To the Graduate Council:

I am submitting herewith a dissertation written by Janet L. Lathey entitled "Production, Characterization, and Immunoregulatory Activity of Anti-idiotypic Antibodies in the Herpes Simplex Virus System." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Barry T. Rouse, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of the Graduate School

PRODUCTION, CHARACTERIZATION AND IMMUNOREGULATORY ACTIVITY OF ANTI-IDIOTYPIC ANTIBODIES IN THE HERPES SIMPLEX VIRUS SYSTEM

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Janet L. Lathey

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To my family and friends

for their continued

support.

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ABSTRACT

Anti-idiotypic antibodies with specificities for herpes simplex virus type 1 (HSV-1) monoclonal antibodies were prepared in rabbits. One, anti-id D, reacted specifically with a monoclonal antibody specific for glycoprotein D (gD) of HSV-1 (MoAb D4.2) and could inhibit its binding to gD, demonstrated by ELISA. The other, anti-id C, although prepared against a monoclonal antibody specific for glycoprotein C (gC) of HSV-1 (MoAb D4.1) reacted with MoAb D4.1, MoAb D4.2, and a monoclonal antibody specific for glycoprotein B of HSV-1 (MoAb D4.8). Furthermore, it could inhibit the binding of each monoclonal to its complementary protein epitope. Thus, anti-id C defined a crossreactive idiotope shared by MoAb D4.1, MoAb D4.2, and MoAb D4.8. Spleen cells from mice primed in vivo with either anti-id D or anti-id C underwent specific proliferation in vitro in response to HSV-1 antigens. Subsequently, intraperitoneally immunized mice were tested for a HSV-1 delayed type hypersensitivity (DTH) response. Neither anti-id C nor anti-id D sensitized mice for a HSV-1 DTH response, conversely, anti-id C immune mice were tolerized for a HSV-1 DTH response. This tolerization could be adoptively transferred to naive X-irradiated mice by splenic T cells, and was specific for HSV-1 DTH. Thus, DTH tolerized mice responded to vaccinia and HSV-2 challenge, while remaining tolerized for HSV-1 DTH. In addition, these animals demonstrated a form of split tolerance such that HSV antibody, CTL, and lymphoproliferative responses were detected in vitro. Thus, anti-id C induced a T cell population capable of specifically

suppressing the HSV-1 DTH response, mimicking the activity of intraperitoneal and intravenous immunization with HSV-1.

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CHAPTER I

INTRODUCTION

Literature Review

Investigations in several systems including viruses make it evident that antibodies can be generated against the combining site of other antibodies (Kennedy and Dreesman, 1985; Sacks et al., 1983). Such anti-idiotypic antibodies, as first theorized by Jerne (1974), may play important regulatory roles in immunity and, since their binding sites may mimic epitopes of antigens, provide alternative reagents as vaccines. With complex antigens, different epitopes may be instrumental in stimulating the various aspects of immunity (Adorini et al., 1979; Naor, 1983; Schwartz et al., 1976) and it is possible that vaccines composed of appropriate epitopes or anti-idiotypic antibodies represent a practical approach to manipulate the type of immunity elicited. Such approaches could be particularly useful in infections caused by herpes simplex virus where immunity is seldom total and frequent episodes of recrudescent disease occur (Shore and Nahmias, 1981). The latter are assumed to result from some change in immunoregulation and it is likely that immunization with appropriate anti-idiotypic reagents would be a valuable means of tailoring immunity to avoid or limit recurrent herpetic infections. The effectiveness of anti-id regulation of an immune response to viral antigens has been shown by other investigators (Kennedy and Dreesman, 1985). Kennedy et al. (1983) purified IgG from two individuals with antibodies to Hepatitis B surface antigen (anti-HbsAg). This IgG was made specific

for HBsAg by collecting eluent from a Sepharose B column coupled with HBsAg, then injected into rabbits. The rabbits produced anti-idiotypic antibodies reactive with anti-HBsAg (anti-id). After extensive purification of anti-id over an anti-HBsAg negative human IgG column, the binding of anti-id to anti-HBsAg could be inhibited by purified HBsAg showing a specificity of anti-id for the antigen-combining site of anti-HBsAg. Anti-HBsAg IgG or serum from 9 different individuals could also inhibit the binding of anti-id to anti-HBsAg, suggesting anti-id detected a common idiotype shared by all nine individuals. This common idiotype was also found in hyperimmune mammal sera, including mice, guinea pigs, rabbits, chimpanzees, swine, and goats, but not in chicken sera (Kennedy et al., 1983b).

The immunogenicity of this Hepatitis B anti-id was tested in mice using various intraperitoneal (IP) immunization procedures (Kennedy and Dreesman, 1984a; Kennedy et al., 1984b, 1984c, 1984e). The injection of anti-id 7 days before HBsAg injection or two anti-id injections 7 days apart increased the number of IgM and IgG producing cells in the spleen as compared to mice injected with normal rabbit IgG (Kennedy and Dreesman, 1984a). Anti-id immunization of mice was also shown to enhance the serum antibody response to HBsAg when alum-precipitated, but not soluble, anti-id was injected before HBsAg (Kennedy et al., 1984e). The anti-id injection was most effective when given 14 days before HBsAg at a dose between 5 and 200 ug. A similar enhancement in anti-HBsAg was observed when alum-precipitated anti-id immunization was followed by injection with a cyclic peptide of HBsAg (Kennedy et al., 1984c). This peptide could inhibit the binding of anti-id to

anti-HBsAg suggesting anti-id and the peptide represented a similar epitope on HBsAg (Kennedy and Dreesman, 1983a). Further experiments have shown that immunization with alum-precipitated anti-id when given as two injections 14 days apart can stimulate the production of anti-HBsAg serum antibodies in mice (Kennedy et al., 1984b). Since antibodies to HBsAg have been shown to confer protective immunity against Hepatitis B, Kennedy et al. (1984b) have suggested that anti-idiotype antibodies may prove to be useful vaccines against Hepatitis B infection.

