Differential T cell response induced by certain recombinant oligopeptides of herpes simplex virus glycoprotein B in mice

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Much attention is presently focused on the quality of the immune response produced by helper T or regulatory cells because of its implications for vaccine development and immunomodulation. Glycoprotein B (gB) of herpes simplex virus (HSV) has been shown to induce a protective T cell response. To further characterize the nature of the T cell response, oligopeptides were expressed from the open reading frame of gB from HSV-2 (gB-2) as fusion proteins with β-galactosidase (GZ) in E. coli. After immunopurification using an anti-GZ affinity column, oligopeptides p59 and p65, spanning amino acid residues 339–394 and 424–484 of gB-2 respectively, were examined for immunogenic response by delayed type hypersensitivity (DTH) in vivo and for antigenic response by T cell proliferation in vitro. p59 but not p65 was able to prime for both DTH and proliferative T cell response to whole HSV-2 and protect against challenge infection. However, when mice were pretreated with cyclophosphamide, p65 primed for a strong DTH response to a level similar to that induced by p59 in mice either pretreated or not treated with cyclophosphamide. This suggests that p65 contains epitopes capable of inducing both DTH and immunosuppression. Thus, when mice were primed with p65 before immunizing with HSV-2, their in vitro HSV-specific proliferative response was suppressed. Therefore, p59 is a good immunogen able to induce significant, though incomplete, protection. It could be considered for inclusion in a cocktail of subunit vaccines against HSV-2 whereas p65 or parts thereof should be excluded for this purpose.

Introduction

There is considerable evidence that cell-mediated immunity (CMI) plays an important role in defence against acute herpes simplex virus (HSV) infection (Nash et al., 1985). Adoptive transfer experiments in mice demonstrate that immune T cells, delayed type hypersensitivity (DTH) and cytotoxic T cells (CTL) consistently clear virus and protect against primary infection (Nash & Gell, 1983; Sethi et al., 1983) whereas immune serum gives variable protection (Nash et al., 1980; Lukic et al., 1985; Chan et al., 1985). Distinct effector T cell subsets appear to limit infection in different host tissues. CD4⁺ T cells act as the principal antiviral T cells in acute herpesvirus infection of the skin (Nash et al., 1987) while CTL were shown to mediate long-term T cell memory of HSV infection (Larsen et al., 1984) and to control spread of virus in the nervous system (Nash et al., 1987).

These protective T cells have been demonstrated to be inducible by various viral glycoproteins. Glycoprotein B (gB) in particular has been shown to induce CD4⁺ protective T cells that are adoptively transferable and this is accompanied by enhanced production of neutralizing antibody in protected recipients upon challenge infection (Chan et al., 1985). Thus, it appears that while gB does not by itself induce virus-neutralizing antibodies, it does induce protection by activating helper T (Th) cells for anti-HSV antibody production. Protective Th cells induced by gB or its non-glycosylated precursor were subsequently demonstrated to produce both IL-2 and IL-3 and protection correlated with the induction of these cells and their early enhanced production of IL-2 and IL-3 on challenge infection compared with control ovalbumin-immunized mice (O’Donnell & Chan, 1991).

Evidence has been accumulating for heterogeneity of CD4⁺ Th cells, the mouse paradigm of Th1 and Th2 being the clearest example (Mosmann et al., 1986). Therefore, the factors that control which Th subsets will be preferentially activated during an immune response have recently also received much
attention, because insight into these regulatory mechanisms during an immune response may be applied to the design of vaccines and strategies for immunomodulation. Apart from cytokines and co-stimulatory signals, antigens and the presenting major histocompatibility complex (MHC) molecules may play a role in determining the type of T cell response generated towards antigens. So far, the relative importance and preferential induction or recruitment of particular T cell subsets that protect against an acute HSV infection is unclear. This is particularly important for vaccine development strategy. Since gB is a well-characterized and highly conserved glycoprotein among the human herpesviruses, is able to induce various T cell subsets responses and is itself a target of the host’s immune response (Chan, 1989), we decided to dissect the T cell response inducible by discrete regions of the gB molecule, with the view that information obtained here may be applied to vaccine strategies for herpesviruses.

