HELPER T CELLS INDUCED BY AN IMMUNOPURIFIED HERPES SIMPLEX VIRUS TYPE I (HSV-I) 115 KILODALTON GLYCOPROTEIN (gB) PROTECT MICE AGAINST HSV-I INFECTION

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Herpes simplex viruses (HSV)[1] are the causative agents of cold sores, encephalitis, and eye and genital infections (1). A series of glycoproteins designated gB, gC, gD, and gE (2) have been identified as structural components of the HSV virion envelope and implicated in virus-induced alterations of mammalian cells (3). These glycoproteins are expressed on the plasma membranes of infected cells, and act as major antigenic stimuli for the cellular and humoral responses of the host (4). It is pertinent to the development of a subunit vaccine effective in controlling primary HSV infection to understand the role of these individual glycoproteins in the induction of the various components of immune reactivity. Most of the recent effort in vaccination against HSV infection has been concentrated on glycoprotein D (gD), culminating in cloning of the gD gene (5) and synthesis of peptides corresponding to the neutralization epitopes (6). The importance of gD is based largely on its ability to induce neutralizing antibody (7–9) and its crossreactivity between type I and type II HSV (10–13). However, since it has been repeatedly demonstrated (14–22) that recovery from HSV infections involves both humoral and cell-mediated immunity, other glycoproteins, which do not induce neutralizing antibodies, may be strongly protective via a different mechanism.

In this paper, we first compared the ability of monoclonal antibodies (mAb) directed against different individual glycoproteins to protect susceptible mice against lethal HSV-I challenge. Secondly, glycoproteins immunopurified by mAb were examined for protective capacity, and the immunological mechanism underlying the protection was analyzed. Our data show that a 115 kilodaltons (kD) glycoprotein (gB), though not possessing epitopes for inducing virus-neutralizing antibodies, contains antigenic determinants capable of activating T cells that are

[1] Abbreviations used in this paper: ADCC, antibody-dependent cellular cytotoxicity; Cy, cyclophosphamide; DEM, Dulbecco's Eagle's medium; DTH, delayed-type hypersensitivity; HSV, Herpes simplex virus; LD_{50}, 50% lethal dose; mAb, monoclonal antibody; m.o.i., multiplicity of infection; PBS, phosphate-buffered saline; pfu, plaque-forming units; RIA, radioimmunoassay.

responsible for the glycoprotein's prophylactic potential. These L3T4$^+$ T cells adoptively protect mice against lethal HSV infection, probably via a linked-recognition effect, the 115 kD glycoprotein acting as a potent carrier for virus-neutralizing epitopes on the whole virus, upon infectious challenge.

Materials and Methods

Mice. CBA mice were bred at The Imperial Cancer Research Fund colony in Mill Hill, London, and at The Wellcome Research Laboratories, Beckenham, Kent.

Cells. BHK-21/C13 cells were maintained in monolayer cultures in Dulbecco's Eagle's medium (DEM) containing 10% calf serum. Vero cells (African green monkey kidney cells) were grown in RPMI 1640 medium containing 10% fetal calf serum.

Virus. HSV type 1 Krueger strain (HSV/P1) was obtained from Beecham Research Laboratories, Brockham Park, Betchworth, United Kingdom. Virus stocks were prepared from BHK cells infected at a multiplicity of infection (m.o.i.) of 0.1 plaque-forming units (pfu)/cell. This was increased to 20 pfu/cell to produce viral glycoproteins. The virus was allowed to absorb at 37°C for 1 h, cells were washed twice and incubation was continued in fresh medium for 2 d (virus stock) or 18 h (viral glycoproteins). Complete HSV-1 particles were purified as previously described (24). Viruses were inactivated by heating at 56°C for 1 h.

Antibodies. Hybrid lines T157, G8D1, and C2D2 were produced as previously described; they have been previously characterized (25–27). Polyclonal sera were raised by injecting CBA mice intraperitoneally with either 5 × 10^6 pfu of heat-inactivated HSV-1 or 10 μg of the 115 kD glycoprotein in saline, at weekly intervals. 5 d after the third injection, mice were bled, and the sera were pooled. Ig fractions of mAb from ascites fluid were purified on a protein A–Sepharose CL 4 B column. Monoclonal Ig were coupled to Sepharose CL 4 B using sodium metaperiodate activation (28).

