAN | 0 | 0 | 0 |
| ICP22/4*02401 | 1475 (A11/A14) | 42RPGFAPCPED | 87 | 2 | 1 |
| | | 42RPGFAPCPED | 88 | 87 | 1 |
| | | 42RPGFAPCPED | 87 | 11 | 1 |

†The responses of CD8 T-cell lines were examined by incubating T cells with three different concentrations of peptide in duplicate for 48 h and then testing the supernatants for their ability to up-regulate class II MHC expression in MDBK cells. The results are presented as the percentage of class II MHC-positive MDBK cells minus background levels obtained with supernatant from unstimulated T cells. Values for the latter varied between 7 and 12 % in different assays. The inferred epitope sequences are highlighted in bold. nt = not tested.
### Table 3. Summary of mapping and in silico prediction of CD8 T-cell epitopes in BHV-1 immediate early proteins

<table>
<thead>
<tr>
<th>Viral protein</th>
<th>Mapped region</th>
<th>BoLA-I</th>
<th>Mapped epitope</th>
<th>Predicted accuracy</th>
<th>F-Rank</th>
<th>Alternative epitope</th>
<th>F-Rank-alt</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICP4</td>
<td>1153–1174</td>
<td>1:00901</td>
<td>1139FVEGEAASH1167</td>
<td>0.694</td>
<td>0.271</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICP4</td>
<td>393–414</td>
<td>3:00201</td>
<td>399AGPDQLQARI408</td>
<td>0.853</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Circ</td>
<td>136–157</td>
<td>3:00201</td>
<td>141TTPHEILIE149</td>
<td>0.853</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICP4</td>
<td>282–303</td>
<td>3:01701</td>
<td>290TGARAGYAA298</td>
<td>0.470</td>
<td>0.078</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Circ</td>
<td>229–250</td>
<td>2:01801</td>
<td>234APAPSGAL242</td>
<td>0.334</td>
<td>0.015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICP22</td>
<td>37–58</td>
<td>4:02401</td>
<td>44PGAFCPEDW52</td>
<td>0.853</td>
<td>0.025</td>
<td>45GAFCPEDW52</td>
<td>0.004</td>
</tr>
</tbody>
</table>

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**Fig. 5.** Antigenic specificity of BHV-1-specific CD8 T-cell lines analysed using an IFN\(\gamma\) ELISpot assay (a, c) and by staining for intracellular IFN\(\gamma\) (b, d), following stimulation with BHV-1 or *T. annulata*-transformed cells pulsed with peptides representing each of the identified CD8 T-cell epitopes. Results are shown for CD8 T-cell lines from animal 3430 (MHC A10/A15) (a, b) and animal 1475 (MHC A11/A14) (c, d). Autologous and allogeneic cells infected with BHV-1 (Aut BHV and Allo BHV, respectively) were tested in the intracellular IFN\(\gamma\) assay to confirm MHC restriction. A control (Cont) consisting of *T. annulata*-transformed cells pulsed with an irrelevant peptide was included in each assay.
Six CD8 T-cell epitopes (three in ICP4, two in Circ and one in ICP22) presented by five different MHCI alleles were identified within the 3 BHV-1 IE proteins. A combination of T-cell screening of synthetic peptides and in silico analysis employing the NetMHCpan prediction method was used for epitope identification. The latter successfully predicted four of the epitopes identified by peptide screening and identified an 8-mer peptide, rather than a 9-mer one, as the more likely identity of the remaining epitope. Further studies using class I MHC tetramers will be required to unequivocally confirm the identity of these epitopes. Nevertheless, the findings highlight the value of these in silico tools for predicting and refining the identity of CD8 T-cell epitopes. In two cases, the in silico method gave low prediction accuracy for the epitope and BoLA restriction element. The distinct nature of the amino acid sequences of the two corresponding BoLA molecules compared to those of the BoLA molecules included in the training of the NetMHCpan method probably account for the overall low prediction accuracy for these molecules. Further data on the nature of the peptides that bind to these alleles are likely to improve the predictive values of NetMHCpan. Such improvements should facilitate more reliable prediction and more rapid identification of epitopes in known CD8 T-cell antigens in future studies.