In this paper, two oligopeptides of gB from HSV type 2 (gB-2) were expressed as fusion proteins with β-galactosidase (GZ) in E coli. The immunopurified fusion proteins were used as immunogens to study DTH responses in vivo or as antigens for proliferative T cell responses in vitro. Our results show that fusion protein p59 is a good immunogen for inducing anti-HSV-2 proliferative T cell and DTH responses and can protect against HSV challenge infection when compared with control GZ. On the other hand, p65 can only induce a DTH response in the presence of cyclophosphamide (CY), suggesting that apart from inducing DTH, it can also induce suppression. In fact, when mice are preprimed with p65 before HSV immunization, their HSV-specific proliferative T cell response in vitro is suppressed. Both antigens can stimulate HSV-primed lymph node cells to proliferate and elicit DTH in HSV-primed mice.

**Methods**

**Mice.** BALB/c mice were obtained from Harlan Olac. They were used at 6–8 weeks of age. All animals were maintained in accordance with Guidelines for the Housing and Care of Laboratory Animals Used in Scientific Procedures (Home Office, UK; 1989) and all experimental procedures were performed in accordance with the guidelines approved by the UK Home Office.

**Virus.** HSV type 2 strain BRY virus stock was prepared from BHK-21/C13 cells as infected cell sonicate or as virions purified on a sucrose density gradient. The virus was inactivated by heating at 56 °C for 1 h or UV-inactivated at 4 °C for 12 min at a distance of 7 cm from a 320 nm UV source.

**Antibodies.** Monoclonal anti-gB antibody (TI57) was prepared and characterized as previously described (Chan et al., 1985). The anti-GZ monoclonal antibody (BG79) coupled to Sepharose CL4B was a kind gift from Glaxo Wellcome.

**Cloning and expression of gB fragments.** Construction of recombinant plasmids and transformation of E coli were carried out essentially as described by Maniatis et al. (1982). An approximately 17 kbp HindIII fragment of the HSV-2 genome containing the gB-2 gene cloned into pAT153 and designated pGR93 (Reyes et al., 1982) was a kind gift from G. S. Hayward via A. Minson. This was subcloned into pUC19 to produce the 4.7 kbp BamHI–KpnI fragment contained in clone pUH10 as shown in Fig. 1. Based on restriction map data of the gB-2 gene location (Person et al., 1985; Baik et al., 1986), a 2.6 kbp XhoI–Xhol fragment containing the extracellular domain of gB-2 was isolated from the 4.7 kbp BamHI–KpnI fragment by electro-elution from agarose gel. This 2.6 kbp XhoI fragment was partially digested simultaneously with the three restriction enzymes Rsal, Alul and Thal to produce fragments in the size range 100–1000 bp. The resultant fragments were used to blunt-
end ligate into the SmaI site of an open reading frame expression vector pXY400 which was subsequently transformed into E. coli strain TG1 to yield blue transformants in the presence of IPTG and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (Sigma). The plasmid pXY460 shown in Fig. 1 was a kind gift from Glaxo Wellcome. The transformants produce gB-2 oligopeptide fusions to GZ between the third and sixth codon of GZ (Winther et al., 1986). The sequence of the inserted gene fragment was confirmed by the dideoxynucleotide chain-termination method of Sanger et al. (1977).