Immunopurification. Purification of viral glycoproteins was performed by a modification of the procedure of Secher and Burke (29), as was previously described (24). Briefly, cells were harvested and disrupted by ultrasonication in E buffer (1% Nonidet P-40 in 150 mM sodium chloride, 2 mM EDTA, 25 mM Tris HCl buffer pH 8.0, 1 mM phenylmethyl sulphonyl fluoride). After centrifugation at 100,000 g for 1 h at 4°C, the extract was applied to the mAb-Sepharose column, sequentially washed with (*a*) phosphate-buffered saline (PBS); (*b*) 25 mM Tris HCl, pH 8.0, 50 mM NaCl, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate; (*c*) 50 mM Tris HCl, pH 6.8; and (*d*) 9 M ethanediol, 0.3 M NaCl, and 0.0075 M sodium phosphate buffer, pH 7.4. Elution of the glycoproteins was achieved at pH 3.0 with 9 M ethanediol, 0.3 M NaCl, and 0.1 M citric acid.

Radiolabelling and Immunoprecipitation. Monolayer cultures of BHK 21/C13 cells were infected with HSV-1 at an m.o.i. of 20 pfu/cell, and the cells were radiolabelled at 5 h postinfection for 24 h with L-[^{35}S]methionine (Amersham International, Ltd., Amersham, England; sp act, >600 Ci/mmol) at 50 μCi/ml in DEM containing one-fifth its normal concentration of methionine. Cell extracts were harvested, washed, and solubilized in E buffer, clarified by centrifugation, and used for immunoprecipitation with the mAb. Immune complexes were collected with Cowan A strain of *Staphylococcus aureus*, repeatedly washed in 1% Nonidet P-40, 650 mM NaCl, 1 mM EDTA, 10 mM Tris HCl buffer, pH 7.5, containing 10% sucrose, reduced, and electrophoresed in a 12.5% acrylamide gel crosslinked with *N,N'*-diallytartardiamide (30). Gels were processed as described by Bonner and Laskey (31), then exposed to Kodak X-omat AR x-ray film at −70°C.

Immunization and Infection of Mice. Young adult CBA mice were injected subcutaneously in four sites in the flanks with a total of 30 μg of immunopurified glycoprotein in saline or 5 × 10^6 pfu heat-inactivated HSV-1 in saline, or saline alone. 7 d after immunization mice were infected with 10 × LD$_{50}$ (50% lethal dose) HSV-1, either 200 μl i.p. or with 20 μl in the left footpad. Disease symptoms and mortality were recorded daily. In our experience, the susceptibility of mice is highly sensitive to factors such as sex, age, and ambient temperature. It is essential that the LD$_{50}$ dose be accurately titrated for each separate experiment. All mortality occurred within 10 d after infection.

Induction and Determination of Delayed-type Hypersensitivity (DTH) Response.   Mice were immunized intradermally with 10 μg of purified, heat-inactivated HSV-I or the glycoproteins 2 d after receiving 150 mg/kg body weight of cyclophosphamide (Cy) intraperitoneally. 5 d after sensitization, they were tested for DTH with injections of antigen (5 μg) into the right hind footpad. An equal volume (50 μl) of saline was injected into the left hind footpad. Footpad swelling was measured 24 and 48 h after injection. DTH was expressed as the difference in thickness of right vs. left hind footpad in 10^{-2} mm.

Fractionation and Adoptive Transfer of Cells.   Spleen and lymph node (inguinal, brachial, axillary, and mesenteric) cells were harvested from normal mice or mice injected subcutaneously in four sites with the 115 kD glycoprotein in saline 7 d before cell harvest. The cells were collected in PBS, and sedimented by centrifugation. Half of the pooled cell population was depleted of B cells by fractionation on an anti-Ig column, as previously described (32). The resulting cell preparations contained <0.5% Ig^+ cells as estimated by anti-Ig fluorescence staining. The other half of the pooled cells were depleted of $L3T4^+$ T cells by resuspending 10^8 cells in 1 ml of neat spent supernatant of mAb clone, GK 1.5 (33). After incubation for 1 h at room temperature, the cells were sedimented by centrifugation, and resuspended in 2 ml of a 1:10 dilution of complement (Low-tox-M rabbit complement; Cedarlane Laboratories Ltd., Hornby, Ontario, Canada), and incubated for an additional 45 min at 37°C. At the end of the incubation, the cells were washed twice, and their viability was estimated by trypan blue exclusion. Graded numbers of viable cells were injected intravenously into syngeneic recipients that were infected 24 h later with an intraperitoneal injection of 10 × LD_{50} of HSV-I.

Virus Neutralization.   Virus (~200 pfu) diluted in RPMI 1640 containing 10% heat-inactivated fetal calf serum was incubated with heat-inactivated antiserum for 1 h at 37°C, with or without complement. Residual viral infectivity, relative to that treated with a control preimmune serum, was determined by plaque assay using vero cells.

