Specificity of the Immune Response of Mice to Herpes Simplex Virus Glycoproteins B and D Constitutively Expressed on L Cell Lines

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(Accepted 11 December 1986)

SUMMARY

Mouse L cell lines have been developed which constitutively express glycoproteins B (gB) and D (gD) of herpes simplex virus type 1. When used to study the immune response of mice to the viral glycoproteins, it was found that both gB and gD induce a delayed type hypersensitivity response and both also induce an antibody response, but only the cell line expressing gD could stimulate the production of neutralizing antibody. Virus-specific cytotoxic T lymphocytes (CTLs) recognized gB expressed by the cell line and this line could also induce CTLs in mice. Recognition of gD by major histocompatibility complex class I restricted CTLs was never seen. Vaccination of mice with the cell lines provided protection from viral challenge and inhibited the establishment of a latent infection, although gD proved to be the better protective immunogen.

INTRODUCTION

The immune response of mice to herpes simplex virus type 1 (HSV-1) is known to involve both antibody and cell-mediated protective mechanisms. In particular, T lymphocytes are considered to play a central role in the recovery from a primary infection (for review, see Nash et al., 1985). However, the particular proteins of the virus recognized by these cells are far from clear. Experiments to date have centred on the use of the virus mutants deficient in the expression of certain glycoproteins to study the nature of the antigens recognized by cytotoxic T lymphocytes (CTLs) (Carter et al., 1981; Lawman et al., 1980; Glorioso et al., 1985), and the affinity purification of glycoproteins from whole virus preparations for use as immunogens to study delayed type hypersensitivity (DTH) responses (Schrier et al., 1983; Chan et al., 1985).

The main problem has been how to separate the various antigens of this complex virus in order to dissect the nature of the immune response. Our approach has been to investigate the immune response to individual glycoproteins of HSV-1 by expressing the appropriate genes in mouse L cells. In this system, the glycoproteins are expressed in isolation but are still presented at the cell surface analogous to infected cells. Our reason for choosing glycoproteins is that they are likely to represent major target antigens of the immune system: this is true for the antibody response (for review, see Norrild, 1985) and there is evidence to suggest that glycoproteins are the major targets for both major histocompatibility complex (MHC) class I and class II restricted T lymphocytes (Carter et al., 1981; Lawman et al., 1980; Glorioso et al., 1985; Schrier et al., 1983).

In this paper we report the establishment of L cells expressing either glycoprotein B (gB) or D (gD) and the use of these lines to study the specificity of the T cell response to HSV-1. The lines were also used to immunize mice against primary virus infections and to prevent the establishment of latent infections.

METHODS

Mice. Female CBA mice, 8 to 12 weeks of age, were obtained from the Department of Pathology Animal House, University of Cambridge, U.K.
Fig. 1. Expression vectors. (a) The plasmid pMI-1 is based on pAT153 and contains the SV40 
HaeIII-C and HindIII-C fragments and the HSV-1 (strain CI(101)) BamHI-Q fragment in the pAT153 EcoRI, 
HindIII and BamHI sites respectively (sites in brackets were lost during cloning). In an anticlockwise 
direction, the plasmid therefore contains the HSV-1 TK promoter, coding sequence and transcription 
termination signal, the SV40 enhancer, early promoter and early transcription initiation site, the 
pAT153 ClaI site and the SV40 early polyadenylation signal. (b) The vector pBB-1 was developed from 
pMI-1 by inverting the KpnI fragment containing the HSV-1 TK gene relative to the SV40 sequences 
and then deleting the non-essential 1.3 kb BamHI fragment which was created (inset). This leaves the 
second HindIII site between the SV40 early promoter and pAT153 ClaI site as an additional cloning 
site. Transcription from the TK promoter is now in the opposite direction to transcription initiated 
from the SV40 early promoter.

Viruses. HSV-1 strains HFEM, KOS, SC16 and CI(101)TK- were all passaged at a low multiplicity of infection 
in BHK-21 cells and stocks of virus were stored at −70 °C. All viruses were titrated on BHK-21 cells.

Cells. LMTK- and BHK-21 cells were grown in Glasgow modified Eagle’s medium (GMEM). Selection of L 
cells containing thymidine kinase (TK) was carried out by supplementing the GMEM with thymidine, adenosine, 
guanosine, glycine and methotrexate and all transfected cell lines were maintained in this medium (Minson et al., 
1978).