In another viral system Koprowski and co-workers (Reagan et al., 1983) stimulated the production of neutralizing antibody by subcutaneous immunization of mice with rabies virus specific anti-idiotypic antibodies. Monoclonal antibodies specific for 5 different epitopes, 4 of which were viral neutralization epitopes, on glycoprotein G (G) of rabies virus were used to immunize rabbits to produce 5 different anti-ids. After extensive absorbtion over a Sepharose B column coupled with a monoclonal antibody of the same isotype but unrelated specificity, the anti-ids proved specific for their complementary monoclonal idiotype. Three of the five idiotype/anti-idiotype interactions could be inhibited by purified G, demonstrating an antigen combining site specificity. These anti-ids were subsequently used to immunize mice. Three immunizations of 40 ug of anti-id IgG emulsified in Freunds adjuvant were given on days 0, 7 and 32. Two of the anti-id immunizations produced antibody that neutralized rabies virus infectivity. Both of these anti-idiotypic antibodies demonstrated antigen-combining site specificity, the

complementary idiotype of one, however, was non-neutralizing, which is curious since the anti-id could stimulate the production of neutralizing antibody. In this system, like the Hepatitis system, anti-ids stimulated the production of protective antibodies and are therefore being considered as possible vaccine candidates (Reagan et al., 1983).

Fields and Greene have investigated the role of anti-idiotypic antibodies in the immune response to Reovirus type 3 (Reo-3) by examining reactions between anti-id and various target cells (Kauffman et al., 1983; Nepom et al., 1982; Noseworthy et al., 1983). The monoclonal anti-id was produced by injecting syngeneic mice with hybridoma cells that produce monoclonal antibodies reactive to Reo-3 hemagglutinin. This anti-hemagglutinin monoclonal antibody could neutralize Reo-3 infectivity and block Reo-3 specific cytotoxic T lymphocyte (CTL) killing of Reo-3 infected targets. The monoclonal anti-id could bind its complementary idiotype and this binding could be inhibited by Reo-3 hemagglutinin. Normal spleen cells were tested for the presence of receptors for anti-id. Fluorescent cell sorter analysis demonstrated that anti-id could bind 45% of the Lyt 1^+ 2^- cells and 15% of the Lyt 1^- 2^+ cells, suggesting the existence of a receptor and making the cells possibly subject to anti-id regulation (Noseworthy et al., 1983). Next, the ability of anti-id to bind and inhibit Reo-3 virion binding to several immune and non-immune cell lines was tested. Reo-3 bound to all 6 lines tested, but anti-id only bound and inhibited Reo-3 binding to 3 of the 6 lines, indicating that anti-id did not represent the only epitope of Reo-3 important in cell

binding (Kauffman et al., 1983). To further demonstrate that the anti-id idiotype was important in the Reo-3 immune response, the hybridoma cell line expressing the anti-id immunoglobulin on its surface was used as a target cell of Reo-3 primed cytototoxic T cells (CTL). These CTL's could lyse the anti-id hybridoma cells and this lysis could be inhibited by the complementary idiotypic antibody (Ertl et al., 1982), demonstrating the ability of the CTL to recognize an anti-idiotypic B-cell determinant. The above data indicate that there are receptors on the B and T cells of the immune system which may render them susceptible to anti-idiotypic antibody regulation. To demonstrate the immunoregulatory ability of this Reo-3 anti-id, mice were immunized subcutaneously with 100 ug of anti-id on day 0 and day 2 and challenged 6 days later in the footpad with Reo-3 (Sharpe et al., 1984). A delayed type hypersensitivity (DTH) response was detected in the footpad 12 to 28 hours following challenge. This DTH response could be transfered to naive mice with spleen cells from anti-id immunized mice suggesting a role in positive immune regulation for anti-idiotypic antibodies.

In the herpes virus system, there are two conflicting reports. Gell and Moss (1985) immunized mice with three anti-ids. These anti-ids were produced against two monoclonal antibodies with a type common specificity for glycoprotein D, and a HSV-1 specific monoclonal against glycoprotein H. Following 5 intradermal injections of 6-18 ug each, mice were primed for a HSV-1 delayed type hypersensitivity response measured in the ear. This immunization procedure, however, had no effect on viral pathogenesis. In comparison to control mice,

mice immunized with anti-id demonstrated no differences in their abilty to clear infectious HSV-1 from the pinna. Conversely, Dreesman and co-workers (Kennedy et al., 1983c; 1984d) have shown that intraperitoneal anti-id immunization can reduce the survival time of mice challenged with herpes simplex virus type 2 (HSV-2). In this study monoclonal IgM antibodies of undefined specificity against HSV-2 were used to immunize rabbits (Kennedy et al., 1983c). The anti-ids produced were purified over a normal mouse IgM Sepharose B column, then bound to and eluted from a complementary idiotype column. Two of the three anti-ids used bound to a cross-reactive idiotope found on both of the immunizing IgM monoclonals but not on 4 other anti-HSV-2 IgM monoclonals or normal mouse IgM. The binding of these anti-ids with their respective idiotypes could be inhibited partially by purified HSV-2 virions, implying some antigen-combining site specificity (Kennedy et al., 1983c). The purified anti-ids were subsequently used to immunize mice 14 days before challenge with a 50% lethal dose of HSV-2. Doses of 5 ug or 50 ug of individual or pooled anti-ids reduced mean survival time of challenged mice by 50%, as compared to mice immunized with normal rabbit IgG, but there was no detectable HSV-2 antibody production (Kennedy et al., 1984d). The results in this study indicate the induction of a suppressor circuit that regulates the anti-HSV-2 immune response.