Radioimmunoassay (RIA) for Viral-specific Antibodies.   HSV-I-specific antibody in the serum was assayed by a solid-phase RIA. All assays were set up in duplicate. Dynatech (Cambridge, MA) U-bottom flexiplates were coated with purified, heat-inactivated HSV-I (50 μg/ml, 50 μl/well) and 50 μl dilution of serum was added and incubated for 1 h at 37°C. After washing, 50 μl of ^{125}I-labelled, affinity-purified rabbit anti–mouse IgG was added and incubated overnight at 4°C. For antibody isotype determination, 50 μl of a 1:100 dilution of rabbit anti–mouse Ig isotype serum (Miles Laboratories, Naperville, IL) was added as second layer. After washing, ^{125}I-labelled, affinity-purified goat anti–rabbit antibody was added, and incubated overnight at 4°C. After extensive washing, each well was counted in a gamma counter. Endpoints were taken as the highest dilutions with twice the cpm of the corresponding samples from control normal mice.

Proliferation Assay.   All assays were set up in 96-well flat-bottom tissue culture plates. Triplicate cultures of 250 μl medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 10 mM Hepes and 5 × 10^{-5} M 2-mercaptoethanol) were set up as previously described (34). Briefly, irradiated normal spleen adherent cells were plated in medium containing 5 × 10^5 pfu/ml of inactivated HSV-I, and incubated in an atmosphere of 6% CO_2 at 37°C. After 4–6 h, 2 × 10^5 primed lymph node T cells were added. 96 h thereafter, cultures were pulsed for 6 h with 1 μCi (37 mBq) ^{125}I-UdR. Results were expressed as mean ± SD. As a source of primed T cells, popliteal lymph node cells were obtained from CBA mice 10–15 d after footpad injection of 5 × 10^6 pfu of inactivated HSV-I or 25 μg of immunopurified glycoprotein emulsified with an equal volume of Freund's complete adjuvant. Cell suspensions were prepared by teasing the tissue through a sterile nylon mesh, and were enriched for T cells by incubation on nylon wool. Antigen-presenting cells were spleen-adherent cells prepared from normal CBA mice by plating spleen cells on plastic petri dishes. After 2 h, nonadherent cells were washed away, and the adherent cells were recovered by treating the plates with 3 mM EDTA for 15 min at 37°C. The cells were irradiated with 1,000 rad before use in the assay.

Statistical Analysis.   Significances of the results were analysed by Student's *t* test; $p <$ 0.05 is considered significant.

Results

Characterization of mAb. Three hybridoma cell lines producing mAb to the envelope glycoproteins of HSV-I were established as described previously (25, 26). Fluorographs of radioimmune precipitates carried out with [^{35}S]methionine-labelled extracts from infected or mock-infected baby hamster kidney (BHK) cells, and electrophoretically separated on N,N'-diallytartardiamide crosslinked gels revealed three different glycoproteins of apparent molecular masses 103 (G8D1), 63 (C2D2), and 115 kD (T157), as shown in Fig. 1, and they are designated gH, gD, and gB, respectively. The 103 kD (G8D1) was previously designated 123 kD (25). However, due to current confusion in HSV glycoprotein terminology, we prefer to refer to the detected glycoprotein species as 103, 63, and 115 kD glycoproteins, respectively. mAb in culture supernatant were iso-typed using rabbit anti–mouse Ig isotype-specific antisera, and they belong to IgG2a (G8D1), IgM (C2D2), or IgG2b (T157) (Table I). Experiments performed to test the capacity of ascitic fluid of the three mAb to neutralize HSV-I in the absence of complement indicate that only C2D2 antibodies neutralize and had a relatively high neutralizing titer (>250; data not shown). Both G8D1 and C2D2 can neutralize HSV-I in the presence of complement, and the titer is >1,280. In
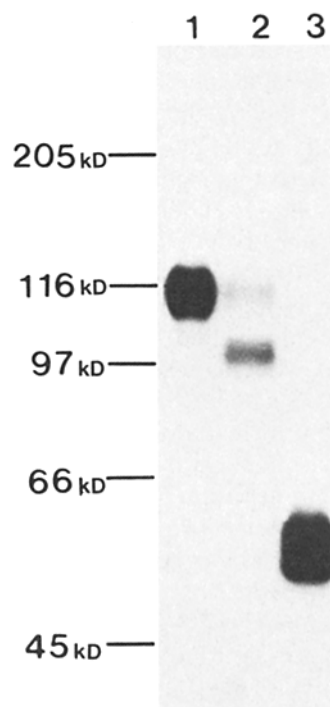

FIGURE 1. Autoradiograms of sodium dodecyl sulfate–polyacrylamide gel electrophoresis of HSV glycoproteins specifically precipitated by mAb. Constant amounts of [^{35}S]methionine-labelled, HSV-I-infected BHK cells were preincubated with Ig from ascites fluid or hybridoma cell supernatant, and the immune complexes were collected with Cowan A strain of *S. aureus.* Immune-precipitated proteins were electrophoresed under reducing conditions on a 12.5% sodium dodecyl sulfate–polyacrylamide gel crosslinked with N,N'-diallytartardiamide, as described in Materials and Methods. *1,* T157; *2,* G8D1; *3,* C2D2.