Injection of mice. The various cell lines were harvested by mechanical displacement from growing surfaces with 
glass beads. The cells were washed three times in phosphate-buffered saline (PBS) and then resuspended to the 
required concentration in PBS. Mice were injected intraperitoneally with 0.1 ml of cells at 2 × 10⁷ to 5 × 10⁷ 
cells/ml or were injected subcutaneously in the hind footpad with 20 μl of cells at 2.5 × 10⁸ cells/ml.

The immunized mice were challenged with SC16 injected into the ear pinna (Hill et al., 1975) or by scarifying 
the virus onto the neck of depilated animals (Simmons & Nash, 1984).

Molecular cloning of gB-1 and gD-1. Fig. 1 shows the expression vectors used to clone the glycoprotein genes. 
Based on pAT153, each contains the simian virus 40 (SV40) enhancer, early promoter, early transcription 
initiation site (SV40 HindIII-C fragment) and early polyadenylation signal (SV40 HaeIII-C fragment) (sequence: 
Fiers et al., 1978). Between the transcription initiation site and polyadenylation signal, the ClaI site of pAT153 
provides a suitable cloning site for the insertion of the structural genes; the unique HindIII site of pBB-1 is also 
between the two SV40 sequences.

In addition both vectors contain the HSV-1 TK gene of strain CI(101) including the promoter, coding sequence 
and transcription termination signal (Wagner et al., 1981) for use as a selectable marker in LMTK- cells. In pMI-1 
(Fig. 1a), TK transcription is in the same direction as that from the SV40 promoter whilst in pBB-1 (Fig. 1b), 
transcription from the two promoters is in opposite directions.

An EcoRI-F clone of HSV-1 (strain KOS) in pBR325 (a gift from M. Levine, Ann Arbor, Mich., U.S.A.) was 
double digested with XhoI and SphI (Boehringer Mannheim) and the 3-0 kb XhoI-SphI fragment containing the 
gB-1 structural gene isolated. The fragment was subcloned into the ClaI site of pMI-1 (fragment and vector blunt-
ended) and a plasmid, pMI-B3, containing the gB gene in the correct orientation to the SV40 promoter and 
polyadenylation signal was isolated.

A BamHI-J clone of HSV-1 (strain Patton) in pBR322 (a gift from R. Watson, Minneapolis, Mn., U.S.A.) was 
double digested with HindIII and NruI (Boehringer Mannheim) and the 1-4 kb fragment containing the gD 
structural gene was isolated. This fragment was subcloned into the HindIII site of pBB-1 and a plasmid, pBB-D1, 
containing the gD gene in the correct orientation to the SV40 sequences was isolated.
Transfection of LMTK^- cells. Calcium phosphate precipitation of DNA onto LMTK^- cells was performed as described by Minson et al. (1978) with a minor modification. In these experiments the DNA dose per dish was made up to 10 µg using LMTK^- DNA as carrier and contained 0.1 µg of linearized plasmid pMI-B3 or pBB-D1 linearized with PvuI or XmnI (Boehringer Mannheim) respectively. Transfected cells were selected in supplemented GMEM. Only one colony per dish was harvested (so each cell line was uniquely derived) and then cloned by limiting dilution to establish a cell line. The cell line, LTK^+, was established by transfection of the parental LMTK^- cells with the expression vector pMI-1.

Polyacrylamide gel electrophoresis of immunoprecipitated antigens. Radiolabelled infected cell lysates were prepared by infecting confluent BHK or LMTK^- cell monolayers (grown in 3.5 cm dishes) at a multiplicity of infection of 10 or 20 p.f.u./cell respectively. Starting at 4 h post-infection, infected or uninfected cells were incubated with methionine-free minimum essential medium supplemented with 1% GMEM containing 50 µCi of [35S]methionine (1315 Ci/mmol; Amersham) for 20 h at 37 °C, harvested and then lysed in 1 ml RIPA buffer (50 mM-Tris pH 7.2, 150 mM-NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100) plus 2 mM-phenylmethylsulphonyl fluoride and 10 units of micrococcal DNase (Sigma) per ml. Lysates were clarified by centrifugation at 100000 g for 1 h at 4 °C. Immunoprecipitations were performed as described by Richman et al. (1986) on 250 µl samples of lysate with 5 µl of ascites fluid containing either anti-gB (gB2) or anti-gD (LP2) monoclonal antibodies (gifts from M. Levine, Ann Arbor, Mich., U.S.A. and A. Minson, Cambridge, U.K., respectively). All samples were boiled for 3 min before use. Samples of 20 µl were electrophoresed for 4 h at 35 mA on 10% acrylamide-0.1% N,N'-methylenebisacrylamide resolving gels with 3% acrylamide-0.13% N,N'-methylenebisacrylamide stacking gels using the SDS buffer system of Laemmli (1970). The gels were then fixed, fluorographed (Bonnet & Laskey, 1974) and dried before exposure to Kodak X-Omat S film at -70 °C.