**Preparation of bacteria lysates and Western blotting.** Overnight liquid cultures grown from individual colonies were seeded 1:5 into 500 ml fresh Luria broth containing 100 µg/ml ampicillin and grown at 37 °C in an orbital shaker to an optical density of 0.6 at 600 nm before induction with 0.5 ml IPTG at 60 µg/ml. Cultures were grown for another 4 h and harvested by centriﬁfugation. Sample buffer was added to bacterial pellets and samples were electrophoresed in 7.5% acrylamide gels, transblotted onto nitrocellulose and probed with rabbit anti-HSV-2 antibody and 125I-labelled protein A as previously described (Chan, 1989).

**Immunopurification.** The bacterial pellet was resuspended in 10 ml of 25 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.2% NP40, 5 mg/ml lysozyme, 1 mM PMSF buffer and incubated overnight at 4 °C. Two ml of 0.1 M MgCl2 and 0.4 ml of DNase at 1 mg/ml were then added and the lysate was incubated at 4 °C for 1 h. The resultant supernatant was pre-cleared by centrifugation and 0.05 vol of 1 M NaCl was added before it was loaded onto the BG79-Sepharose CL4B column, which had been pre-equilibrated at room temperature with ST buffer (50 mM NaCl, 1 M sodium borate, 1 mM xylitol, 1 mM EDTA buffer pH 7.4). After thorough washing with ST buffer, the bound protein was eluted with 0.1 M sodium borate, 1 mM xylitol, 1 mM EDTA buffer pH 10.5. The eluate was dialysed extensively against 2 mM sodium phosphate buffer pH 7.4, lyophilized and reconstituted in distilled water before use. gB-2 was immunopurified from HSV-2-infected BHK cell lysates as previously described (Chan et al., 1985).

**Proliferation assay.** All assays were set up in triplicate in 96-well flat-bottomed tissue culture plates as previously described (Chan, 1989). Draining lymph node (DLN) cell suspensions obtained from antigen-primed mice were dispensed at 4 x 10^5 cells per well in 100 µl of Click’s Eagle’s high amino acid medium supplemented with 2 mM l-glutamine, 2 x 10^-5 M 2-mercaptoethanol and 0.5% normal mouse serum. Each culture, with or without antigen, was incubated in an atmosphere of 5% CO2 at 37 °C for 4 days, pulsed for 18 h with 1 µCi per well of [3H]thymidine (Amersham) and harvested with a cell harvester; radioactivity was determined in a β-counter. Cell proliferation is expressed as SI (stimulation index = c.p.m. with antigen/c.p.m. without antigen). An SI of 2 or less is considered not significant.

**Induction and determination of the DTH response.** Mice were immunized intradermally with 20 µg purified heat-inactivated HSV-2 BRY, immunopurified recombinant antigens or PBS. In some experiments the mice were pretreated with 150 mg/kg body weight of CY intraperitoneally 2 days before priming. Seven days after sensitization, they were tested for DTH with 2 x 10^5 p.f.u. UV-inactivated HSV-2 BRY or 10 µg of recombinant antigen in 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 mm.

**Statistical analysis.** Signiﬁcance of the results were analysed by Student’s t-test; *P < 0.05* is considered signiﬁcant.

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**Results**

**Cloning and expression of fusion proteins**

The expression vector pXY460 shown in Fig. 1 is modified from pXY410 (Winther et al., 1986) by the insertion of an AluI site between the ribosome-binding site (Shine–Dalgarno sequence) and the initiating ATG codon. Transcription initiation at this start site is not in the correct reading frame to produce GZ and colonies containing the parent plasmid are white on the appropriate microbiological indicator plates (Miller, 1972). When an insert which corrects the reading frame is placed into this plasmid at the SmaI site by blunt-end ligation, large quantities of the protein are produced and the colonies are blue on indicator plates. Thus, recombinants producing hybrid proteins are easily identified and selected.