TABLE I

Passive Protection of mAb to HSV-I Glycoproteins

mAb isotype	Glycoprotein (kD)	Neutralizing titer*	Survivors (day 21)
G8D1 (G2a)	103	>1,280	20/20
C2D2 (M)	63	>1,280	19/20
T157 (G2b)	115	<40	0/10
Normal mouse serum	—	<40	2/20

Groups of mice were injected in the hind footpads with $10 \times LD_{50}$ of HSV-I (Krueger strain, HSV/P1) and 24 h later with 0.3 ml i.p. of ascitic fluid containing the mAb or normal mouse serum. They were observed daily for disease symptoms and mortality until 21 d postinfection. Similar results were obtained when the viruses were administered intraperitoneally.
* Reciprocal dilutions of 50% neutralization of infective virus by antibody in the presence of complement.

contrast, mAb T157 has no detectable neutralizing activity, even in the presence of complement (Table I).

Effect of Passively Transferred mAb on Host Resistance to HSV-I Infection. It has been shown previously (35, 36) that passive transfer of mAb directed against virion envelope glycoproteins protected mice from a lethal challenge with HSV-I. It was also reported that the capacity of glycoprotein-specific mAb to neutralize HVS-I, to promote complement mediated cell lysis and antibody dependent cellular cytotoxicity (ADCC) in vitro did not correlate with the protective activity of antibody in vivo (37). In fact, one monoclonal antibody devoid of all the three immunologic reactions in vitro was highly effective in promoting recovery from HSV-I induced ocular disease (37). Therefore, we tested the capacity of all three mAb to protect susceptible mice from HSV-I infection, using a standard protocol (25, 35). Ascitic fluid (0.3 ml) was injected intraperitoneally into nine 9-wk-old male CBA mice 24 h after footpad or intraperitoneal infection with $10 \times LD_{50}$ HSV-I. Mice injected with mAb G8D1 or C2D2 were completely protected against the HSV-I infection, whereas antibody T157 was ineffective in conferring passive protection (Table I). Such ineffectiveness was not improved by increasing the amounts of ascitic fluid transferred to 0.6 ml (data not shown).

These results are in agreement with previous reports (35) that neutralizing mAb against the type-common epitope of gD and the type-specific epitope of gC were protective when given after subcutaneous virus infection. They also demonstrated a good correlation between neutralizing activity and protective capacity of antibodies specific for glycoproteins.

Protective Effects of Immunopurified Glycoproteins. The demonstration of passive protection clearly indicates that individual viral glycoproteins are important and vary in the generation of protective immunity. Differences in biological activities of mAb in vivo may be related to the topographical location or temporal expression of viral glycoprotein epitopes on the surface of infected cells, to which a particular mAb binds. Therefore, it was of interest to compare the effectiveness of the three immunopurified glycoproteins in inducing immune responses capable of interfering with HSV-I infection in vivo. Mice were immunized subcutaneously with 30 µg of the glycoproteins in saline. Control mice were injected

TABLE II

Protective Immunity and Antibodies Induced by Immunopurified HSV-1 Glycoproteins

Glycoproteins	Survivors	Antibody titer (RIA)	
		Prechallenge	Postchallenge
103 kD	4/8	20	20
63 kD	3/8	20	20
115 kD	8/8	320	2,560
Inactivated HSV-1	8/8	320	5,120
Saline	1/8	<20	<20

Groups of mice were injected with 30 μg s.c. glycoprotein in saline or 5 × 10^6 heat-inactivated HSV-I. They were bled (prechallenge sera) 6 d later, and challenged in the footpad with 10 × LD$_{50}$ of HSV-124 h thereafter. All mice were bled again 3 d after challenge (postchallenge sera) and mortality recorded until 21 d after infection.

TABLE III

Analysis of Antibody Isotypes Induced by Injection of HSV-I and Immunopurified 115 kD Glycoprotein

Antibody iso-type	Inactivated HSV-I		115 kD glycoprotein	
	Prechallenge	Postchallenge	Prechallenge	Postchallenge
A	<10	160	<10	80
M	320	2,560	320	2,560
G1	<10	320	<10	160
G2a	<10	<10	<10	10
G2b	<10	320	<10	160
G3	80	320	80	320

Same serum samples as those described in Table II.

with saline or 5 × 10^6 pfu of heat-killed HSV-I, also diluted in saline. 7 d later, all animals were challenged in the footpad with 10 × LD$_{50}$ dose of live virus.