It is not surprising that conflicting data resulted from the immunization of mice with viral anti-idiotypic antibodies, since discrepancies have been observed in other systems. Xenotypic and syngeneic anti-ids generated from antibodies specific for the organic

hapten 4-hydroxy-3-nitrophenyl-acetyl (NP) were shown to be both stimulatory (10-100 ng immunizations) and suppressogenic (10 ug immunization) for idiotope expression according to the dose of anti-id administered in vivo (Kelsoe et al., 1980; 1981).

A subclass specific effect was noted in xenotypic anti-ids generated from A5A, a recurrent Streptococcus A idiotype. IgG 2a antibodies could prime for idiotypic expression, whereas IgG 2b antibody immunization was suppressive (Eichmann, 1974). This observation was at first supported by an isologous monoclonal NP system (Reth et al., 1981), but later put into doubt by studies in the same NP system with isotype variant monoclonal antibodies (Radbruch, 1980). In this study, anti-id antibodies of the same variable region on different heavy-chain constant regions were used to immunize mice. Anti-idiotopes of the IgG 1, IgG 2a, and IgG 2b subclass all enhanced idiotope expression at nanogram doses and were suppressive at microgram doses. Since these preliminary findings are contradictory it is possible that the target idiotope may be as important to immune regulation as is the immunogen.

The route of immunization may also effect the outcome of anti-id prophylaxis. Mice immunized once subcutaneously (SC) with various amounts of rabbit or mouse monoclonal antibodies directed against the major cross-reactive idiotype (anti-CRI) on A/J anti-p-azobenzenearsonate (ABA) antibodies developed an ABA specific T cell-mediated delayed type hypersensitivity response (DTH) (Sy et al., 1980; Thomas et al., 1981). The priming was either given SC in two sites or four sites plus the footpad. Mice were challenged either in

the footpad or ear, respectively, 5 days later. Conversely, intraveneous (IV) administration of anti-id to mice in the ABA system results in the induction of T suppressor cells (Ts) capable of suppressing DTH and CTL response to ABA. This suppression has been observed using rabbit and mouse monoclonal anti-CRI antibodies (Sy et al., 1979; Thomas et al., 1983).

The T suppressor cells involved in the anti-id stimulated suppression of ABA DTH have been studied extensively. Thomas et al. (1983) have observed that immunizing A/J mice IV with 2 ug of monoclonal anti-id on 5 consecutive days stimulated spleen cells that suppressed an ABA specific response. The anti-id primed Ts mediating the DTH suppression could be adsorbed to ABA or anti-id coated dishes and their suppressor activity abrogated when treated with anti-Thy 1.2 antibody plus complement. Thus, anti-id primed DTH suppression resulted from a T cell with a receptor recognizing antigen and/or anti-id. Since binding of anti-id to Ts before adsorbing to ABA coated plates, blocked the adsorption of these Ts to ABA, the recognition of ABA and anti-id may be mediated through the same receptor. The ABA specific suppression after IV anti-id immunization was also observed in CBA mice which have undetectable levels of the CRI determinant on serum antibodies. Therefore, antibody idiotope expression was not a prerequisite for anti-id primed DTH suppression.

A similar ABA DTH and CTL suppression initiated by rabbit anti-CRI has been compared to suppression resulting from IV administration of ABA conjugated spleen cells (Monroe et al., 1985). Whereas, adoptive transfer of anti-CRI primed spleen cells could suppress a DTH or CTL

response in the efferent stage, ABA primed splenocytes could only suppress when present during the afferent phase of the DTH or CTL response. This type of efferent suppression was observed for 6 out of 7 rabbit anti-CRI preparations following IV immunization of A/J mice. The efferent anti-CRI suppression was shown to be mediated by a soluble T cell suppressor factor. The ability of this factor to transfer suppression to antigen primed mice was observed to be cyclophosphamide insensitive, Igh, but not MHC, restricted and antigen nonspecific. This activity pattern parallels the activity of Ts_3 which is produced in the Ts circuit after IV immunization of mice with ABA conjugated splenocytes. Consequently, IV immunization with anti-CRI appears to mimic the efferent phase of ABA induced DTH and CTL suppression.

Suppression has been observed in the herpes virus system, also, following IV immunization with virus or virus infected splenocytes. Intraveneous immunization in the HSV system results in a form of split tolerance in which virus specific DTH responses are suppressed but antibody production, CTL and spleen cell proliferative responses, as well as clearance of infectious virus, are unaffected (Nash, 1985). This DTH specific expression appears to be related to the route of immunization, since injection of virus by the intradermal (ID) or subcutaneous route results in a DTH response following virus challenge (Nash, 1985). However, virus injections by the ID and SC routes also resulted in the development of a suppressor B cell in draining lymph nodes (Nash and Gell, 1980). This cell was observed 2-3 weeks after HSV-1 injection and could transfer virus specific DTH suppression for up to 4 months. It was suggested that this suppression could be

mediated via idiotype-anti-idiotype reactions between B and T cells (Nash, 1985).

