The CD4$^+$ T-cell response to adenovirus is focused against conserved residues within the hexon protein

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Adenovirus is a significant pathogen in immunocompromised patients and is widely utilized as a gene delivery vector, so a detailed understanding of the human immune response to adenovirus infection is critical. This study characterized the adenovirus-specific CD4$^+$ T-cell response of healthy donors by incubation with whole virus or with individual hexon and fiber proteins. Adenovirus-specific CD4$^+$ T cells averaged 0.26% of the CD4$^+$ T-cell pool and were detectable in all donors. T cells recognizing the highly conserved hexon protein accounted for 0.09%, whereas no response was observed against the fiber protein. A panel of hexon-specific CD4$^+$ T-cell clones was generated and shown to lyse targets infected with adenovirus from different serotypes and species. Three CD4 T-cell epitopes are described, which map to highly conserved regions of the hexon protein.

INTRODUCTION

Human adenoviruses (Ads) comprise six species of the genus Mastadenovirus (family Adenoviridae) and many of the 51 serotypes are endemic in the human population. Infection is often asymptomatic and rarely causes severe disease in immunocompetent individuals (Horwitz, 2001). However, Ad has been associated with morbidity and mortality in the immunocompromised (Leen & Rooney, 2005), most frequently in paediatric haematopoietic stem cell transplantation (HSCT) recipients (Flomenberg et al., 2005), and recent reports of safety and efficacy of adoptive T-cell therapy for Ad disease in HSCT recipients by donor lymphocyte infusion (Hromas et al., 1994; Hale et al., 1999; Chakrabarti et al., 2002; Lion et al., 2003) and AIDS patients (Kojaoghlanian et al., 2003). In HSCT recipients, a correlation between lack of T-cell reconstitution and Ad infection indicates a role for cellular immunity in the control of Ad infection (Chakrabarti et al., 2002; Feuchtinger et al., 2005). The greatest risk of Ad infection is when the donor is HLA mismatched and the graft has to be T-cell depleted to reduce graft versus host disease. There are examples of clearance of Ad infection in HSCT recipients by donor lymphocyte infusion (Hromas et al., 1994; Miyamoto et al., 1998; Bordigoni et al., 2001) and recent reports of safety and efficacy of adoptive T-cell therapy for Ad disease in HSCT recipients (Feuchtinger et al., 2006; Leen et al., 2006). Current drug therapy for Ad infection is suboptimal, so there is an urgent need for the development of new therapeutic approaches such as T-cell-mediated immunotherapy.

Adoptive T-cell therapies for the treatment of Epstein–Barr virus (EBV) and cytomegalovirus (CMV) disease in immunocompromised patients are showing encouraging results in clinical trials (Burns & Crawford, 2004; Einsele & Hebart, 2004). The design of these therapies has been underpinned by a detailed knowledge of dominant antigens and epitopes recognized by virus-specific T cells (Bollard et al., 2004). Whilst antiviral T-cell therapies focused initially on the delivery of virus-specific CD8$^+$ T cells, there is increasing evidence of the importance of CD4$^+$ T cells in the control of viral diseases (Gamadia et al., 2003; Long et al., 2005). Despite the interest in using adoptive T-cell therapy for Ad disease, there are no similar detailed studies characterizing Ad-specific cellular immunity. Murine models are limited because Ad infections are at best semi-permissive and consequently cellular immune responses are dominated by CD8$^+$ cytotoxic T cells (CTLs) specific for early proteins (Mullbacher et al., 1989; Rawle et al., 1991). Most studies of human immunity in healthy volunteers have reported a predominant CD4$^+$ response (Olive et al., 2001; Sester et al., 2002; Heemskerk et al., 2003; Leen et al., 2004; Tang et al., 2006; Veltrop-Duits et al., 2006) but the relative importance of CD8 and CD4 T cells in Ad disease remains unclear. A study of target antigens recognized by Ad-specific memory T cells without in vitro selection bias is lacking. However, eight HLA class I-restricted epitopes (Leen et al., 2004; Tang et al., 2006) and ten HLA class II-restricted epitopes have been identified (Olive et al., 2002; Haveman et al., 2006; Heemskerk et al., 2006) through the use of T-cell lines or peptide screening. Importantly, Ad-specific T cells that recognize target epitopes within the hexon, the major coat protein, are
often serotype and species cross-reactive (Olive et al., 2002; Leen et al., 2004; Veltrop-Duits et al., 2006). The immunodominance of Ad antigens and epitopes remains to be defined.