The results of this study are consistent with previous evidence that CD8 T-cell responses to viral infections are frequently focused on a limited number of dominant epitopes, which differ depending on host MHCI genotype (reviewed in [42]). In this regard, it is of note that the two MHCI-identical, but otherwise unrelated, animals in the current study had responses to the same three epitopes (two in ICP-4 and one in Circ), whereas T cells from the third animal, which had a different MHCI type, recognized different epitopes in ICP4 and Circ and additional epitopes in ICP22 and UL49. Given the influence of MHCI type on epitope specificity, it will be necessary to extend these studies to additional animals of different MHCI types to determine whether the same IE gene products are consistently immunodominant.

CD8 T-cell responses specific for IE gene products are considered to be particularly relevant for immunity, because their early expression, before the full effect of virus-mediated down-regulation of class I MHCI, should theoretically enable rapid induction of CD8 T-cell responses following virus challenge. However, studies of responses to HSV-1 in humans have identified CD8 T cells that are specific for a variety of proteins. Laing et al. [12] list 23 CD8 T cell target HSV-1 proteins, including IE and E gene products as well as glycoproteins, although individual people only showed responses to a subset of these, which varied depending on their MHCI type. As pointed out by these authors, the summarized findings represent the results of a large number of studies of T-cell specificities from both healthy and lesioned HSV-1-infected people, using different antigen-screening systems. Both the stage of infection of donors and the T-cell assays employed could have influenced the particular

in recombinant vaccinia viruses. The involvement of CD8 T cells was inferred from ablation of cytotoxicity following depletion of CD8 T cells by antibody- and complement-mediated lysis. Since CD8 is also known to be expressed on bovine NK cells and γδ T cells [36, 37], which can exhibit cytotoxic activity, the attribution of the cytotoxicity measured in these studies to classical CD8 T cells remains open to question. Nevertheless, it is possible that CD8 T cells specific for additional antigens, including the glycoproteins, were present at low frequency in our T-cell lines, but were not detected by our screening method. The T-cell lines used in the present study were depleted of γδ T cells and, although they were not examined for expression of NK cell markers, the demonstration of exquisite MHC restriction of the detectable responses clearly showed that they were mediated by classical CD8 T cells.

It is particularly striking that 3 of the 4 IE gene-encoded proteins included in the screening panel were recognized strongly by the CD8 T-cell lines, whereas only 1 of the remaining 12 E and L gene products gave a positive result in 1 animal. One of the IE genes, ICP4, is only expressed in the IE phase, whereas the others (Circ and ICP22) also continue to be expressed during later phases. ICP4 and ICP22 are known to be components of the protein complex that initiates transcription of E genes [38], and HSV-1 orthologues of both have been identified as CD8 T-cell antigens in humans [12]. The third IE gene (Circ), which does not have a known orthologue in HSV-1 and is of unknown function, encodes a myristylated protein found in the cytoplasm of infected cells [39]. The E gene UL49 encodes a tegument protein [40], the orthologue of which in HSV-1 and HSV-2 has also been identified as a CD8 T-cell antigen that is particularly dominant in people expressing the HLA-B^*0702 class I allele [12]. Although antigen screening was confined to proteins encoded by 16 of the 73 BHV-1 open reading frames, the results indicated pronounced dominance of IE gene products among the viral proteins that were screened. All three animals examined recognized epitopes in the ICP4 and Circ proteins and one animal recognized ICP22. Moreover, CD8 T cells specific for these epitopes represented a substantial component of the CD8 T-cell lines, with 20–24% of CD8 T cells being found to respond specifically to the strongest epitopes, based on an intracellular IFNγ staining assay. Given that not all CD8 T cells specific for a given epitope produce detectable levels of IFNγ [41], these figures are almost certainly an underestimate of the true frequencies. The use of T. annulata-infected cells as antigen-presented cells to generate the CD8 T-cell lines is unlikely to have resulted in a preferential bias in the specificity of the detected response. They express high levels of both class I and class II MHC proteins on their cell surface and support fully productive infection with BHV-1, resulting in cell lysis and release of infectious virus; they also show surface expression of BHV-1 glycoproteins following infection with the virus [25]. Hence, they are expected to express the full repertoire of BHV-1 proteins and present them efficiently to CD8 T cells.
antigens identified. As in the current study, early studies to screen for HSV-1 antigens used immune CD8 T cells stimulated in vitro with cells incubated with whole live or UV-irradiated virus (eg. [43]), whereas several more recent large-scale screening studies examined the ability of large panels of viral peptides or recombinant proteins to elicit CD8 T-cell responses in vitro [7, 8]. While the latter undoubtedly identified genuine antigenic specificities, the epitopes may not all be presented efficiently by virus-infected cells and hence the findings do not necessarily reflect the frequencies of cells capable of recognizing infected cells and acting as effectors. An extreme example of this is the EBV EBNA1 protein, which inhibits its own proteasomal degradation, resulting in the failure of CD8 T cells to recognize epitopes from this protein on virus-infected cells [44], yet CD8 T cells specific for the antigen can be detected by other screening methods [45]. The frequencies of CD8 T-cell antigen specificities might also be influenced by whether they are examined during active infection or following convalescence, and whether subjects have received repeated viral challenge. In this regard, the CD8 T-cell lines examined in the current study were derived from blood T cells harvested 15 days after virus challenge of animals previously vaccinated with a live attenuated vaccine. Previous longitudinal studies of CD8 T-cell responses to human CMV and EBV indicated that the range of CD8 T-cell specificities remains relatively stable over time [13, 46, 47], although in some instances a less pronounced dominance hierarchy has been observed in the memory pool compared to the active response [48]. It is possible that the detection in the current study of prominent responses to BHV-1 IE proteins reflects preferential expansion of pre-existing memory T cells specific for the IE antigens in the 15-day period immediately following virus challenge.