Induced culture lysates from clones identiﬁed as expressing fusion proteins were run in duplicate on SDS–PAGE for Coomassie staining and for Western blotting (Fig. 2a,b). As shown in Fig. 2a, there is variation in electrophoretic mobility and intensity of staining with Coomassie blue between fusion proteins. Only five selected clones (pXH59, 65, 71, 73 and 85) showed HSV-2 specific immunostaining and therefore express gB residues on Western blot analysis using an anti-HSV antibody as probe (Fig. 2b). Clone pXH84 was not recognized by the anti-HSV serum used, although, like the control clone pXY461, it expresses GZ. pXY461 was used purely to produce control GZ for immunological studies. Additional confirmation of the expression of gB residues by these five clones was obtained by comparing our nucleotide sequence data (data not shown) with that of the published sequence for gB-2 (Bzik et al., 1986). In this report, two fusion proteins, p59 and p65, containing residues 339–394 and 424–484 respectively of the amino acid sequence of gB-2 were tested for antigenicity.

Before they were used in *in vitro* and *in vivo* experiments, the two expressed gB-2 fragments were immunopurified with BG79-Sepharose CL4B and their purity was checked by electrophoresis (Fig. 3). As shown in the gel, there is insignificant contamination of the purified preparations even though they are visualized as doublets. The doublets could be due to degradation of fusion proteins by proteases during the extraction procedure because insufficient protease inhibitors were added to the extraction buffer.

**Antigenicity and immunogenicity of the fusion proteins**

p59 and p65 were examined for T cell antigenicity by using them to stimulate HSV-primed DLN cells to proliferate *in vitro*. Like gB-2, both p59 and p65 were able to stimulate, at 2 and 10 µg per well, HSV-2-primed DLN cells to proliferate *in vitro* and are therefore antigenic; control antigen p461 could not stimulate proliferation (data not shown). Since p59 and p65 stimulated HSV-2-primed T cells to proliferate *in vitro*, the possibility that they may prime T cells for a response to HSV-
SDS–PAGE analysis of IPTG-induced culture lysates of control pXY461 (GZ) and pXH02–85. Two gels were run simultaneously. Gel (a) was stained with Coomassie blue and gel (b) was Western blotted and bands were detected with rabbit polyclonal anti-HSV-2 antisera and 125I-labelled protein A. Immunoblots were exposed to X-ray film.

SDS–PAGE analysis of immunopurified samples of control p461 (GZ), p59, p65 and p71. Molecular mass markers used (lane SS) are 200, 115, 92 and 68 kDa.

2 was examined. While both p59 and p65 are equally antigenic, their immunogenicities differ. As shown in Fig. 4, p59 can prime for a proliferative T cell response to either gB-2 or inactivated HSV-2 but p65-primed DLN cells could not be stimulated to proliferate in vitro by either of the antigens. However, with CY pretreatment, p65-primed mice can induce a significant proliferative response (SI = 3.5) stimulated by HSV-2 antigen (data not shown). Although p65 could not induce an HSV-specific proliferative T cell response, it could, like p59 and control antigen p461, induce a significant but reduced proliferative response to GZ. This reduction in proliferative response to GZ could be due to the gB-2 portion of the molecule. We next investigated whether p65 can prime for a DTH response.

**Induction of an HSV-specific DTH response by the fusion proteins**

When mice were pretreated with CY, both fusion proteins could prime as well as elicit an HSV-specific DTH response of approximately 0.4 mm which was significantly higher than the 0.15 mm footpad swelling produced in mice primed or elicited with control antigen p461 (Fig. 5). Since CY pretreatment has
T cell responses induced by HSV gB oligopeptides

Fig. 4. Proliferative T cell response to gB-2 of DLN cells primed with immunopurified fusion proteins. Groups of three mice were primed subcutaneously with various antigens at 20 µg per mouse with aluminium hydroxide as adjuvant and boosted with 10 µg per mouse of the appropriate antigen 7 days later. DLN cells were harvested 5 days later and 4 x 10^5 cells per well were stimulated with 10 µg per well control antigen ovalbumin ( ), control GZ p461 ( ) or immunopurified gB-2 ( ).