Genetically modified human adenovirus serotype 5 (Ad5; species Human adenovirus C) is widely used in gene therapy clinical trial protocols (Young et al., 2006) and is under development as a vector for vaccination (Vanniasinkam & Ertl, 2005; Xing et al., 2005). However, most trials using Ad vectors have not investigated vector-specific cellular immune responses, in part because Ad-specific cellular immunity has been relatively poorly defined. As a consequence, it is still not known whether pre-existing Ad5 cellular immunity reduces the efficacy of vectors. Recent trials of an Ad5-based human immunodeficiency virus vaccine have correlated lack of efficacy with high levels of pre-existing neutralizing antibodies (Kahn, 2003); as yet, it is not known whether Ad-specific T cells also impair Ad5-based vaccine vectors by destroying transduced antigen-presenting cells (APCs) or whether vector-derived antigen might compete for antigenic dominance over transgene product(s). The identification and characterization of novel T-cell epitopes from Ad5 will provide tools to monitor trials and also predict the likely immunogenicity of newer vectors from a range of Ad serotypes.

Given the lack of information on the dominance of antigen recognized by Ad-specific T cells in the peripheral blood, we studied the T-cell response to two of the major antigenic components of the Ad5 capsid: the hexon and fiber proteins. We established that the hexon protein is the major target of CD4+ Ad-specific T cells. Using clonal analysis, these Ad5 hexon-specific T cells were shown to be serotype and species cross-reactive. Three HLA class II-restricted epitopes were identified and T cells recognizing these epitopes were shown to be cytotoxic. These results have wider implications for the use of adoptive T-cell therapy for the treatment of Ad infection and for the efficacy of Ad-based gene-delivery vectors.

### METHODS

**Cell preparations and cell lines.** Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood of healthy volunteers by Ficoll-Hypaque centrifugation (Lymphoprep; Axis Shield) into RPMI 1640 supplemented with 2 mM glutamine, 100 IU penicillin ml⁻¹, 100 μg streptomycin ml⁻¹ and 10% fetal bovine serum (FBS). Where specified, PBMCs were depleted of CD4+ or CD8+ T cells with CD4 or CD8 Dynabeads (Dynal) in accordance with the manufacturer’s recommended protocol and shown to be >97% free of the depleted subset by flow cytometry. An EBV-transformed lymphoblastoid cell line (LCLs) was prepared with prototype 1 strain B95-8 (Miller & Lipman, 1973) and cultured in medium as described above. Primary human fibroblasts derived from skin biopsies were grown in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine, 100 IU penicillin ml⁻¹, 100 μg streptomycin ml⁻¹ and 10% FBS, and activated to upregulate HLA expression by the addition of 300 U interferon (IFN)-γ (Sigma) ml⁻¹ to the culture medium 3 days prior to use as APCs.

**Virus and antigen.** Human Ad2 and Ad5 (species C), Ad3 (species B) and Ad4 (species E) were propagated in A549 cells until a cytopathic effect was observed. Cells were harvested and lysed by three freeze–thaw cycles before cellular debris was removed by centrifugation (800 g, 10 min). Infected cell lysates were used as antigen in subsequent assays and contained both viral protein and infectious virus (measured by plaque assay). Virus was also further purified from cell lysates by CsCl banding (Engler et al., 1999). Infectivity was quantified by plaque assay and particle number measured using a DNA-binding PicoGreen assay (Molecular Probes) (Murakami & McCaman, 1999). Excess Ad5 soluble antigen separated by CsCl banding was used as a rich source of hexon and fiber proteins.