The DTH suppression investigated in the HSV model for split tolerance mentioned above is mediated by a T cell (Nash and Ashford, 1982). There appears to be two populations of suppressor cells induced. Seven days after intraveneous immunization of mice with HSV-infected splenocytes suppressor cells could be transferred to HSV-1 primed mice which inhibited both the afferent and efferent phases of the HSV DTH response. Conversely, intraveneous immunization with HSV virus particles stimulated cells only capable of suppressing the afferent phase of the HSV DTH response (Schrier et al., 1983). Nash and Gell (1983) have further defined the kinetics and cell phenotype of this HSV specific DTH suppression. T cells with the phenotype Thy 1.2^+, Lyt 1^+2^-, and IJ^+ are first detected in the spleen of Balb/c mice 7 days after IV immunization with a TK^- strain of HSV-1. These Ts are involved in afferent suppression of DTH. By 28 days post IV immunization, there is an additional population of Lyt 1^-2^+ suppressor cells which are resistant to cyclophosphamide treatment and appear to mediate lifelong suppression of the HSV-1 DTH response (Nash, 1985). T suppressors from both populations mediate a type specific HSV DTH suppression (Nash, 1985). Thus, Ts from HSV-1 IV immunized mice can suppress a HSV-1 DTH response, but have little effect on an HSV-2 DTH response. Since DTH is the only response compromised in this HSV split tolerance model, immunity against the herpes virus does exist in the IV immunized mice. This is in contrast to the ABA hapten system described, since IV immunization of mice with ABA-conjugated

splenocytes or anti-CRI results in depression of DTH and CTL responses (Monroe et al., 1985).

Rationale

Various methods of immune regulation by anti-idiotypic antibodies have been described. Anti-id immunization has the ability to enhance or suppress CTL, DTH, and antibody responses; all of these effects can alter the pathogenesis of a subsequent viral infection. The consequence of anti-id immunization can be related to route of immunization, the idiotope specificity of the anti-id, or the concentration of the immunogen. All the studies described involved the use of exogenous anti-id. However, auto-anti-idiotypic antibodies have been detected in the human system after antigen injection. Specifically, Geha has shown that 2 to 3 weeks following booster immunization with tetanus toxoid antigen, auto-anti-idiotypic antibodies are detectable in serum which react with anti-tetanus antibodies (Geha, 1982). Therefore, it is reasonable to believe anti-ids directed against specific immune cells are present during an antigen specific immune response and contribute to the regulation of that immune response. Similarly, aspects of the HSV immune response may be regulated by anti-idiotypic antibodies.

The exogenous use of anti-id sera has been described previously in the HSV system. Gell and Moss (1985) observed in mice a DTH stimulatory role for anti-ids directed against anti-glycoprotein antibodies. Conversely, after anti-id immunization Kennedy et al. (1984d) observed increased pathogenicity, as demonstrated by decreased

survival time after lethal challenge. These anti-ids were also directed against anti-glycoprotein antibodies (Kennedy et al., 1983c).

The pathogenesis of HSV is complex. After initial infection HSV recedes to the local ganglia, where it remains latent until reactivated by an external stimulus. Following reactivation, the virus replicates and travels down the ganglia returning to the area of the initial infection. The recrudescent disease which ensues is the result of viral replication and perhaps immunopathology (Shore and Nahmias, 1981). A reagent which could inhibit HSV replication and/or immunopathology could limit the disease caused by the virus.

The purpose of this project is to investigate the involvement of anti-idiotypic reagents in HSV pathogenesis. Since a stimulatory and a suppressive role have been demonstrated for HSV anti-ids (Gell and Moss, 1985; Kennedy et al., 1984d), it will be necessary to investigate positive and negative regulatory mechanisms resulting from anti-id immunization. One possible outcome of this research is the tailoring of a reagent which can alter the pathogenesis of HSV by inhibiting viral replication or immunopathology, thus affecting the outcome of infection.

Specific Aims

1. Anti-idiotypic antibodies will be produced in rabbits against two monoclonal antibodies specific for glycoprotein C (gC) and glycoprotein D (gD) of HSV-1.
2. The distribution of the gC and gD idiotypes in human, mouse and rabbit sera will be examined.

3. The binding specificities of the two anti-ids will be characterized.
4. The humoral immune response in mice following anti-id immunization will be monitored for virus specific antibodies.
5. The ability of the anti-ids to prime mice *in vivo* for a HSV lymphoproliferative response *in vitro* will be determined.
6. The *in vivo* immunoregulatory activity of the anti-ids will be investigated using a HSV DTH model.

CHAPTER II

PRODUCTION AND CHARACTERIZATION OF AN ANTI-IDIOTYPIC ANTIBODY SPECIFIC FOR A MONOCLONAL ANTIBODY TO GLYCOPROTEIN D OF HERPES SIMPLEX VIRUS

Introduction

Investigations in several systems including viruses make it evident that antibodies can be generated against the combining site of other antibodies (Kennedy and Dreesman, 1983a; Kennedy et al., 1983c; Nepom et al., 1982; Reagan et al., 1983; Sacks et al., 1983). Such anti-idiotypic antibodies, as first theorized by Jerne (1974), may play important regulatory roles in immunity and, since their binding sites may mimic epitopes of antigens, provide alternative reagents as vaccines. With complex antigens, different epitopes may be instrumental in stimulating the various aspects of immunity (Adorini et al., 1979; Naor, 1983) and it is possible that vaccines composed of appropriate epitopes or anti-idiotypic antibodies represent a practical approach to manipulate the type of immunity elicited. Such approaches could be particularly useful in infections caused by herpes simplex virus (HSV) where immunity is seldom total and frequent episodes of recrudescent disease may occur (Shore and Nahmias, 1981). The latter are assumed to result from some change in immunoregulation and it is likely that immunization with appropriate anti-idiotypic reagents would be a valuable means of tailoring immunity to avoid or limit recurrent herpetic infections. The present investigation describes the

production and characteristics of an anti-idiotypic reagent against a monoclonal anti-glycoprotein D antibody (anti-id D).

Material and Methods

Viruses, Cell Cultures, and Media

The KOS strain of HSV-1 and the 186 strain of HSV-2 were used throughout these studies. The production and titration of these virus stocks was previously described by Eberle and Courtney (1980). UV-inactivated virus was prepared by exposing 0.5 ml HSV stock virus to a germicidal lamp at a distance of 3 cm for two minutes. HEp-2, Vero, or MRC-5 cells were propagated at 37°C in Eagle's medium supplemented with glutamine, 0.075% $NaHCO_3$, 100 IU of penicillin, 100 ug of streptomycin, and 10% newborn calf serum, (HEp-2), or 10% fetal calf serum, (Vero and MRC-5). In most experiments cells were grown in 75 cm^2 flasks or roller bottles. After viral infection, medium with 2% serum was used. For immunofluorescence, cells were grown on glass coverslips in 60 mm plastic petri dishes at 37°C in a humidified atmosphere of 5% CO_2 in growth medium containing 0.225% $NaHCO_3$.