Determination of antibody titres in serum

Two methods to determine antibody titre were used.

Radioimmunoassay. This assay was based on the method reported by Colombatti & Hilgers (1979). Briefly, antigen plates contained HSV-1 strain HFEM-infected or uninfected BHK-21 cells whose non-specific binding sites were blocked with heat-inactivated foetal calf serum. Serum and 125I-labelled Protein A (30 mCi/mg; Amersham) dilutions were made in PBS plus 10% heat-inactivated foetal calf serum. Endpoint titres of antibody were taken as the dilution at which the counts from infected wells were double that of uninfected wells.

Plaque reduction assay. Neutralizing antibody titres were determined by incubating 5 × 10^5 p.f.u. HFEM with serum and exogenously added rabbit serum as a source of complement before titrating the remaining viable virus on BHK-21 cells. Antibody endpoint titres were taken as the dilution that gave 50% reduction in virus titre.

Detection of virus-specific CTLs. CTLs were prepared as described by Pfizenmaier et al. (1977) with modifications. Briefly, the cervical and auricular draining lymph nodes or spleens from experimental mice were removed and made into single cell suspensions as previously described by Nash et al. (1980a). Lymph node or spleen cells were cultured for 5 days in RPMI 1640 medium with SC16-infected, X-irradiated spleen cells (2:1 cells:feeder ratio) and then harvested for use in a cytotoxicity assay.

Assay. 2 × 10^6 target cells were labelled with 100 µCi 51Chromium (sodium chromate in aqueous solution, 250 to 500 µCi/mg Cr; Amersham) as in Nash et al. (1980b). Target cells were then incubated with effectors for 8 to 10 h at 37 °C in 5% CO₂ and the percentage specific 51Chromium release was determined. All values were obtained from triplicate samples.

DTH response. Mice were injected subcutaneously in the left hind footpad with 20 µl PBS containing 5 × 10^6 cells or 5 × 10^4 p.f.u. Cl(101)TK^- . Seven days later 5 × 10^3 p.f.u. Cl(101)TK^- was injected into the left ear and the ear thickness was measured at 24, 48, 72, 96 and 120 h post-infection as previously described (Nash et al., 1980a). All results are expressed as the difference in thickness between infected and uninfected ears.

Titration of virus in mouse tissues. Ear pinnae were removed 5 days post-infection, homogenized in 1 ml 10% tryptose phosphate broth (ETC) and stored at −70 °C before dilution for independent assay of virus on BHK-21 cells (Nash et al., 1980a).

To determine latent virus in ganglia, mice received 10^5 p.f.u. SC16 scarified onto the neck. The mice were sacrificed during the latent phase of infection (i.e. >30 days post-infection) and the left cervical ganglia (CII, III and IV pooled) were removed. Ganglia were placed into 0.5 ml GMEM with 10% tryptose phosphate broth and stored at −70 °C before dilution for independent assay on BHK-21 cells. For the purpose of these experiments, if any infectious virus was recovered from the ganglia this was scored as positive for a latent infection.

RESULTS

Cloning the structural genes for HSV-1 gB and gD

The cloning vectors used to express the HSV-1 glycoprotein genes are shown in Fig. 1. Each vector contains the SV40 early promoter from which cloned genes are expressed and the HSV-1 TK gene as a selectable marker.
(a) gB-1 (strain KOS)

0.35 m.u.  \( \text{SphI} \)  Polyadenylation signal 3.1 kb mRNA mRNA initiation site ATG

\( XhoI \)-0.37 m.u.