In conclusion, the current study has for the first time provided unambiguous information on the identity of BHV-1 viral proteins recognized by specific CD8 T-cell responses, yielding evidence that three of the IE gene products are dominant targets. Since memory CD8 T cells specific for IE gene products would be expected to be effective in minimizing virus production following challenge of immune animals and upon reactivation of latent infection, these antigens provide potentially useful candidates for the generation of a subunit vaccine for BHV-1, should such a vaccine be required.

METHODS

Viruses

The Cooper and Iowa strains of BHV-1 used in the study (kindly provided by Moredun Scientific, Roslin, UK) and the methods used to culture and quantify the virus are described elsewhere [25]. All of the in vitro analyses of T-cell responses used the Cooper strain.

Animals and immunization

Three Holstein cattle aged approximately 18 months and screened as BHV-1-negative at the outset of the experiments were used in the study. Their MHC class I types were determined using a combination of serological typing with monoclonal antibodies [49] and allele-specific PCR [50].

The animals were vaccinated with a live attenuated BHV-1 vaccine (Tracherine IBR marker live, Pfizer Animal Health, Surrey, UK). One of them (1475) was challenged intranasally 2 months later with wild-type BHV-1 (2 x 10^7 TCID_{50} Cooper strain). The remaining two animals were challenged twice with wild-type BHV-1, first with 2 x 10^7 TCID_{50} Iowa strain 4 weeks after vaccination, and then after a further 2 years with 2 x 10^7 TCID_{50} Cooper strain, both administered intranasally. CD8 T-cell responses were studied using blood leukocytes collected 15 days after the final challenge. Cell lines transformed by infection with *Theileria annulata* were established from each animal at the outset of the study by in vitro infection of peripheral blood mononuclear cells (PBMC) with the sporozoite stage of the parasite, as described previously [51].

BHV-1-specific CD8 T-cell lines

The method used for establishing CD8 T-cell lines specific for BHV-1 is described in detail elsewhere [25]. Briefly, aliquots of 4 x 10^6 PBMC from immunized animals were cultured in 24-well plates with 2 x 10^5 gamma-irradiated autologous *T. annulata*-infected cells infected with BHV-1. Viable cells harvested after 7 days were subjected to a second stimulation with irradiated BHV-1-infected cells and, after a further 7 days of culture, viable cells were again harvested and enriched for CD8 T cells by antibody- and complement-mediated depletion of CD4 and γδ T cells. The CD8-enriched populations (>97% pure) were then subjected to a further stimulation at a ratio of 10:1 BHV-1-infected cells to T cells, with addition of 100 U ml^-1 recombinant human IL-2 (Chiron, Emeryville, CA, USA).