Monoclonal Antibodies

The technique of Kohler and Milstein (1975) as modified by Harmon et al. (1982) and Fleming et al. (1983) was followed. Balb/c mice, 6-8 weeks of age, were inoculated by intraperitoneal inoculation with approximately 10^4 plaque forming units (PFU) of HSV-1 strain McKrae which had been grown in rabbit kidney cells. Secondary immunization

with 10^4 PFU of virus was performed 5 weeks later by the intravenous (IV) route. Four days after IV immunization, splenocytes were fused with M5 cells and cultured in 24-well plates. Positive cells were selected by enzyme-linked immunosorbent assay (ELISA) on microtiter plates coated with HSV-1 infected Vero cell lysate (250 ug of protein/well), as previously described (Nesburn, Willey, and Trousdale, 1983). These cells were cloned by limiting dilution, expanded, and characterized. Monoclonal antibodies anti-gD (D4.2), anti-gC (D4.1) and anti-gB (D4.8) are subclassed as IgG2a, IgG2a, and IgG2b, respectively, as determined by Ouchterlony immunodiffusion using antibodies to murine immunoglobulin isotypes (Litton Bionetics, Charleston, SC). The antigenic specificity of these antibodies was determined by radioimmunoprecipitation (Eberle and Courtney, 1980). As shown in Figure 2.1, D4.1 and D4.8 precipitate glycoprotein C (gC) and glycoprotein B (gB), respectively, from HSV-1 infected cell extracts. Monoclonal antibody (MoAb) D4.2 will be described in the following section. Ascites fluid was produced by intraperitoneal (IP) injection of Balb/c mice with 1 x 10^7 hybridoma cells; 10-14 days later, ascites were collected in a centrifuge tube by IP insertion of an 18 gauge needle.

Antibody Purification

MoAb D4.2 and MoAb D4.1 were purified from ascites fluid by adsorption to a protein A-Sepharose 4B column (Pharmacia Fine Chemicals, Inc., New Jersey). The column was washed with 20 gel volumes of 0.1 M sodium phosphate buffer (PB), pH 8.4. Four volumes of

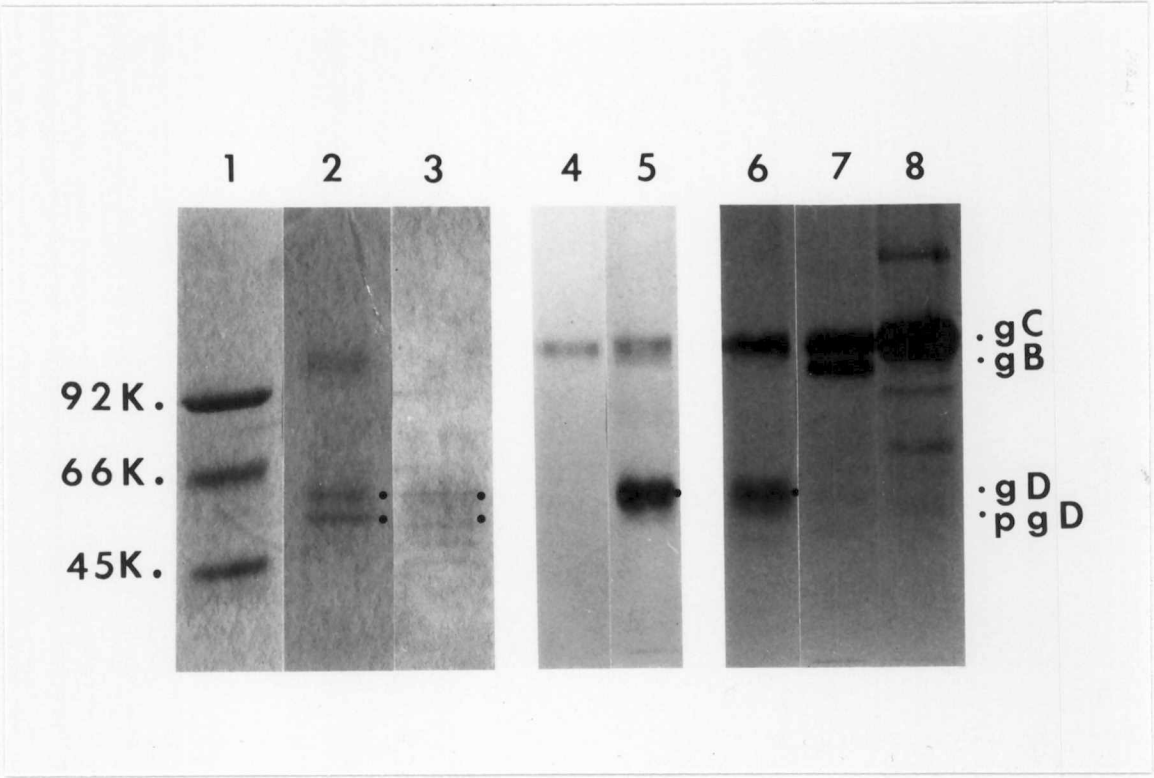

Figure 2.1. SDS-PAGE of purified proteins and immunoprecipitations. Lane 1, molecular weight standards; Lane 2, affinity purified native gD, 95 K protein represents gD dimer (Eisenberg et al., 1982); Lane 3, denatured gD after HTP chromatography. Lanes 1-3 were stained with coomassie blue. Lanes 4-8, immunoprecipitation of HSV-1 or HSV-2 infected, ^{3}H-glucosamine labeled MRC-5 cells after autoradiography. Lane 4, negative hybridoma tissue culture fluid with HSV-1; Lane 5, MoAb D4.2 with HSV-2; Lane 6, MoAb D4.2 with HSV-1; Lane 7, MoAb D4.8 with HSV-1; Lane 8, MoAb D4.1 with HSV-1.