Proteins were purified as described previously (Haase et al., 1997; Engler et al., 1999) using a POROS 10 HQ ion-exchange perfusion chromatography column under the control of a BioCad Vision WorkStation (Perceptive Biosystems). Purity was confirmed by SDS-PAGE and Western blot analysis using antibody R1/99, a hyperimmune serum from a rabbit immunized with Ad5.

**ELISPOT assays.** Unmodified or CD4- or CD8-depleted PBMC preparations were stimulated with A549 cell lysates produced from Ad5-infected or mock-infected cells (60 μg protein per 10⁶ cells) for 1.5 h (37 °C, 5% CO₂). Cells were washed and plated in triplicate at 4 x 10⁴ and 1 x 10⁵ cells per well onto PVDH-backed 96-well plates (Millipore) pre-coated with 15 μg anti-IFN-γ monoclonal antibody (mAb) (Mabtech) ml⁻¹. Alternatively, unmodified PBMCs were plated as above and peptide (5 μg ml⁻¹) or DMSO was added directly to the well. After overnight incubation (37 °C, 5% CO₂), spot-forming cells (s.f.c.) were detected as described previously (Leen et al., 2001). Spots were counted using an automated ELISPOT reader (AID) and the results given as s.f.c. per 10⁶ cells in the test wells, minus the background response to mock-infected lystate or DMSO as appropriate.

**Intracellular IFN-γ staining.** PBMCs (2 x 10⁶) were incubated with Ad5-infected or mock-infected cell lystate (60 μg protein per 10⁶ cells) or purified Ad5 hexon or fiber protein (5 μg per 10⁶ cells) at 37 °C for 12 h. Brefeldin A (10 μg ml⁻¹; Sigma) was added after the first hour of stimulation. Cells were washed with PBS and stained for 30 min on ice with anti-CD3, anti-CD4 and anti-CD8 antibodies (BD Pharmingen). Cells were then fixed and permeabilized using an Intraprep kit (Immunotech) according to the manufacturer’s instructions. Cells were stained with anti-IFN-γ antibody (BD Pharmingen). Samples were analysed on an Epics XL flow cytometer (Beckman Coulter).

**Generation of polyclonal and clonal T-cell lines.** Freshly isolated PBMCs were stimulated with Ad5-infected A549 cell lystate (60 μg per 10⁶ cells) by incubating them with antigen for 90 min before washing and plating at 10⁶ cells ml⁻¹ in 2 ml wells. Cells were fed twice weekly by a half medium change and from day 14 onwards cells were supplemented with 50 μ recombinant human interleukin (IL)-2 (Chiron) ml⁻¹. Cells were restimulated on day 14 by the addition of autologous PBMCs or LCLs that had been stimulated with Ad5 as above and subsequently γ-irradiated (exposure to 40 Gy γ-irradiation from a Cs¹³⁷ source). The ratio of T cells to irradiated APCs was 5:1. On days 14 and 28, the T-cell lines were cloned by limiting dilution at 0.3 and 3 cells per well with allogeneic γ-irradiated, phytohaemagglutinin-treated PBMCs (10⁷ per well) in RPMI 1640 supplemented with IL-2 (200 U ml⁻¹), 10% FBS and 1% human AB serum. After 2–3 weeks, growing microcultures were screened for Ad5 reactivity by IFN-γ ELISA using Ad5-infected or mock-infected autologous LCLs as APCs. Selected clones were expanded in 2 ml wells using the same medium and feeder cells as described above for cloning.

**ELISAs of IFN-γ release and mAb blocking.** Cloned T cells were incubated in V-bottomed microtest plate wells with autologous,
HLA-matched or HLA-mismatched LCLs or fibroblasts that were either unmanipulated or pre-pulsed for 2 h with 5 μg peptide ml⁻¹ (or DMSO as a control) or infected with Ad (or mock infected) for 1.5 h and then washed. The supernatant medium harvested after 18 h was assayed for IFN-γ by ELISA (Endogen) in accordance with the manufacturer’s recommended protocol. In blocking assays, fibroblasts were pre-incubated with mAbs specific for HLA-DR (L243; ATCC clone HB-55), HLA-DQ (SPV-L3; Serotec), and HLA-DP (B7.21; kindly provided by Dr G. Taylor, University of Birmingham, UK), for 1 h before the addition of T cells to the assay.