(b) gD-1 (strain Patton)

0.90 m.u.  \( \text{HindIII} \)  NruI 0.93 m.u. mRNA initiation site 3.0 kb mRNA

\( \text{ATG} \)

Fig. 2. Structural genes for gB-1 and gD-1. (a) HSV-1 strain KOS (Bzik et al., 1984) was sequenced between 0.345 and 0.370 map units (m.u.) in the UL region of the HSV-1 genome. The sequence spanned the gB gene which is transcribed from right to left in the prototypic form of the viral genome. The \( XhoI \)-\( \text{SphI} \) sites used to excise the structural gene from an \( \text{EcoRI-F} \) clone are indicated and the fragment generated contains the coding region and polyadenylation signal. (b) HSV-1 strain Patton (Watson et al., 1982) was sequenced between 0.90 and 0.93 m.u. of the US region, spanning the 5' non-coding and coding regions of the gD gene. The \( \text{HindIII} \) and \( \text{NruI} \) sites used to excise only the coding region of the gene are shown. In the prototypic arrangement of the HSV-1 genome, the gD gene is transcribed from the left to right.

Using the published sequences for gB-1 (strain KOS: Bzik et al., 1984), suitable restriction sites for subcloning the structural gene of gB-1 (strain KOS) from an \( \text{EcoRI-F} \) clone were chosen. The \( XhoI \) site 5' of the coding region was used. This is 252 bases from the initiation codon and is 3' of the putative TATA box and mRNA initiation site of gB. The chosen 3' terminal of the cloned fragment was the \( \text{SphI} \) site which is 48 bases 3' of the predicted polyadenylation signal. Therefore the cloned \( XhoI \)-\( \text{SphI} \) fragment contains the complete gB coding sequence, some 5' leader sequence (but not the promoter) and some 3' flanking sequence (including the putative gB polyadenylation signal) (Fig. 2a). This 3 kb fragment was cloned into the \( \text{ClaI} \) site of pMI-1 and a plasmid containing the gB gene in the correct orientation to the SV40 promoter was identified by restriction enzyme analysis.

The published sequence of gD-1 (strain Patton: Watson et al., 1982) was used to choose suitable restriction sites to excise the structural gene of gD-1 (strain Patton) from a \( \text{BamHI-J} \) clone. The 5' and 3' restriction sites chosen were the \( \text{HindIII} \) and \( \text{NruI} \) sites respectively (Fig. 2b). The \( \text{HindIII} \) site is 75 bases 5' of the initiation codon and the \( \text{NruI} \) site is 188 bases 3' of the termination codon. Therefore the \( \text{HindIII-NruI} \) fragment chosen contains only the gD-1 coding sequence and some 5' and 3' flanking sequence (Fig. 2b). This 1-4 kb fragment was cloned into the \( \text{HindIII} \) site of pBB-1 and all recombinant plasmids isolated contained the gD gene in the correct orientation to the SV40 promoter. The \( \text{HindIII-NruI} \) fragment has also been cloned into the \( \text{ClaI} \) site of pMI-1 to give a plasmid, pMI-D2.

**Establishment of L cell lines expressing HSV-1 glycoproteins**

L cell lines recovered after transfection with the vectors all contained plasmid DNA integrated into the genome of the cells. Two cell lines have been used in this study: LTKgB (clone 22) and LTKgD (clone 2). LTKgD contained the complete gD gene at about three copies/genome whilst LTKgB contained the complete gB gene at about five copies/genome (determined by Southern blot analysis, data not shown). Plasmid DNA was maintained stably in the cell genome throughout passage (LTKgB passed 20 times and LTKgD passed 23 times which is the equivalent of approximately 5 to 6 months in culture).

To demonstrate the expression of the glycoprotein genes in the cell lines, radiolabelled cell
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Fig. 3. Immunoprecipitates from LTKgB and LTKgD. LTKgB (clone 22) and LTKgD (clone 2) are cell lines established from LMTK− cells transfected with pMI-B3 (gB gene) and pBB-D1 (gD gene) respectively. [35S]Methionine-labelled cell lysates were immunoprecipitated with (a) gB2 and (b) LP2 monoclonal antibodies and precipitates were electrophoresed on 10% SDS-polyacrylamide gels cross-linked with N,N'-methylenebisacrylamide. Fluorograms of gels were exposed to Kodak X-Omat S film for (a) 4 and (b) 3 days. MI, Mock-infected LMTK− cells; HFEM, HFEM-infected LMTK− cells; 2, LTKgD; 22, LTKgB. Positions of molecular weight marker proteins are shown.