ascites fluid plus one volume of 0.5 M PB was added to the column and the gel mixture was transferred to 15 ml centrifuge tube and agitated overnight at 4°C. The gel mixture was poured into the column and washed with 0.1 M PB until no absorbancy was detectable at 280 nm. Anti-gD was eluted by the addition of 0.1 M citrate buffer, pH 4.2, and immediately neutralized in 0.5 M PB (pH 9.6). The eluted immunoglobulin was concentrated by ultrafiltration against phosphate-buffered saline, pH 7.4 (PBS). Protein concentrations were determined by absorbance at 280 nm using an IgG standard curve.

Production of Anti-id D

Two New Zealand white rabbits were injected subcutaneously with 300 ug of protein A Sepharose-purified MoAb D4.2 emulsified in Freund's complete adjuvant. Two intramuscular boosters of 200 ug and 170 ug of IgG in Freund's incomplete adjuvant were given on days 7 and 14, respectively, and sera collected 7 days later. Anti-id D was absorbed by 2 passages over a Sepharose 4B column coupled with normal mouse sera (NMS). Effluent from the second passage was collected and concentrated by one 40% ammonium sulfate precipitation. After overnight dialysis against PBS at 4°C, the concentrate was passaged over a second column coupled with MoAb D4.1 IgG. Effluent from this column was passaged over a protein A-sepharose column and IgG eluted with 0.1 M citrate buffer (pH 2.5). As a final step anti-id D was bound to, then eluted from a column coupled with MoAb D4.2.

Antigen Preparation and Purification

ELISA cell extracts were prepared from MRC-5 or HEp-2 cells infected with a MOI of 50 PFU/cell of HSV-1 or HSV-2, respectively. After 24 hours, cells were washed three times with PBS, suspended at a concentration of 10^8 cells per ml in PBS, sonicated for 3 minutes, and stored at -70°C. Denatured gD was prepared by sodium dodecyl sulfate (SDS)-hydroxylapatite (HTP) chromatography as previously described (Eberle and Courtney, 1980). Presence of gD was determined by SDS-polyacrylamide gel electrophoresis (PAGE) (Powell and Courtney, 1975). Native gD was purified by affinity chromatography, similar to the method described by Eisenberg et al. (1982), with the following modifications. An HSV-1 infected HEp-2 cell lysate labeled with ^{3}H-glucosamine was mixed with Sepharose 4B with bound MoAb D4.2 and agitated overnight at 4°C. The column was poured and washed extensively with buffer containing 0.01 M Tris, 0.5 M NaCl, and 0.1% NP40 (pH 7.5). The gD was eluted with 0.1 M citrate-phosphate buffer, pH 2.75. This eluent was collected in 2.0 M Tris (pH 10.6), concentrated and dialyzed against PBS, pH 7.4, with an Amicon ultrafiltration cell. Purity was monitored by SDS-PAGE, followed by staining with Coomassie blue (Powell and Courtney, 1975) (Figure 2.1). The eluted gD was also tested for gB and gC contamination by ELISA. There was no reactivity with either MoAb D4.1 or MoAb D4.8 (data not shown).

ELISA

An enzyme-linked immunosorbent assay was used to characterize MoAb D4.2. Polyvinyl chloride microtiter plates (Dynatech Laboratories,

Alexandria, VA), were coated with 50 ul per well of specific antigens [purified gD or cell extracts diluted in carbonate buffer, pH 9.6 (Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g per liter H_2O)] overnight at 4°C. The concentration of each antigen and antisera was pre-determined by block titration against specific positive control sera (Voller et al., 1979). Plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T) between this and each successive step. PBS containing 3% gelatin (PBS-G) was added to each well (50 ul) and incubated one hour at 37°C. This was followed by 50 ul of MoAb 4.2 conjugated with peroxidase (Sigma, St. Louis, MO) (Voller et al., 1979) diluted 1:1250 in PBS-T for one hour at 37°C. Development with O-phenylene diamine (1 mg/ml) (Sigma) was allowed at room temperature for twenty minutes, then stopped with 2.5 M H_2SO_4 and read on a Fisher ELISA Reader. The titer was defined as the highest dilution of serum with an absorbance (488 nm) of at least 0.1 and 2 times greater than negative controls. ELISA specificities of anti-id D were determined by a similar assay except antibody (NMS, MoAb D4.1 or MoAb D4.2) was pre-coated on microtiter plates and PBS-G was followed by anti-id D, then goat anti-rabbit IgG peroxidase labeled serum (Cappel Laboratories, West Chester, PA). The ELISA used to determine gD purity contained the following modifications. MoAb D4.1 or MoAb D4.8 was added, after gD coated plates were incubated with PBS-G, followed by anti-mouse IgG peroxidase labeled serum (Cappel Laboratories).