Epitope prediction and synthetic peptide preparations. The Ad5 hexon protein sequence (NCBI Entrez protein database accession no. P04133) was input into the online epitope prediction software SYFPEITHI (www.syfpeithi.de) (Rammensee et al., 1999) to predict HLA-DRB1*0101-restricted 15mer epitopes. Epitope predictions were analysed to determine the conservation of epitope sequence among serotypes by comparison of hexon amino acid sequences predicted from nucleotide sequences of representative members of all six species of human Ad (Ad3, -4, -5, -12, -40 and -48) from the NCBI Entrez protein database. Twelve peptides scored 27 or higher by the SYFPEITHI criteria. Of these, nine were conserved throughout Ad species; any variation in sequence amongst these serotypes did not give rise to a significantly reduced score relative to that predicted for the Ad5 sequence as assessed by SYFPEITHI criteria. These nine epitope peptides and the previously identified DEP910 (Olive et al., 2002) were synthesized by 9-fluorenylmethoxycarbonyl chemistry (Alta Bioscience), dissolved in DMSO and their concentrations determined by biuret assay. The position within the Ad5 hexon protein of the peptides synthesized is identified by the amino acid number of the N-terminal residue. Epitopes recognized by T cells were also given a three-letter code referring to the first three N-terminal residues.

Chromium-release assays. CD4⁺ T-cell clones were tested for killing of target cells at defined effector : target ratios in 16 h chromium-release assays; the results were expressed as the percentage specific lysis of the target line. Targets were IFN-γ-activated autologous fibroblasts pre-exposed for 2 h to 5 μg peptide ml⁻¹ (or DMSO as a negative control) or pre-infected overnight with Ad2, -3, -4 or -5 (10⁷ particles per cell or mock infected). Supernatants were sampled and γ-emission quantified using a Packard Cobra gamma counter.

RESULTS

High frequency of Ad5 hexon-specific but not fiber-specific T cells in peripheral blood

To determine the levels of pre-existing Ad-specific cellular immunity, PBMCs from ten donors were left unmodified or depleted of CD8⁺ or CD4⁺ T cells before analysis in an IFN-γ ELISPOT assay. Fig. 1(a) shows that Ad-specific T cells were enriched in the CD8-depleted population and greatly reduced in the CD4-depleted cells in comparison with unmodified PBMCs, indicating that the response was dominated by CD4⁺ T cells. Low-frequency CD8⁺ T-cell responses were detected in five out of ten donors. To define the antigen recognized by the predominant memory CD4⁺ T cells with the minimum of in vitro selection bias, freshly isolated PBMCs from a separate cohort of 15 donors were stimulated with Ad5 virus or with purified Ad5 capsid hexon or fiber protein. Responding cells were enumerated by intracellular staining for IFN-γ. Fig. 1(b) shows that all
15 donors tested had CD4⁺ T cells that responded to virus (mean 0.26%, range 0.04–0.71%) and to hexon (mean 0.09%, range 0.03–0.21%), but not to the fiber protein. Representative plots of intracellular IFN-γ staining are shown following antigenic stimulation with wild-type Ad5-infected cell lysate (Fig. 1c), purified Ad5 hexon (Fig. 1d), purified Ad5 fiber (Fig. 1e) or A549 cell lysate (Fig. 1f). Veltrop-Duits et al. (2006) recently showed that responses to sections of the hexon protein were detectable in the majority of donors after stimulation and culture in vitro for 4 days. The results presented here are the first analysis of CD4⁺ T-cell responses to whole Ad proteins performed immediately ex vivo using fresh peripheral blood to avoid antigen bias caused by prolonged culture, and show that the hexon protein is an immunodominant target for Ad-specific T cells.