Lysates were immunoprecipitated with type-common monoclonal antibodies to gB and gD (gB2 and LP2 respectively). The two cell lines used, LTKgB (Fig. 3a, lane 22) and LTKgD (Fig. 3b, lane 2), expressed gB and gD respectively as clearly precipitable bands which co-migrated with infected cell gB (Fig. 3a, lane HFEM) and gD (Fig. 3b, lane HFEM). Uninfected cell peptides which cause background bands upon immunoprecipitation are shown in mock-infected (MI) lanes of Fig. 3. In Fig. 3(a), lanes 22 and HFEM show bands that do not correspond to background bands. These are approx. 49K, 33K and 27K in lane 22 and approx. 110K, 87K and
Table 1. Serum antibody titres from mice immunized with gB or gD

<table>
<thead>
<tr>
<th>Group</th>
<th>Radioimmunoassay</th>
<th>Neutralization</th>
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<tbody>
<tr>
<td>LMTK⁻</td>
<td>10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>LTKgB</td>
<td>10⁴ to 10⁵</td>
<td>&lt;10</td>
</tr>
<tr>
<td>LTKgD</td>
<td>10³ to 10⁵</td>
<td>10 to 10³</td>
</tr>
<tr>
<td>LMTK⁻ + HFEM</td>
<td>10² to 10⁶</td>
<td>10² to 10³</td>
</tr>
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</table>

*Endpoints were expressed as the reciprocal of the highest dilution of antiserum which gave an infected:uninfected BHK binding ratio of 2 for radioimmunoassay, or 50% reduction in plaque number for neutralization assay.

From a series of cell lines studied, pMI-1 was found to be the more efficient expression vector; for example 100% of the cell lines screened expressed gD in pMI-1 compared to 33% in pBB-1. Previous evidence suggests that this effect may be due to the orientation of the TK gene compared to the glycoprotein gene (Darby et al., 1981; Minson et al., 1978).

Antibody response induced by cell lines

The immune response of mice to individual HSV-1 glycoproteins, gB and gD, was studied using the cell lines established in the previous section. Firstly, the antibody response of mice to the cells was investigated. Mice received two injections of 5 × 10⁶ live cells (LMTK⁻, LTKgB, LTKgD, or LMTK⁻ infected with HFEM, m.o.i. 2) intraperitoneally, 14 days apart. Seven days after the second injection, serum samples were taken and the antibody levels determined by radioimmunoassay or by a plaque reduction assay (Table 1). Sera were analysed individually and the results for each immunization group were expressed as the range obtained from individual mice in the group. Each group contained five mice. As can be seen in Table 1, immunization of mice with live cells expressing gB or gD induced comparable antibody titres, when measured by radioimmunoassay. Non-specific binding of the sera to uninfected and infected BHK-21 cells caused a high background at high serum concentrations and this caused the apparent ‘HSV-1-specific antibody’ titre seen in mice immunized with only the parental LMTK⁻ cells. Subsequent immunoprecipitation of radiolabelled HSV-1-infected BHK cell lysate with the sera has shown this binding to be non-specific (data not shown). Immunoprecipitation of infected BHK lysates has also shown that the anti-HSV-1 antibody in sera from mice immunized with LTKgD or LTKgB was specific for gD and gB respectively (data not shown).

No detectable neutralizing antibody was found in sera from mice immunized with LTKgB. In contrast, mice immunized with LTKgD produced clearly detectable neutralizing antibody (Table 1). This neutralizing antibody was complement-independent.