Anti-id Inhibition ELISA

Polyvinyl chloride microtiter plates were incubated overnight at 4°C with 50 ul of a 1:200 dilution of native gD per well. This dilution and MoAb D4.2 dilution were determined by an ELISA block titration (Voller et al., 1979). Wells were then washed three times in PBS-T and coated with 50 ul of PBS-G and incubated at 37°C for one hour. Thirty minutes before addition to the above plate, 4-fold dilutions of anti-id D or preimmune serum in PBS-T were combined with equal volumes of a 1:1250 dilution of MoAb D4.2 conjugated with peroxidase and incubated at 37°C. The anti-id D/MoAb D4.2 (50 ul per well) was added to microtiter wells after washing with PBS-T and incubated one hour at 37°C. After washing, O-phenylene diamine (50 ul per well), diluted in 0.2 M citrate phosphate buffer with 0.03% H_2O_2, pH 6 (1 ml/ml), was added and development allowed at room temperature for 20 minutes. The reaction was stopped with 2.5 M H_2SO_4 and absorbance determined on a Fisher ELISA reader at 488 nm. Controls consisted of MoAb D4.2 preincubated with PBS-T and added to wells precoated with gD or carbonate buffer. Results are expressed as percent inhibition of MoAb D4.2 activity which was calculated as follows:

$$\frac{\text{(Absorbance of MoAb D4.2 Control)-(Absorbance of sample)}}{\text{Absorbance of MoAb D4.2 Control}} \times 100$$

where sample is either anti-id D or control serum. The serum idiotype inhibition assay contained the following modification. The test serum and MoAb D4.2 labeled with peroxidase were added to the gD coated microtiter plate without a thirty minute pre-incubation.

Immunoprecipitation

HSV-1 and HSV-2 cell lysates were prepared and labeled with ^{3}H-glucosamine as previously described (Eberle and Courtney, 1980). The lysate (20 ul) was combined with 20 ul of 1:5 dilution of ascites fluid and 100 ul of TNE buffer (0.15 M NaCl, 0.005 M EDTA, 0.05 M Tris, pH 7.4) with 0.1% Triton X-100 and incubated overnight at 4°C. Rabbit anti-mouse serum (Cappel Laboratories), diluted 1:250, was added for one hour at 37°C followed by 20 ul of Pansorbin (Calbiochem, San Diego, CA), for one hour at 37°C. Subsequently, each tube was centrifuged at 750 g for 10 minutes, aspirated, and 1 ml of cold TNE added. The precipitate was washed three times in TNE buffer, suspended in 30 ul of 2% SDS-2% B-mercaptoethanol-1M urea and incubated at 37°C for 20 minutes. Following boiling for 30 seconds, the precipitate was removed by centrifugation at 750 g for 5 minutes and the supernate was analyzed by SDS-PAGE. Resulting gels were treated with Enlightning (New England Nuclear, Boston, MA) for 20 minutes dryed and autoradiography performed.

Immunofluorescence

Membrane immunofluorescence was conducted as previously described with the following modifications (Eberle and Courtney, 1980). Nine hours after infection with HSV-1 or HSV-2, MRC-5 coverslips were fixed with 3.7% formalin in PBS, then reacted with normal goat serum. MoAb D4.2 ascites fluid or NMS diluted 1:5 was incubated with coverslips for 30 minutes followed by fluorescein-isothiocyanate-conjugated goat anti-mouse IgG serum for 30 minutes (Cappel Laboratories). Coverslips were then mounted in 50% glycerol and observed for fluorescence.

Neutralization

MoAb D4.2 ascites fluid was heated 45 minutes at 56°C to inactivate any complement then diluted in growth medium (2% serum). Next, 0.2 ml of each dilution was added to 0.2 ml of HSV-1 or HSV-2 virus stock diluted to 4×10^3 PFU per ml. Each virus/antibody mixture was incubated one hour at room temperature, then 0.1 ml was added per well to 6 well plates seeded with Vero cells. After adsorption for one hour at 37°C, the monolayers were overlayed with Eagle's medium supplemented with 2% fetal calf serum and 0.5% methyl cellulose. After 4 days incubation at 37°C in a humidified atmosphere of 5% CO_2, monolayers were stained with neutral red as previously described (Bone and Courtney, 1974).

Lymphocyte Proliferation

Spleen cells were processed essentially as described by Lawman et al. (1980). Four mice from each treatment gruop were killed by cervical dislocation and their spleens removed aseptically. Single-cell suspensions were prepared by teasing cells through 80-mesh sieves into Hank's balanced salt solution (HBSS). After one wash, erythrocytes were lysed by brief exposure to 0.83% NH_4Cl at 37°C. Cells were then washed in HBSS twice and the viable cells enumerated. The lymphocyte proliferation assay was performed as described by Horohov, Moore and Rouse (1985a). Splenocytes were cultured in RPMI-1640 containing 5% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 ug/ml), gentamicin (50 ug/ml), and 5×10^{-5} mM 2-mercaptoethanol.

Microcultures consisted of 4 x 10^5 cells in 0.2 ml of medium per well of a 96 well microtiter plate (Costar, Cambridge, MA). Cultures were incubated with UV-inactivated HSV at a multiplicity of infection of 1.0 PFU/cell, calculated before inactivation. On the fifth day of culture incorporation of tritiated thymidine ([^{3}H]-TdR) into cellular DNA was used as a measure of lymphocyte proliferation. Typically, 0.5 uCi of [^{3}H]-TdR (New England Nuclear) was added to the appropriate wells of the 96 well plate during the last 4 hours of incubation. The cells were then harvested onto glass fiber filter paper (Skatron Inc., Sterling, VA) with a semi-automated cell harvester (Flow Laboratories, McLean, VA). The filter papers, immersed in 0.5 ml of Scinti Verse E (Fisher Scientific Co., Louisville, KY) were counted in a Beckman LS 700 liquid scintillation spectrometer.

Mouse Immunizations

Six week old female Balb/c mice were obtained from Cumberland View Farms, Clinton, Tennessee. Four groups, anti-id IgG, preimmune IgG, HSV, and normal, were set up containing 4 mice per group. Mice immunized with IgG received 4 subcutaneous injections of either anti-id D IgG or preimmune IgG on days 0, 14, 35, and 51. These injections contained 10 ug, 5 ug, 10 ug, 5 ug of IgG, respectively, emulsified in complete Freund's adjuvant. HSV immune mice were injected with 1 x 10^7 PFU of KOS on day 0. Normal mice received no injection. All mice were sacrificed on day 58 and the lymphocyte proliferation assay performed. Before each injection and before sacrifice mice were bled from the retroorbital sinus and the sera tested by ELISA.