Mice immunized with heat-killed virus and the 115 kD glycoprotein were completely protected while 103 and 63 kD glycoproteins induced only partial protection (Table II). We therefore attempted to analyze the immunological mechanisms underlying the effectiveness of 115 kD glycoprotein as a subunit HSV-I vaccine.

Antibody Response and Protection. Although TI57 was neither neutralizing nor passively protective, it is possible that the protective effects of immunopurified 115 kD glycoprotein are due to humoral antibody directed against other epitopes on the same molecule. Antibody titers against HSV-I in mice immunized with the three glycoproteins were measured by RIA. As shown in Table II, antibody titers to HSV-I before infection and early postinfection were significantly elevated only in mice immunized with inactivated virus or 115 kD glycoprotein. Thus, the prophylactic capacity of the 115 kD glycoprotein correlates with an early induction of specific antibody, which is even more evident 3 d after virus challenge. Table III analyzes the isotypes of HSV-specific antibodies in the serum of mice induced by inactivated HSV or the 115 kD glycoprotein before and after challenge infection. There was remarkable similarity between

TABLE IV

*Passive Transfer of Polyclonal Antibodies from Mice Immunized with
115 kD Glycoprotein*

Antigen*	Neutralizing titer[‡]	Survivors[§]
115 kD glycoprotein	<20	0/10
HSV-1	>640	9/10
Control (NMS)	<20	0/10

* Polyclonal hyperimmune sera were raised as described in Materials and Methods. Control serum was from unimmunized mice (NMS).
‡ Reciprocal dilutions of 50% neutralization of infective virus by serum in the absence of complement.
§ Groups of 10 mice were injected with 10 × LD_{50} of HSV-1 intraperitoneally, and 24 h later injected intraperitoneally with 0.35 ml of the serum. Mortality was recorded daily up to 21 d after infection.

the antibody, responses to the two different priming antigens in total antibody titers and isotype distribution. IgM was the predominant class of antibody before challenge, and remained so even after challenge. IgG3 was detected in relatively high levels before and after challenge, while IgG1, IgG2b, and IgA were evident only after infection. IgG2a was not detected throughout the experiment. It is possible, therefore, that the 115 kD glycoprotein stimulates the production of high levels of protective early antibody, and that, in this respect, is superior to 103 or 63 kD glycoproteins in inducing protective immunity.

To test this possibility, we examined the neutralizing ability and the passive protective capacity of the polyclonal hyperimmune antibodies induced by the 115 kD glycoprotein. Polyclonal anti-115 kD glycoprotein antibodies and control anti-HSV-I and normal CBA sera (0.35 ml) were injected intraperitoneally into syngeneic recipients 24 h after a lethal intraperitoneal challenge with HSV-I. In contrast to the inactivated virus, the 115 kD glycoprotein did not induce antibody with substantial neutralizing activity or protective capacity (Table IV). Thus, early antibody response alone is probably not responsible for the prophylactic effect of the 115 kD glycoprotein.

DTH Response and Protection.   DTH to HSV-I has been previously (38) shown in mice. Conventionally purified glycoproteins, gC and gD, were able to induce and elicit DTH response to HSV (21). Mice immunized with gC developed DTH reactivity and were protected from lethal challenge with HSV-I. However, serum neutralizing or precipitating antibodies were not detected before HSV-I challenge. It was suggested (21) that the protective effect of gC may be ascribed to a DTH response. We therefore examined the DTH reactivity in animals immunized with immunopurified glycoproteins. In repeated experiments, where mice were immunized with 10–30 µg of glycoproteins subcutaneously or intradermally, none of the glycoproteins induced a significant DTH response (data not shown). Pretreatment of mice intraperitoneally with cyclophosphamide (Cy) 2 d before immunization, however, significantly enhanced the DTH response. Under this regimen, the 115 kD glycoprotein was clearly the most efficient glycoprotein in the induction of DTH, which was comparable to that induced by the whole inactivated HSV-I (Fig. 2). However, in contrast to HSV-I, the 115 kD glycopro-
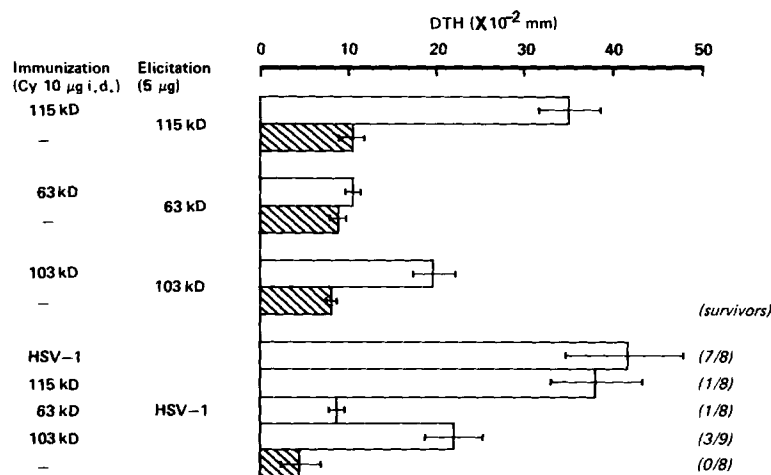
FIGURE 2. DTH and protective immunity induced by purified inactivated HSV-I or specific glycoproteins. Groups of five mice were injected intraperitoneally with 150 mg/kg of Cy, and 2 d later immunized subcutaneously with 10 μg of purified heat-inactivated HSV-I or immunopurified glycoproteins. 5 d after sensitization, and together with control unimmunized mice (hatched columns), they were tested for DTH in the footpad with 5 μg of the antigens indicated. Separate groups of eight mice were also similarly primed but challenged intraperitoneally with 10 × LD_{50} of HSV-I 5 d after sensitization without DTH testing. DTH is expressed as footpad thickness increase (in 10^{-2} mm) 24 h after injection. Vertical bars represent SEM. Survivors were recorded daily until 21 d after infection.