Specificity of HSV-1-induced cytotoxic T lymphocytes

CTLs induced in vivo by infection of mice with strain CI(101)TK⁻ were re-stimulated with virus antigen in vitro and tested against infected or transfected cells in a ⁵¹Chromium release assay. The effector cell population induced by this method has previously been shown to be Thy1⁺ L3T4⁻ Lyt2⁺ (Nash et al., 1980b; A. A. Nash, unpublished observation). As shown in Fig. 4, lysis of SC16-infected cells was 39% at an effector : target ratio of 50 : 1. Although gB is
The T cell response to HSV glycoproteins

Fig. 4. Specificity of HSV-1-induced cytotoxic T cells. Mice were injected twice with \(5 \times 10^4\) p.f.u. Cl(101)TK- in the ear at a 7 day interval. Four days after the last injection, draining lymph nodes were removed and lymph node cells were cultured with virus antigen for 5 days before use in the cytotoxicity assay. Target cells were (O) LMTK-, (■) LTKgB, (●) LTKgD and (▲) SC16-infected LMTK- cells. The assay mixture was incubated for 10 h at 37°C and the \(^{51}\)Cr release determined. Minimum \(^{51}\)Cr release into the supernatant was 15% or less of the maximum values obtained. Error bars show one standard deviation of experimental values.

Fig. 5. Specificity of cytotoxic T cells induced in vivo with LTKgB. Mice were injected in the ear with \(5 \times 10^4\) p.f.u. Cl(101)TK- 7 days before injection intraperitoneally with \(5 \times 10^6\) LTKgB cells (two doses at a 7 day interval). Five days after the last immunization spleens were removed and spleen cells cultured for 5 days with HSV-1 antigen. Cells were harvested, separated on lymphocyte separation medium (Flow Laboratories), and the lymphocyte fraction was used as effectors in the cytotoxicity assay. Target cells were (O) LTK+, (■) LTKgB, (▲) SC16-infected LTK+ and (●) KOS-infected LTK+ cells. The assay mixture was incubated for 9 h at 37°C and the \(^{51}\)Cr release determined. Minimum \(^{51}\)Cr release values obtained were 20% or less than the maximum values. Error bars show one standard deviation of experimental values.

recognized by the HSV-1-specific CTL (14% specific \(^{51}\)Chromium release at 50:1; LTKgB, Fig. 4), the low level of killing indicates that gB-specific CTLs constitute only a minor population. There was, however, no recognition of the cell line expressing gD (LTKgD, Fig. 4).

Specificity of CTLs stimulated in vivo with gB

As only a minor population of HSV-1-specific CTLs recognized LTKgB, it was decided to amplify this population by stimulating with antigen in vivo. Mice were injected with live virus [strain Cl(101)TK-] and then LTKgB (twice) at 7 day intervals before removal of the spleens and re-stimulation of the lymphocytes in vivo with SC16-infected spleen cells. Fig. 5 shows that CTLs generated by this method now recognized LTKgB as their major target (25:1, 71% specific \(^{51}\)Chromium release) and SC16- and KOS-infected cells were also recognized (45% and 43% specific \(^{51}\)Chromium release at 25:1 respectively). The effector T lymphocytes in these experiments were characterized as Thyl+ L3T4- Lyt2+ (data not shown). (KOS-infected cells were included as targets as the gB gene cloned into pM1-1 was originally from strain KOS and therefore any strain-specific recognition of gB would be shown as a difference in recognition between the SC16- and KOS-infected targets.)
Fig. 6. DTH response to gB and gD. Mice were injected subcutaneously in the hind footpad with 5 × 10⁶ cells (O, LTK⁺; ■, LTKgB; □, LTKgD) or 5 × 10⁴ p.f.u. CI(101)TK⁻ (▲). Seven days after the injection, the mice were challenged with 5 × 10⁵ p.f.u. CI(101)TK⁻ in the ear. Ear thickness was measured at 24, 48, 72, 96 and 120 h post-challenge and the difference between infected and uninfected ears was calculated. Values were expressed as the mean differences in ear thickness ± 1 standard deviation of five mice/group.

Induction of CTLs specific for gD

In view of the results of the above experiment we investigated whether LTKgD could stimulate a CTL response. However stimulation of CTLs in vivo with this line by the method used to induce gB-specific CTL (Fig. 5) produced lymphocytes which gave a 7%, 5% and 3% specific ⁵¹Chromium release from LTK⁺, SC16 infected LTK⁺ and LTKgD cells respectively at a 50:1 effector:target ratio. In this and other experiments CTLs specific for gD were never observed. A cell line expressing HSV-1 TK was included as a target in many experiments (see above) and the presence of the transfected TK gene had no apparent effect on the recognition of infected cell proteins by HSV-1-specific CTLs.