Clonal analysis of CD4⁺ Ad5-specific T cells

To characterize the Ad5-specific T cells further, Ad5-specific polyclonal T-cell lines from four donors (donors 1, 5, 6 and 15) were generated in vitro by repeated stimulation of PBMCs with virus-loaded γ-irradiated autologous PBMCs or LCLs. These lines were then cloned by limiting dilution and the clones screened by IFN-γ ELISA for their response to Ad5-infected autologous LCLs. Thirty-two out of 34 CD4⁺ T-cell clones isolated were capable of recognizing Ad5 hexon protein and recognized epitopes conserved amongst adenovirus of species B (Ad3), C (Ad2 and Ad5) and E (Ad4). Representative data from one such clone are illustrated in Fig. 2. The magnitude of IFN-γ release in these assays is influenced by a number of factors relating to the efficiency of infection and amount of antigen loaded and processed, and should not be taken as indicative of the T-cell clone’s avidity for its target peptide–MHC complex.

Identification of HLA class II-restricted epitopes within the Ad hexon protein

A large number of T-cell clones generated from donor 6 were hexon specific (17/19) and shown to be restricted through the HLA DRB1*0101 allele (7/11 tested) by IFN-γ ELISA utilizing autologous, HLA class II-mismatched and partially mismatched fibroblasts as APCs in combination with HLA class II blocking antibodies (data not shown). SYFPEITHI epitope prediction software (Rammensee et al., 1999) was used to identify HLA DRB1*0101-restricted epitopes within the Ad5 hexon protein. With the knowledge that hexon-specific T-cell clones were serotype and species cross-reactive, predicted epitopes conserved amongst human Ads were identified. Nine 15mer peptides were synthesized (Table 1) and a panel of T-cell clones derived from donor 6 were screened for their ability to secrete IFN-γ in response to autologous IFN-γ-activated fibroblasts loaded with individual peptides. Three different T-cell clones recognized three novel epitopes derived from the Ad5 hexon protein. The three peptides recognized were among the highest four predicted binding affinities for HLA DRB1*0101, confirming the accuracy of the predictions (Table 1). The isolation of a VDC513-specific T-cell clone confirmed a recent report identifying VDC513 as a target for CD4⁺ T cells (Haveman et al., 2006).

Fig. 3 shows that T-cell clones recognizing peptides GTA117 and VDC513 were restricted through HLA DRB1*0101, as predicted; however, QWS8 was restricted through HLA DR16, for which the HLA-binding motif is poorly defined.

An important determinant in the ability of a T-cell clone to recognize presented antigen is the affinity with which it can bind peptide–MHC complex on target cells. Whilst a number of factors contribute to this interaction, it can be assessed in functional assays such as those measuring IFN-γ release. Fig. 4 shows the functional avidity of T-cell clones.

Table 1. T-cell epitopes predicted to bind HLA DRB1*0101 located within conserved regions of the hexon protein

<table>
<thead>
<tr>
<th>Position (aa)*</th>
<th>Epitope residues</th>
<th>Predictive score</th>
</tr>
</thead>
<tbody>
<tr>
<td>117†</td>
<td>GTYNALAPKGAPNP</td>
<td>36</td>
</tr>
<tr>
<td>513†</td>
<td>VDCYINLRWSLDY</td>
<td>36</td>
</tr>
<tr>
<td>554</td>
<td>YVPHFHVQPKFVFA</td>
<td>34</td>
</tr>
<tr>
<td>81</td>
<td>QWSYMHSQDASEY</td>
<td>32</td>
</tr>
<tr>
<td>341</td>
<td>TGNMGVLQAGASQIN</td>
<td>32</td>
</tr>
<tr>
<td>89</td>
<td>DRNVLDMASTYDFDIR</td>
<td>29</td>
</tr>
<tr>
<td>867</td>
<td>RTLWIPFSSNMNSM</td>
<td>29</td>
</tr>
<tr>
<td>99</td>
<td>YFDIRGVLDRGPTEK</td>
<td>27</td>
</tr>
<tr>
<td>824</td>
<td>NSGFVGYLAPTRE</td>
<td>27</td>
</tr>
</tbody>
</table>

*Based on the Ad5 sequence.
†Recognized by T-cell clones from donor 17 as tested by IFN-γ ELISA.
defined in peptide titration assays as the concentration of peptide mediating 50% maximal IFN-γ response. The avidity of these CD4+ T-cell clones was similar to EBV-specific CD4+ T cells restricted through the HLA DR1 allele (Khanna et al., 1995) and others restricted through a range of DRB1 alleles (Long et al., 2005).