tein under such immunizing conditions was unable to confer effective protection against a lethal challenge of HSV-I (Fig. 2). The Cy pretreatment has been shown to drastically reduce B cells and suppressor T cell precursors (39, 40), and none of the mice pretreated with Cy produced a significant amount of specific antibody before or 7 d after challenge infection (data not shown). It thus appears that DTH alone induced by 115 kD glycoprotein is insufficient for protection.

Induction of Proliferative T Cells by the 115 kD Glycoprotein. The high early postinfection antibody response in mice primed with 115 kD glycoprotein prompted us to investigate the possibility that this glycoprotein may contain antigenic determinants recognizable by nonneutralizing antibodies, and capable of activating helper T cells that may be responsible for the glycoprotein's prophylactic capacity. To test this, we first examined the ability of the three glycoproteins to induce T cells that proliferate upon stimulation with inactivated HSV-I in vitro. Results shown in Fig. 3 demonstrate that all three glycoproteins induced T cells that show significant proliferation against HSV-I, the efficiency of proliferative T cell induction being in the order of HSV-I > 115 kD > 63 kD > 103 kD. These results are in striking correspondence with the superior protection, as well as the ability to induce a postchallenge antibody response by whole HSV and 115 kD glycoprotein (compare Fig. 3 and Table II). It appears that the proliferative T cells induced by 115 kD glycoprotein may have a prophylactic role. This possibility is further investigated in adoptive transfer experiments.

Adoptive Transfer of Protective Immunity with T cells from Donor Mice Immunized with 115 kD Glycoprotein. Donor mice were injected subcutaneously with 30 μg
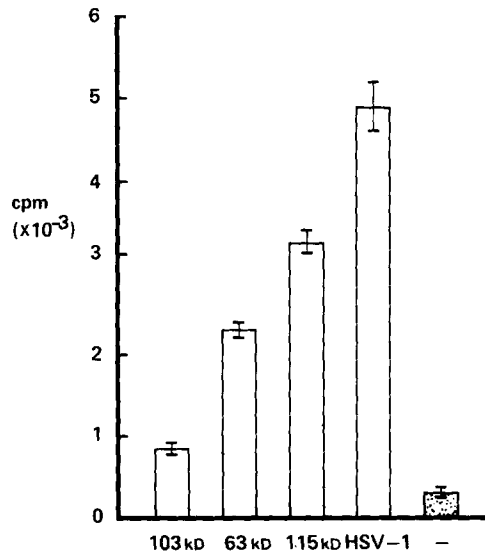
FIGURE 3.   In vitro proliferative responses against inactivated HSV-I of lymph node cells from mice immunized with affinity-purified glycoproteins or heat-inactivated HSV-I. For details, see Materials and Methods.

TABLE V

Adoptive Transfer of Protective Immunity Against HSV-I Infection with L3T4⁺ T Cells from Donor Mice Immunized with 115 kD Glycoprotein

Donor cells	Number of cells transferred	Survivors*	Postchallenge neutralizing antibody titer‡
Immune, Ig⁻	6×10^7	9/10	160
	6×10^6	7/10	80
Immune, treated with GK 1.5	6×10^7	5/10	40
plus complement	6×10^6	2/10	<20
Normal, Ig⁻	6×10^7	2/10	<20
Normal, treated with GK 1.5 plus complement	6×10^7	1/10	<20

* 21 d after infection.
‡ 6 d postchallenge, serum virus-neutralizing antibody titers in the absence of complement.