DTH response of mice primed with gB or gD

To study the DTH response to HSV-1 glycoproteins, mice were injected subcutaneously with live cells (LTK⁺, LTKgB or LTKgD) or virus and challenged 7 days later in the ear pinna with live virus. Fig. 6 clearly shows that priming with whole virus, LTKgD or LTKgB all produce a maximal response at 48 h post-infection. The swelling induced by immunization with gB or gD was as great as that induced by whole virus antigen. Background swelling caused by viral replication is not seen (Fig. 6, LTK⁺) as the virus strain used to challenge the mice was TK⁻ negative and replicates poorly in vivo.

Protection of mice against viral challenge by immunization with gB or gD

Two methods were used to look at the protection afforded to mice by immunization with the glycoproteins: virus clearance from the site of infection and protection from establishment of a latent infection.

Virus clearance from the site of infection in mice immunized with gB or gD

Mice were injected once with 2 × 10⁶ live cells (LTK⁺, LTKgB or LTKgD) or 8 × 10⁴ p.f.u. SC16 subcutaneously in the hind footpad 7 days before challenge in the ear with 5 × 10⁴ p.f.u. SC16. Virus remaining in the ear at 5 days post-infection was determined and is expressed in Table 2 as the virus titre from individual mice. Previous immunization of animals with live virus
**The T cell response to HSV glycoproteins**

Table 2. *Virus clearance from the ear pinna of mice immunized with gB or gD*

<table>
<thead>
<tr>
<th>Group</th>
<th>Virus titre* (log$_{10}$ p.f.u./ear)</th>
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<tbody>
<tr>
<td>LTK$^+$</td>
<td>5.10, 5.38, 5.39, 5.58, 5.70</td>
</tr>
<tr>
<td>LTKgB</td>
<td>3.26, 3.37, 4.10, 4.30, 4.75</td>
</tr>
<tr>
<td>LTKgD</td>
<td>&lt;1.0, 1.00, 1.30, 1.30, 2.38</td>
</tr>
<tr>
<td>SC16</td>
<td>&lt;1.0, &lt;1.0, &lt;1.0, &lt;1.0, 1.00</td>
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* Virus titres of less than 1 log$_{10}$ were not determined.

Table 3. *Effect of immunization with gB or gD on establishment of latent infections*

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of immunizations</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>LTK$^+$</td>
<td>100*</td>
</tr>
<tr>
<td>LTKgB</td>
<td>100</td>
</tr>
<tr>
<td>LTKgD</td>
<td>100</td>
</tr>
<tr>
<td>CI(101)TK$^-$</td>
<td>78</td>
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* Values given are the percentage of mice latently infected.

In direct contrast to protection as measured by virus clearance, one immunization of antigen (LTKgB or LTKgD) did not protect the mice from establishment of a latent infection (Table 3). However, two doses (at a 14 day interval) of gB or gD (Table 3) did provide protection against establishment of latency. Again gD induced the greatest protection with only one of 10 mice showing a latent infection compared with gB in which four of 10 mice showed latency (although it must be noted that only 80% of the unimmunized mice showed a latent infection).

**DISCUSSION**

In view of the complexity of HSV-1 it is necessary to separate its antigenic determinants in order to unravel the nature of the immune response to this virus. In this report, we describe the use of L cell lines constitutively expressing gD or gB of HSV-1 as probes to study the type of immune response induced to these glycoproteins and their effect as immunogens in the prevention of infection and the establishment of latent infections.

An intriguing observation with LTKgD (clone 2; cell line expressing gD) was that CTLs obtained from virus-infected animals would not recognize this cell line, neither could this cell line induce a CTL response when used to immunize mice. Nevertheless, mice injected with
LTKgD were able to eliminate virus rapidly from the skin and when mice were immunized twice with LTKgD they were protected from the establishment of a latent infection following challenge with a high dose of wild-type virus. Vaccination with gD (whether synthetic peptides, purified protein or vaccinia virus recombinants expressing gD) has also previously shown that this protein is a potent protective immunogen (Eisenberg et al., 1985; Berman et al., 1985; Long et al., 1984; Cremer et al., 1985; Paoletti et al., 1984).