In addition to secreting IFN-γ, CD4+ Ad-specific T cells have been shown previously to mediate lysis of antigen-loaded cells directly (Smith et al., 1996). The ability of T-cell clones specific for the three novel Ad epitopes to lyse peptide- and virus-infected cells was determined by chromium-release assays. Fig. 5 shows that all T-cell clones specific for novel peptides were able to lyse Ad-infected or peptide-loaded autologous IFN-γ-treated fibroblasts at low effector to target ratios in overnight killing assays.

In order to determine what proportion of individuals generated memory T cells recognizing these epitopes, ELISPOT assays were performed on fresh PBMCs of four HLA DR1+ donors and five DR1+2 donors. Fig. 6 shows that all four DR1+ donors had T cells specific for the peptides identified in this study, as well as the previously identified peptide DEP910 (Olive et al., 2002). No responses were detected in the DR1+2 donors to the peptides identified in this study (data not shown). Interestingly, responses were identified to peptide QWS8, which was a predicted HLA DRB1*0101-binding peptide, although the T-cell clone originally isolated recognized it through HLA DR16. Of the four DR1+ donors that had T cells specific for the peptides identified in this study, as well as the previously identified peptide DEP910 (Olive et al., 2002). No responses were detected in the DR1+ donors to the peptides identified in this study (data not shown). Interestingly, responses were identified to peptide QWS8, which was a predicted HLA DRB1*0101-binding peptide, although the T-cell clone originally isolated recognized it through HLA DR16. Of the four DR1+ donors with T cells recognizing peptide QWS8, the only common HLA DR or DQ allele was DR1 (DP status was unknown for three of the donors) and only donor 6 was DR16 positive. In donor 19, who had the strongest response to QWS8, blocking antibodies to HLA DR (but not DQ, DP or HLA ABC) were able to eliminate this response (data not shown). This suggests defined in peptide titration assays as the concentration of peptide mediating 50% maximal IFN-γ response. The avidity of these CD4+ T-cell clones was similar to EBV-specific CD4+ T cells restricted through the HLA DR1 allele (Khanna et al., 1995) and others restricted through a range of DRB1 alleles (Long et al., 2005).

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that the QWS8 peptide is able to bind multiple HLA alleles and may be presented in the context of both HLA DR1 and DR16 alleles.