of 115 kD glycoprotein, and their spleen and lymph node cells were retrieved 7 d later. The pooled cell populations were either depleted of B cells by passing through an anti-Ig column, or depleted of L3T4⁺ cells by treatment with mAb GK1.5 and complement, as detailed in Materials and Methods. These subpopulations of cells were then transferred intravenously into groups of 15 normal CBA mice at either 6×10^7 or 6×10^6 cells per recipient, which were subsequently challenged with a lethal intraperitoneal dose of HSV-I 24 h after cell transfer. 10 mice per group were observed for mortality while the remaining 5 were bled for titration of virus-neutralizing antibody in the serum. The results presented in Table V show that 6×10^7 and 6×10^6 Ig⁻ (T cell–enriched) spleen and lymph node cells from 115 kD glycoprotein–immunized donors conferred sub-

stantial protection against HSV-I infection (survivors were 9 of 10 and 7 of 10, respectively) compared with mice receiving 6×10^7 of Ig$^-$ cells from normal donors (2 of 10 surviving). Mice injected with 6×10^7 cells treated with GK1.5 and complement also showed significant (5 of 10 surviving) protection, which, however, largely disappeared when the cell dose was reduced to 6×10^6 cells per recipient (2 of 10 surviving). These results therefore suggest that the protective immunity induced by the 115 kD glycoprotein is adoptively transferable by L3T4$^+$ T cells and not B cells. The protection seen in the high dose of GK1.5 and complement–treated cells could be due to residual L3T4$^+$ cells, which titrated out at a lower cell dose. Table V also shows that the protection is correlated with the induction of neutralizing antibody in the serum of recipients upon challenge infection. Thus, mice receiving L3T4$^+$ T cells and recovered from the lethal infection developed significant levels of HSV-I-neutralizing antibody in their serum. In contrast, such antibody was not detectable in sei a from similarly challenged mice receiving 6×10^6 immune L3T4$^-$ cells or normal cells.

Discussion

The finding (20) that athymic (nu/nu) mice are more susceptible than their normal litter mates to HSV infections implies that T cell–mediated responses are essential for antiviral immunity. Such observations are consistent with the severity of primary and recurrent HSV infection encountered (41) in patients with predominantly T cell immunodeficiencies. Furthermore, mice rendered wholly antibody deficient by the regular administration of anti-μ antibody from birth (42) were able to recover from an HSV-1 infection in the ear. However, such B cell–suppressed animals developed higher incidence of latency and a more florid primary infection of the dorsal root ganglia than did controls. This suggests that specific antibody was essential for restricting the spread of HSV to the nervous system, a finding that was confirmed (42) by the passive transfer of specific neutralizing antibody to nude mice. Nonneutralizing antibodies, on the other hand, were ineffective in restricting entry of virus to the nervous system. These reports emphasize the importance of both humoral and cell-mediated immunity in preventing and controlling HSV infections.

Data reported in this paper confirm the critical role of neutralizing antibodies. They also provide direct evidence that antigenic determinants activating T cells are essential, and sometimes more potent in stimulating prophylactic immunity than those inducing neutralizing antibodies. Thus, the 115 kD glycoprotein, which lacks antigenic determinants for stimulating virus-neutralizing antibodies, can induce stronger prophylactic immunity than that produced by 63 and 103 kD glycoproteins, though the latter glycoproteins are good inducers of virus-neutralizing antibodies. This finding suggests that neutralization and passive protection should not be the sole criteria for selecting mAb for the identification and isolation of protective antigens for prophylactic immunization against infectious diseases. A precedent for this was noted in the experimental malaria system (43). In contrast to the 63 and 103 kD glycoproteins, the 115 kD glycoprotein induced strong DTH and proliferative T cell responses to intact HSV. Furthermore, the protective immunity was adoptively transferred with T cells bearing

the L3T4$^+$ surface marker, characteristics of helper T cells. Such transferance of protection was accompanied by a parallel enhancement of production of neutralizing antibody in protected recipients upon challenge infection (Table V). It thus appears that a major protective mechanism induced by immunization with the 115 kD glycoprotein in this system is the induction of helper T cells that facilitate the production of neutralizing antibody upon infectious challenge via a linked-recognition effect; the 115 kD glycoprotein acting as potent carrier for enhancing the production of neutralizing antibodies directed against epitopes present elsewhere on the intact virus. A similar linked-recognition effect has been demonstrated in the influenza virus system, where T cells from mice primed with matrix protein were found to enhance both the serum hemagglutination-inhibiting antibody titer (44), and protection (45) upon heterologous challenge infection.