Although human (T4+) CTL clones have been produced which will proliferate in response to cloned, truncated forms of gD-1 (Zarling et al., 1986a) and are cytotoxic for cells infected with vaccinia virus recombinants expressing gD (Zarling et al., 1986b), these T4+ cells are MHC class II restricted (Zarling et al., 1986a, b). Classical (MHC class I restricted) T cell killing specific for gD-1 has not been observed in man or the mouse. This could reflect a failure of gD to associate with MHC class I molecules or could represent a 'hole' in the T cell repertoire for this antigen–MHC combination. Whatever the explanation for the failure to induce CTLs to the glycoprotein, it is obvious that the mice are not compromised by this. So in the prevention of infection, CTLs may be considered a redundant force. Support for this suggestion is found in mice deficient in Lyt2+ cells where these animals are well able to control a primary HSV-1 infection (Nash et al., 1987).

The protective mechanism stimulated by gD-1 is uncertain; LTKgD induces a good neutralizing antibody response and a DTH response. There is strong evidence favouring either or both in the control of a primary cutaneous infection (Simmons & Nash, 1985; Nash et al., 1980a). However, mice deficient in B cell function and hence the ability to make antiviral antibodies, were able to resist re-infection with HSV-1 which was scarified into the skin (Simmons & Nash, 1987). This would suggest that the antiviral activity here could be mediated predominantly via MHC class II restricted 'helper' T cells.

In contrast to LTKgD, lymph node cells from virus-infected mice were able to recognize and lyse LTKgB (clone 22; cell expressing gB) albeit weakly. That gB is a target for Lyt2+ CTLs was shown clearly when this clone was used to immunize mice. Both the clone and infected syngeneic cells were recognized. Why the infected cells are lysed to a lesser extent than LTKgB is unclear. One possible explanation is that on the membrane of infected cells, gB interacts with other glycoproteins and is unavailable for interacting with MHC class I gene products. Alternatively, in the transfected cell, antigen 'processing' may occur in a different way than in the infected cell. These arguments may also be applied to the recognition of LTKgD by virus-specific CTLs but this would not explain why LTKgD will not induce CTLs which recognize itself.

Using HSV-1 mutants temperature-sensitive for gB, it has previously been inferred that gB is recognized by CTLs (Lawman et al., 1980). However, Glorioso et al. (1985) have shown that gC is the major target antigen recognized by MHC class I restricted T cells. This is particularly intriguing in view of the evidence in other viruses where there is a dominant recognition of non-membrane proteins, such as nucleoprotein of influenza (Townsend et al., 1984), immediate early gene product of murine cytomegalovirus (Reddehase & Koszinowski, 1984), and the nuclear protein of vesicular stomatitis virus (Yewdell et al., 1986). It remains to be determined conclusively which is the dominant antigen recognized by HSV-1-specific CTLs.

LTKgB also induces a protective response which is less efficient than that to LTKgD. This difference could be related to the amount of antigen expressed by the two cell lines, although preliminary evidence suggests that there is little difference between the two. Unlike LTKgD, LTKgB does not induce a neutralizing antibody response. As these mice are able to clear the virus from the inoculation site, this supports the arguments above, that neutralizing antibodies are not an absolute requirement for protection. Nevertheless, there is evidence in murine HSV infection for a role of non-neutralizing antibodies in protection against ocular (Rector et al., 1982), subcutaneous (Balachandran et al., 1982) and intracerebral infection (Kumel et al., 1985).

By using cell lines expressing individual glycoproteins, a start has been made on dissecting the nature of the immune response to this complex virus. There are at least seven glycoproteins of HSV-1 so far determined (for review, see Spear, 1985; Richman et al., 1986; Frame et al., 1986; Buckmaster et al., 1984), and gD and gB have already shown that the type of immune response to different glycoproteins may be as varied as the type of immune response to different viruses. We
The T cell response to HSV glycoproteins

are now undertaking a systematic dissection of the immune response to other HSV-1 glycoproteins for comparative purposes. Protective responses induced by these glycoproteins will also be studied as this information will be important for the development of subunit vaccines to HSV-1.

However, it should be borne in mind that these are just seven genes out of more than 50 encoded by the virus genome, many of whose proteins may prove to be important target structures for the immune system.

The expression vector, pMI-1, was developed and kindly given to the authors by Dr M. Inglis, Department of Pathology, University of Cambridge, U.K. The authors would like to thank the Medical Research Council of Great Britain for funding the work reported in this paper. B.B. was a recipient of a MRC research studentship.

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(Received 25 September 1986)