Isolation and cloning of BHV-1 genes

PCR primers containing a KOZAK sequence were designed to amplify full-length sequences of the selected BHV-1 genes, such that the stop signal was removed. A PCR protocol designed to amplify GC-rich DNA was employed, using the conditions described by Hansen and Justesen [52]. PCR reactions were performed in a volume of 50 µl containing 1 µl 25 mM DNTP mix, 1 µl 20 pmol ml^-1 primer stock, 1 µl 50 mM MgCl2, 5 µl x 10 PCR buffer, 1 µl template DNA and 0.5 µl Taq DNA polymerase (Bioline, UK). PCR reactions were tested with or without DMSO at 5% (v/v) and with Betaine solution (Sigma, Dorset, UK) at 1 or 2 M concentration in order to obtain the most PCR product. The conditions that worked best for most reactions were 5% DMSO and 1 or 2 M Betaine.

PCR reactions were performed on a Technec Thermal cycler (Bibby Scientific, UK) with the following settings: 95° for 1 min, then 30 cycles of 95° 1 min, annealing for 1 min,
72 °C for 1 min/kb, followed by 72 °C for 10 min. If the PCR product contained a single band, the product was purified using a Qiagen PCR purification kit (Qiagen Ltd, UK) as described. If the product contained multiple bands, the band of the correct size was gel-purified using a Qiagen gel purification kit (Qiagen Ltd, UK) as described.

The purified PCR product was then cloned into the expression vector pcDNA3.1V5 His TOPO according to the manufacturer’s instructions. Positive colonies were selected by colony PCR and plasmid DNA for transfection was prepared using a Qiagen mini prep kit according to the manufacturer’s instructions (Qiagen Ltd, UK).

**Antigen screening**

Antigen screening was carried out essentially as described previously [53], and was based on measurement of interferon gamma (IFNγ) release by CD8 T-cell lines incubated with COS-7 cells transfected with individual BHV-1 genes along with a pool of cDNAs encoding the class I heavy chains expressed by the respective animals. COS-7 cells were plated at 2 x 10⁶ cells per well in 96-well flat-bottom plates in Dulbecco’s modified Eagle’s medium (DMEM) with 10% foetal bovine serum, 2 mM L-glutamine, 100 u penicillin and 10 µg streptomycin/ml. The following day the cells were transfected with 100 ng of BHV-1 DNA, 100 ng bovine class I DNA and 0.9 µl of FuGENE (Roche Diagnostics) in 50 µl of DMEM without additives for 4 h at 37 °C. The transfection medium was then replaced with 200 µl DMEM and the cells were incubated for 24 h at 37 °C. 1 x 10⁶ CD8 T cells in 150 µl of RPMI growth medium were then added to each well and the plates incubated for a further 24 h prior to the removal of 100 µl of medium for IFNγ detection.

**Mapping of CD8 T-cell epitopes**

A combination of two methods was used to locate and identify the epitope(s) recognized by immune CD8 T cells. First, cloned fragments of the positive cDNAs obtained by PCR amplification were screened for recognition, as described for the full-length cDNAs, in order to localize the epitopes to one discrete region. Overlapping synthetic peptides (17mers overlapping by 12 residues; Pepscan Ltd, Netherlands) representing the positive region were then screened to identify the epitope using T. annulata-infected cells as antigen-presenting cells (APC), as described previously [53]. Assays were conducted in 96-well flat-bottomed plates using 2 x 10⁴ CD8 T cells/well, with 10⁴ peptide-pulsed APC, in a total volume of 200 µl and incubated for 48 h at 37 °C. Supernatants were assayed for IFNγ as a measure of recognition by the CD8 T-cell line. The minimum peptide length recognized by the T cells was then determined by measuring responses to 10-fold dilutions of peptides of different lengths covering the region identified by the positive 17-mer peptides. Mapping of one of the epitopes (restricted by the 1*00901) used peptide-pulsed APC, as described above, whereas mapping of the remaining epitopes measured responses to peptides added directly to the CD8 T cells. Preliminary experiments indicated that these methods had similar sensitivity (data not shown).

**Epitope prediction**

An in silico validation of the identified epitopes and corresponding BoLA restriction elements was performed using the NetMHCpan prediction method [28]. This method is trained on an extensive set of close to 140 000 MHC-peptide binding values covering 158 MHC molecules, including 13 bovine MHC class I (BoLA-I) alleles, and allows for prediction of peptides capable of binding to any MHC class I molecule of known protein sequence. NetMHCpan version 2.9 [30] was used rather than the recently updated version 3.0 [54], as the former was trained on an extended set of BoLA-I binding data that are not available in the public-domain data used to train version 3.0.