The fact that 6 × 10^7 spleen and lymph node cells treated with mAb GK 1.5 and complement were still capable of transferring significant protection raises the possible involvement of other cells in the protection induced by 115 kD glycoprotein. The L3T4$^-$ cytotoxic T cell would be a natural candidate. However, it has been shown in many viral systems (46) that significant levels of cytotoxic activity were only induced with infectious virus and, in our system, no detectable cytotoxic T cell reactivity was obtained when mice were immunized with inactivated HSV or purified glycoproteins (W. L. Chan and A. A. Czitrom, unpublished). Another possibility is the transferance of immune B cells that secrete specific nonneutralizing antibodies involved in ADCC. Although ADCC is highly cytolytic in vitro, its significance in vivo remains to be established. Furthermore, the fact that monoclonal and polyclonal antibodies against the 115 kD glycoprotein did not confer significant protection renders the involvement of ADCC highly unlikely. Thus, the most straightforward interpretation would be that the modest protective effect of GK 1.5 plus complement–treated cells was due to the helper activity of residual T cells that express fewer L3T4 antigens. This is consistent with the low but significant enhancement of neutralizing antibody titers in the recipients upon challenge infection (Table V). Futhermore, such modest antibody enhancement and protection largely disappeared when the number of cells transferred was reduced to 6 × 10^6.

DTH is a widely used assay for cell-mediated immunity, which is thought to reflect pathological processes involved in infectious diseases. DTH to HSV has been demonstrated in seropositive humans (47), guinea pigs (48), and mice (38). Strong correlation was obtained between protection and DTH reactivity induced by infectious virus (38) or conventionally-purified glycoprotein C (21), though direct evidence for a protective role of DTH is still lacking. In our system, using the three immunopurified glycoproteins, no detectable DTH reactivity could be induced unless the mice were pretreated with Cy. Under such conditions, DTH induced by the 115 kD glycoprotein was comparable to that induced by the intact HSV, but unlike the latter, it was unable to confer effective protection against a lethal challenge infection. These results indicate that DTH alone induced by the 115 kD glycoprotein is insufficient for protection.

Although our data suggest the involvement of helper T cells for neutralizing antibody in the protective immunity induced by 115 kD glycoprotein, they do

not exclude a similar helper effect for cytotoxic T cells (49). Major histocompatibility complex–matching shows that at least two T cell subsets determine resistance to HSV (18), class II–restricted helper cells, and class I–restricted cytotoxic T cells. Thus L3T4$^+$ helper T cells for effector cytotoxic T cells may conceivably be induced by 115 kD glycoprotein, and adoptively enhance specific cytotoxic T cell reactivity activated by challenge infection, hence reinforcing the host's recovery.

The prophylactic immunity induced by 115 kD glycoprotein is nontrivial. A single subcutaneous injection of 10–30 μg of the glycoprotein in saline could produce complete protection against a lethal (10 × LD$_{50}$) challenge infection administered intraperitoneally or in the footpad (Table II and our unpublished data). Further experiments using isolated and structurally defined peptide fragments of the 115 kD glycoprotein may localize specific epitopes and lead to chemically defined anti-HSV vaccines.

Summary

Three herpes simplex virus type I (HSV-I) glycoproteins of apparent molecular masses 103, 63, and 115 kD have been purified using virus-specific monoclonal antibodies (mAb) G8D1, C2D2, and T157, respectively. Both G8D1 and C2D2 neutralize HSV-I in vitro and passively protect CBA mice against HSV-I infection in vivo, whereas T157 is neither neutralizing nor passively protective. However, mice given a single subcutaneous injection of 30 μg 115 kD glycoprotein in saline were completely protected against lethal challenges of HSV-I administered intraperitoneally or in the footpad 7 d after immunization. In contrast, mice similarly immunized with 103 or 63 kD glycoproteins were only partially protected. The prophylactic immunity was correlated with an early induction of specific antibody, which became even more evident 3 d after virus challenge. There was a remarkable similarity in antibody isotype distribution between the responses to 115 kD glycoprotein and to heat-inactivated intact HSV-I. However, the prechallenge sera from 115 kD glycoprotein hyperimmunized mice were again neither virus-neutralizing nor passively protective. All three glycoproteins induced only low levels of delayed-type hypersensitivity (DTH). Pretreatment of mice with cyclophosphamide significantly enhanced DTH to 115 kD and 103 kD glycoproteins in the absence of antibody, but failed to confer significant immunity, indicating that DTH alone is insufficient for protection. Splenic and lymph node Ig$^-$ (B cell–depleted) cells from mice protectively immunized with 115 kD glycoprotein could adoptively transfer effective protection and enhance a virus neutralizing antibody response in normal recipients challenged with a lethal dose of HSV-I. Both the protection and the ability to enhance neutralizing antibody were diminished when the cells were treated with mAb GK 1.5 and complement. These results therefore demonstrate that the 115 kD glycoprotein, though not apparently containing accessible epitopes for the induction of virus-neutralizing antibody, possesses determinants capable of activating helper T cells. These L3T4$^+$ cells confer strong protective immunity by enhancing protective antibody upon challenge infection, probably through associative help.

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