**DISCUSSION**

The identification of antigens recognized by virus-specific T cells is important in understanding the interplay between virus infection and persistence within the host. Ads are known to be common causative agents of pharyngitis, pneumonia and gastroenteritis in the young (Horwitz, 2001). In this context, Ad is regarded as a lytic virus, where acute infection is resolved following a strong adaptive immune response. In murine models, Ad infection induces both a cellular and humoral immune response; the T-cell repertoire in this setting focuses on early proteins such as E1 and is predominantly mediated by CD8\(^+\) T cells leading to rapid clearance of virus (Mullbacher et al., 1989; Rawle et al., 1991). However, this bias towards recognition of early antigen may be misleading, as human Ads are unable to complete replication in murine cells, leading to decreased expression of late proteins. There have been no published studies of the T-cell response to active Ad infection in humans; thus, the only indication of which antigen(s) may be important targets for T cells come from studying memory responses in healthy individuals. Consistent with recent literature, we were able to detect memory CD4\(^+\) T cells recognizing Ad antigen in 100% of individuals; CD8\(^+\) T cells were detected at low frequency in five out of ten donors. Publications to date have not investigated the immunodominance of Ad proteins without the use of prolonged in vitro culture. In order to address this, we studied T-cell responses to two of the major components of the Ad capsid: the hexon and fiber proteins. Using fresh peripheral blood from healthy donors, it was possible to detect CD4\(^+\) cells responding to both a virus lysate and to purified hexon protein, but not to the fiber protein. This indicates that after viral infection there is induction of long-term immunity to virus through maintenance of CD4\(^+\) cells recognizing a structural protein. The uniform presence of detectable levels of CD4\(^+\) T cells recognizing Ad does not fit the profile of immunity induced by other lytic viruses (Klenerman & Hill, 2005) such as influenza.
(Brown et al., 2004; Lucas et al., 2004) and acute lymphocytic choriomeningitis virus infection (Homann et al., 2001) where virus-specific CD4+ T cells decrease to very low numbers in memory. It would therefore seem likely that levels of Ad-specific CD4+ cells recognizing viral structural proteins are maintained by repeat or persistent exposure to antigen. This could be explained either by virus reactivating from a site of persistence or by infection with different serotypes of Ad. The idea of sequential infection with Ad of different serotypes leading to selection of T cells that recognize epitopes conserved among serotypes has been proposed by Leen et al. (2004) and the identification of further conserved epitopes in this work lends weight to this argument. Since Ad was first isolated from adenoid and tonsil tissue in 1953 (Rowe et al., 1953), there have been reports of Ad persisting in an as yet undefined state. There are reports of Ad DNA being present in adenoid- and tonsil-derived tissue (Neumann et al., 1987), including T cells (Garnett et al., 2002), and epidemiological studies have shown that Ad can be detected in faecal specimens months to years after initial infection (Fox et al., 1969, 1977), suggesting that the virus may establish a persistent/ latent infection in its host. The role of adaptive immunity in containing these latent or persistent viruses remains to be defined, as does their influence on antigen specificity of Ad-specific T cells.

In order to characterize further the dominant hexon-specific CD4+ T cells found in the peripheral blood of healthy individuals, we analysed cells cloned by limiting dilution from four donors. The majority of clones generated by stimulating PBMCs with virus-infected cell lysate were hexon specific and all recognized antigen conserved among the serotypes tested (Ad2, -3, -4 and -5). Seventeen out of 19 clones generated from donor 6 were shown to be hexon specific and these included clones specific for the HLA class II-restricted epitope DEP910 (Olive et al., 2002).

The development of epitope-prediction algorithms has assisted the analysis of antigen recognition by T cells. Whilst most work has focused on the HLA class I alleles, there is now a growing body of evidence underpinning peptide-binding predictions for some of the common class II alleles. In this study, of nine epitopes predicted and synthesized, clones were identified recognizing three of the nine epitopes predicted and synthesized. In this study, the HLA class II molecule DPB1*0101. Interestingly, one clone recognizing peptide QWS8 was restricted through the HLA DRB1*16 allele, for which a peptide-binding consensus has yet to be defined. However, the QWS8 epitope was recognized by three HLA DR1*16+ donors, suggesting that it is able to bind both alleles. T cells were able to recognize peptide-loaded targets with an avidity similar to other reported CD4+ T-cell clones (Khanna et al., 1995; Long et al., 2005), demonstrating that they are potentially important targets in an immune response to Ad. As well as secreting IFN-γ, T cells recognizing the identified epitopes were able to kill target cells infected with virus or loaded with peptide. The role of cytotoxic CD4+ T cells in controlling Ad infection is unclear, as epithelial cells at the primary site of infection – the respiratory tract (for species C) – are unlikely to express HLA class II. Through studies of other human viruses such as CMV and EBV, it is becoming apparent that cytotoxic IFN-γ-secreting CD4+ T cells do have an important role in controlling viral infection (Gamadia et al., 2003; Long et al., 2005; Adhikary et al., 2006); whether cytotoxicity is critical for this role is still undetermined. In this study, Ad-specific CD8+ T cells were only detected at low frequency in concordance with previous reports (Leen et al., 2004; Veltrop-Duits et al., 2006); however, it is likely that, in an acute infection, Ad-specific CD8+ T cells expand and play an important role in virus clearance. In addition to cytotoxicity, the CD4+ T cells described in this study may play a role in licensing of dendritic cells to prime Ad-specific CD8+ CTLs (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998) and/or maintenance of CD8+ T cells by secretion of IFN-γ (Janssen et al., 2003). The secretion of IFN-γ by Ad-specific CD4+ T cells may also help to sensitize infected cells to lysis by CD8+ T cells by inducing upregulation of HLA class I expression. Indeed, exposure of Ad-infected fibroblasts to IFN-γ has been shown to overcome the inhibitory effects of E3 gp19k on the class I presentation pathway and allow CD8+ T-cell-mediated lysis (Flomenberg et al., 1996).