Fig. 5. DTH response to HSV antigens in cyclophosphamide-pretreated mice. Groups of five mice pretreated 48 h earlier with cyclophosphamide (150 mg/kg) were primed intradermally in the flanks with 20 µg per mouse immunopurified gB-2, p59, p65, control p461 (GZ), heat-inactivated (HI) purified HSV-2 BRY (pHSV-2) or 4 x 10^5 p.f.u. HI-infected cell sonicate of HSV-2 BRY or PBS. DTH response was elicited 7 days later by injection into the right hind footpad with 10 µg per mouse immunopurified gB-2, p59, p65, p461 ( ) or 2 x 10^5 p.f.u. HI-infected cell sonicate of HSV-2 ( ) and footpad swelling measured 24 h later. Vertical bars represent 1 SEM.

Fig. 6. Effect of CY on the DTH response to HSV-2 in mice primed with various antigens. Groups of five mice with ( ) or without ( ) CY pretreatment 48 h before priming, were primed intradermally in the flanks with 20 µg per mouse gB-2, p461, p59 or p65. DTH response was elicited 7 days later with 2 x 10^5 p.f.u. UV-inactivated infected cell sonicate of HSV-2 BRY. Footpad swelling was measured 24 h later. Vertical bars represent 1 SEM.

Fig. 7. Effect of prepriming with the fusion proteins on the induction of immune response to HSV. Groups of three mice were injected with 20 µg per mouse p461 (GZ), PBS, p59, p65 or 2 x 10^5 p.f.u. UV-inactivated HSV-2 BRY intradermally in the flanks. They were challenged 7 days later with 20 µg per mouse heat-inactivated (HI) HSV-2 BRY subcutaneously in the footpads. DLN cells were harvested 8 days later and 4 x 10^5 cells per well were stimulated with 10 µg per well control GZ p461 ( ) or HI HSV-2 BRY ( ) and medium ( ).
been shown to drastically reduce suppressor cell precursors and a subset of suppressor cells is sensitive to CY pretreatment (Liew, 1982) the pretreatment could bring about a significant increase in the DTH response. So we next investigated whether p65 could prime for a DTH response without pretreatment with CY.

**Cyclophosphamide pretreatment abrogates suppression of the DTH response by p65**

To test whether the HSV-specific DTH responses induced by the fusion proteins and gB-2 were influenced by the co-induction of suppression, parallel groups of mice pretreated with CY or left untreated were immunized with each of the antigens. As shown in Fig. 6, with the exception of p59 the HSV-specific DTH response induced in mice pretreated with CY is significantly higher than that in untreated mice. p65 induced a DTH response in CY-pretreated mice that is comparable to that induced by p59 but failed to induce a significant response in untreated mice.

**p59 primes for suppression of HSV-specific proliferative response**

When mice preprimed with p65 are immunized with HSV antigen, they fail to mount an *in vitro* proliferative response to stimulation with HSV antigen (Fig. 7). Control p461- or PBS-preprimed mice, however, had significant responses to HSV antigen, as did p59-preprimed mice.

**p59 but not p65 can protect mice against HSV-2 infection**

Purified heat-inactivated HSV-2 antigen conferred complete protection to mice, while 10/11 mice immunized with gB-2 survived (Fig. 8). With p59, 4/11 mice survived compared with 1/11 for the groups immunized with p65 or p461 (GZ). Although mice immunized with p65 were not protected, their prechallenge sera had a mean anti-HSV-2 antibody titre of 1/40 compared with 1/20 for mice immunized with p59, suggesting that the antibody induced is not protective, as previously demonstrated (Chan *et al.*, 1985). Mice immunized with GZ had no detectable anti-HSV antibody response (< 1/20).