**Measurement of IFNγ release**

All of the experiments conducted with full-length viral genes to screen for antigen recognition and MHC restriction, as well as those using peptides to map the 1*00901-restricted CD8 epitope, measured IFNγ release in culture supernatants using a sandwich ELISA in Immulon 4HBX flat-bottomed microtitre ELISA plates (Thermo Fisher Scientific, Roskilde, Denmark), using non-competing monoclonal antibodies specific for bovine IFNγ (clones CC330 and CC302; AbD Serotec, Kidlington, UK). The latter was conjugated with biotin, and binding was detected with horseradish peroxidase-conjugated streptavidin (AbD; Serotec, UK), followed by addition of TMB (3, 3′, 5′-tetramethylbenzidine) substrate solution (BD Biosciences, Oxford, UK). A standard curve for determining IFNγ concentrations was generated using a series of doubling dilutions of bovine IFNγ (AbD; Serotec, UK). The IFNγ concentration was then calculated by correlation to the standard curve generated by the IFNγ standards. In the remaining epitope-mapping experiments, IFNγ release by T cells was measured using a biological assay performed as described previously [53], based on the ability of the IFNγ in culture supernatants to up-regulate surface expression of MHC class II on Madin–Darby bovine kidney (MDBK) cells. In preliminary studies, we showed that this assay had slightly greater sensitivity than the ELISA (data not shown).

**Interferon γ ELISpot**

ELISpot analysis was performed using Multiscreen IP filter plates (Millipore with PVDF membrane) using the protocol described by Millipore (EDM Millipore, UK). The plates were coated using an anti-IFNγ monoclonal antibody (CC330, AbD Serotec, UK) at 10 µg ml⁻¹ and incubated overnight at 4 °C. After the membrane was washed and blocked, two-fold dilutions of the effector CD8 T cells were added to triplicate wells, ranging from 5 x 10⁴ to 1.25 x 10⁵ cells per well. Stimulator cells, consisting of T. annulata-infected cells either infected with BHV-1 or pulsed with peptide, were added at an effector:stimulator ratio of 1:10; effector cells alone and T. annulata-infected cells without peptide or pulsed with an irrelevant peptide were included.
as negative controls. After 24 h incubation at 37 °C, the cells were removed and the plates washed before the secondary biotinylated anti-IFNγ monoclonal antibody (CC302, Serotec) was added at 2 µg ml⁻¹. After incubation at 37 °C for 2 h, the plates were washed and an alkaline phosphatase-conjugated streptavidin (Serotec, UK) was added at a dilution of 1/1000. After 45 min incubation at room temperature, the streptavidin was removed and the wells washed before addition of BCIP/NBT substrate (Sigma fast BCIP/NBT tablet, Sigma). The wells were then incubated for 5–10 min before being washed under running water and dried overnight in the dark. Spots on the plates were counted and analysed using an Elispot reader (AID Strassberg, Germany).

**Intracellular cytokine staining for IFNγ**

Intracellular cytokine staining for IFNγ was used in order to quantify the number of cells responding to particular viral antigens. The responding CD8 T cells were incubated for 2 h with stimulator cells (T. annulata-infected cells infected with BHV-1 or pulsed with peptide) at a responder: stimulator ratio of 1:5–1:10 before Brefeldin A was added at a concentration of 10 µg ml⁻¹. After a further 2 h incubation at 37 °C, the cells were washed twice in PBS and (if required) stained for CD8 using a specific FITC-conjugated antibody (CC63; Serotec, UK) or an isotype control, before being fixed in 2 % paraformaldehyde. The cells were then permeabilized in permeabilization solution 2 (Becton–Dickinson, CA, USA) and stored overnight at 4 °C in PBS containing 0.1 % sodium azide. The following day, the cells were stained using an RPE-conjugated anti-IFNγ antibody (CC302, serotec) or an isotype control antibody and analysed using a FACScalibur flow cytometer (Becton–Dickinson, CA, USA).

**References**

mutants with a deletion in the gC, gG, gI, gE, or in both the gI and gE gene. *Vaccine* 1998;16:802–809.


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