The hexon protein is the most abundant protein in the capsid, and its tertiary and quaternary structure are critical for stable particle formation. Variability occurs in the loop domains of the hexon exposed on the surface of the capsid. The loops have more flexibility than other domains of the protein and so can tolerate greater variation in sequence and structure. Hexon subunits must fold correctly to form stable homotrimers and successfully interact with the other proteins of the capsid to allow viral particles to form. Structural and phylogenetic analysis of the hexon protein from all 51 human Ad serotypes have revealed three variable regions that can withstand mutation without adverse effects on the structure of the molecule (Rux et al., 2003; Ebner et al., 2005). Importantly, the three T-cell epitopes identified in this study, as well as the previously identified epitopes, lie within the conserved hexon core and none are in the variable regions. This has direct implications for the use of Ad as a gene-delivery vector; it is unlikely that any of these T-cell epitopes could be deliberately mutated in order to make a less immunogenic vector without seriously compromising the structure of the virus. Memory T-cell immunity induced by exposure to the more common species C viruses will effectively induce broad-spectrum T-cell immunity including immunity to Ad vectors based on less-prevalent human serotypes and possibly Ad from other host species, which are currently under development as gene delivery vectors (Vogels et al., 2003; Stone et al., 2005; Thirion et al., 2006).

The identification of immunodominant proteins and mapping of T-cell epitopes is critical for the development of adoptive T-cell therapy for Ad disease in immunocompromised individuals. They not only provide reagents
to monitor immune recovery but could also be used for selection and HLA DR1 of one of the few class II alleles where synthesis of HLA class II tetramers is possible, allowing isolation of high-purity CD4+ T cells for adoptive immunotherapy without antigenic stimulation (Scriba et al., 2005).

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REFERENCES


pathogen in immunocompromised patients.

served human CD4
toid cell lines.

The adenovirus capsid protein hexon contains a highly con-
(2002).


Takenaka, K., Tanimoto, K., Horiuchi, T., Asano, Y. & other authors

Early viral complications following CD34-selected autologous
peripheral blood stem cell transplantation for non-Hodgkin's

pathogen in immunocompromised patients. Br J Haematol 128,
155–171.

Leen, A., Meij, P., Redchenko, I., Middeldorp, J., Bloemaena, E.,
of Epstein–Barr virus latent-cycle proteins for human CD4+

Leen, A. M., Sili, U., Vanin, E. F., Jewell, A. M., Xie, W., Vignali, D.,

Conserved CTL epitopes on the adenovirus hexon protein expand subgroup cross-
reactive and subgroup-specific CD8+ T cells. Blood 104, 2432–2440.

Leen, A. M., Myers, G. D., Sili, U., Huls, M. H., Weiss, H., Leung, K. S.,

Monoculture-derived T lymphocytes specific for multiple viruses
expand and produce clinically relevant effects in immunocompro-

pathogen in immunocompromised patients. Br J Haematol 128,
135–144.

pathogen in immunocompromised patients. Br J Haematol 128,
135–144.

pathogen in immunocompromised patients. Br J Haematol 128,
135–144.

pathogen in immunocompromised patients. Br J Haematol 128,
135–144.

pathogen in immunocompromised patients. Br J Haematol 128,
135–144.

pathogen in immunocompromised patients. Br J Haematol 128,
135–144.

pathogen in immunocompromised patients. Br J Haematol 128,
135–144.