**Discussion**

The aim of this study is to further characterize gB with a view to identifying immuno-potentiating and regulatory determinants using fusion proteins. Data reported here demonstrate that (a) fragments of gB purified as fusion proteins can be recognized by HSV-primed DLN cells and (b) one of the fusion proteins, p59, can prime for both proliferative T cell and DTH responses and protect against challenge infection with HSV while the other, p65, can prime for little or no T cell proliferation or DTH response. The inability of p65 to prime for proliferative T cell or DTH responses can be overcome by pretreatment with CY. The isolation of clones like pXH84 that produce non-gB-2 fusion proteins may result from fragments originating from regions of non-coding DNA sequences flanking the gene. Although such regions are expected to contain numerous stop codons, they can contain untranslated open reading frames which can be expressed in this system. Alternatively, the inserted coding sequences may be out of frame for gB-2. Comparison of our unpublished sequencing data with that of Bzik *et al.* (1986) showed that in the case of pXH84, the latter possibility prevails. Immunopurification with BG79 provided a simple and efficient general method for producing high yields of each of the fusion proteins and GZ from pXY461 with good purity and there was no detectable non-specific mitogenic effect on the HSV-specific T cell response *in vitro* at optimal concentrations.

Both p59 and p65 contain antigenic determinants recognizable by HSV-primed DLN cells and they stimulated HSV-primed DLN cells to a similar extent as gB-2 (data not shown). In contrast, only p59 is immunogenic in inducing proliferative T cell responses stimulated by HSV-2 and gB-2 *in vitro*. There are two possible reasons for the lack of either a gB-2- or HSV-specific T cell response in p65-primed DLN cells. Firstly, although p65 can associate with class II antigen in vitro, it is not able to be processed and presented to induce T cell response suggesting a different requirement for presentation of antigen to be recognized by activated memory T cells and for the induction of immune response in naive T cells. Townsend *et al.* (1986) demonstrated that some peptides can associate with
class I antigen as targets for CTL but were not able to induce CTL. This is unlikely to be the case as p65 can induce a response to its fusion partner, GZ (Fig. 4). Alternatively, p65 may induce suppressor cells and the existence of T cells that function to down-regulate the immune response is a well-documented phenomenon (Sercarz & Kryczk, 1991). Thus, it is possible that cells from p65-primed mice did not proliferate in response to HSV-2 because of the induction of a proportion of cells that suppress the proliferative T cell response to HSV-2. This population is likely to be diluted out in HSV-2-primed animals, thereby allowing the antigenic part of p65 to stimulate a response. Although for a while, the nature and mode of action of suppressor cells were a highly controversial issue, recent data show that they are antigen-specific (Modlin et al., 1995) or CD4+ T cells (Muts et al., 1994). The presence of a suppressor epitope within the mycobacterial hsp65 protein has recently been identified by Muts et al. (1994).

Data presented in Figs 6 and 7 suggest that p65 may contain a suppressor epitope. Kardys & Hashim (1981) have shown that CY preferentially abrogates suppression of disease-inducing T cells induced by bovine myelin basic protein (aa 75–84) and pretreatment of mice with CY has been shown to enhance DTH responses (Liew, 1982). This is indeed the case here. As p65 can prime for DTH when the mice were pretreated with CY, the data suggest that in addition to the presence of a suppressor epitope, p65 also contains an epitope for memory T cells for the DTH response. It has been demonstrated that the protective T cell response to HSV is sensitive to the effect of suppressor cells (Schrier et al., 1982), and that could partly account for the inability of p65, compared with p59, to induce a protective response in the mice (Fig. 8). Although p65 induced a slightly higher antibody response than p59, the antibody induced is not protective. Since the antibody induced by p65 is IgG, the response is likely to be T cell dependent and is probably due to p65 inducing a population of cells that will suppress proliferative T cell and DTH responses but is able to help antibody response. Clearly, for the purpose of vaccination, it would be desirable to identify and exclude regions of gB with the suppressor characteristics of p65. Therefore, future work includes the identification and fine mapping of the suppressor epitope and investigation of its immunomodulatory effects.

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