Statistical Analysis

The lymphoproliferation experiment in this paper included four treatment groups, consisting of four mice per group. Splenocytes from the four mice within each group were pooled and the assay performed using twelve replicates for each treatment group. Data from the four treatment groups were evaluated using analysis of variance and the Welsch Step-up procedure (Sokal and Rohlf, 1981).

Results

Characteristics of Monoclonal Antibody D4.2

The monoclonal antibody (D4.2) to glycoprotein D used to prepare anti-id D was selected because of its binding and neutralizing characteristics. Thus, MoAb D4.2 containing ascites fluid neutralized the infectivity of HSV-1 and HSV-2 to titers of 1×10^4 and 1×10^5, respectively (Table 2.1). Further analysis showed that MoAb D4.2 immunoprecipitated gD from HSV-1 and HSV-2 infected cell lysates (Figure 2.1, Table 2.1) and was reactive with both HSV-1 and HSV-2 infected cell lysates as demonstrated by ELISA (Table 2.1). However, the gD epitope was present only on affinity purified native gD, not gD denatured by SDS and dithiothreitol. Thus, MoAb D4.2 bound to ELISA plates coated with native gD but not denatured gD (Table 2.1). The above data indicate that the gD epitope bound by MoAb D4.2 may be a conformational determinant that is involved in the infectious process, since the binding of MoAb D4.2 neutralizes viral infectivity.

By means of immunofluorescence, MoAb D4.2 was shown to bind to the surface of MRC-5 cells infected with HSV-1 or HSV-2 (Table 2.1). This

Table 2.1. Characteristics of Monoclonal Antibody D4.2

Test Evaluated	Virus Type	
	HSV-1	HSV-2
ELISA Titers[b]		
Infected cell extract	6,075	675
Native gD	>54,675	NT[a]
Denatured gD	<25	NT
Neutralization titer[c]	10,000	100,000
Immunoprecipitation[d]	gD	gD
Immunofluorescence[e]	Positive	Positive

[a]NT, not tested.

[b]Determined by standard ELISA procedure. Plates were coated with an optimum dilution of HSV-1 or HSV-2 infected cell extracts, native gD, or denatured gD. Titers were determined as the reciprocal of the dilution with an absorbance (488 nm) of at least 0.1 and 2-fold above negative control values.

[c]The endpoint was determined as the highest antibody dilution which reduced the number of plaques by 50%.

[d]HSV-1 or HSV-2 infected MRC-5 cell extracts labeled with ^{3}H-glucosamine were reacted with MoAb D4.2 and then analyzed by SDS-PAGE (Figure 2.1).

[e]HSV-1 or HSV-2 infected MRC-5 cells were reacted with MoAb D4.2 ascites followed by fluorescein-conjugated anti-mouse serum.

indicated that the epitope of gD under study was accessible on the cell surface. We sought to determine if this gD epitope induced antibody production as the result of an acquired infection or hyperimmunization with HSV-1 or HSV-2. To determine the presence of such antibodies in various sera, an ELISA inhibition approach was used which measured the inhibition of binding of MoAb D4.2 to gD. With one exception human, rabbit, and mouse (BALB/c and C3H/HeJ) sera which were positive by ELISA for HSV antibodies could inhibit the binding of MoAb D4.2 to gD (Table 2.2). None of the seronegative samples tested inhibited the binding of MoAb D4.2 to gD (Table 2.2). Consequently, our results show that the gD epitope was immunogenic in all species tested and, moreover, that antibodies with a binding specificity similar to that of MoAb D4.2 (perhaps a similar paratope) are produced in response to an infection by HSV.

Generation and Activity of Anti-idiotypic Antiserum

The IgG fraction of MoAb D4.2, purified by adsorption to and elution from a protein A-sepharose column, was used to immunize rabbits. After three injections, antiserum was collected and absorbed with normal mouse serum and MoAb D4.1. The latter antibody was of the same isotype and allotype as MoAb D4.2. The absorption procedure was done to remove antibody activity to constant region determinants and was monitored by assaying with ELISA plates coated with NMS, MoAb D4.2, and MoAb D4.1. As shown in Figure 2.2, MoAb D4.2 binding activity was retained while activity against NMS was eliminated. The residual activity against MoAb D4.1 could be explained either by incomplete

Table 2.2. Inhibition of Binding of MoAb D4.2 to gD by Anti-HSV Sera of Different Origins

Sera Tested[a]	Positive Inhibition[b]
Human	
HSV Seropositive	14/14
HSV Seronegative	0/10
Rabbit	
HSV-1 Immune[c]	4/4
HSV-2 Immune[d]	3/4
HSV Normal	0/3
Mouse	
BALB/c	
HSV Immune	5/5
HSV Normal	0/5
C3H/HeJ	
HSV Immune	6/6
HSV Normal	0/6

[a]Determined by standard ELISA procedure. Plates were coated with optimum dilution of HSV-1 infected cell extracts, followed by test serum, then anti-species IgG conjugated with peroxidase.

[b]Determined by inhibition ELISA procedure. Plates were coated with optimum dilution of affinity purified gD, followed by test serum and a pretitrated amount of MoAb D4.2 conjugated with peroxidase. Absorbances were measured at 488 nm and percent inhibition calculated as described in Material and Methods. Values of 30% or greater were considered positive. Positive values ranged from 86% to 100% inhibition. Negative values ranged from 0%-27% inhibition.

[c]Rabbits immunized with HSV-1 antigen only.

[d]Rabbits immunized with HSV-2 antigen only.

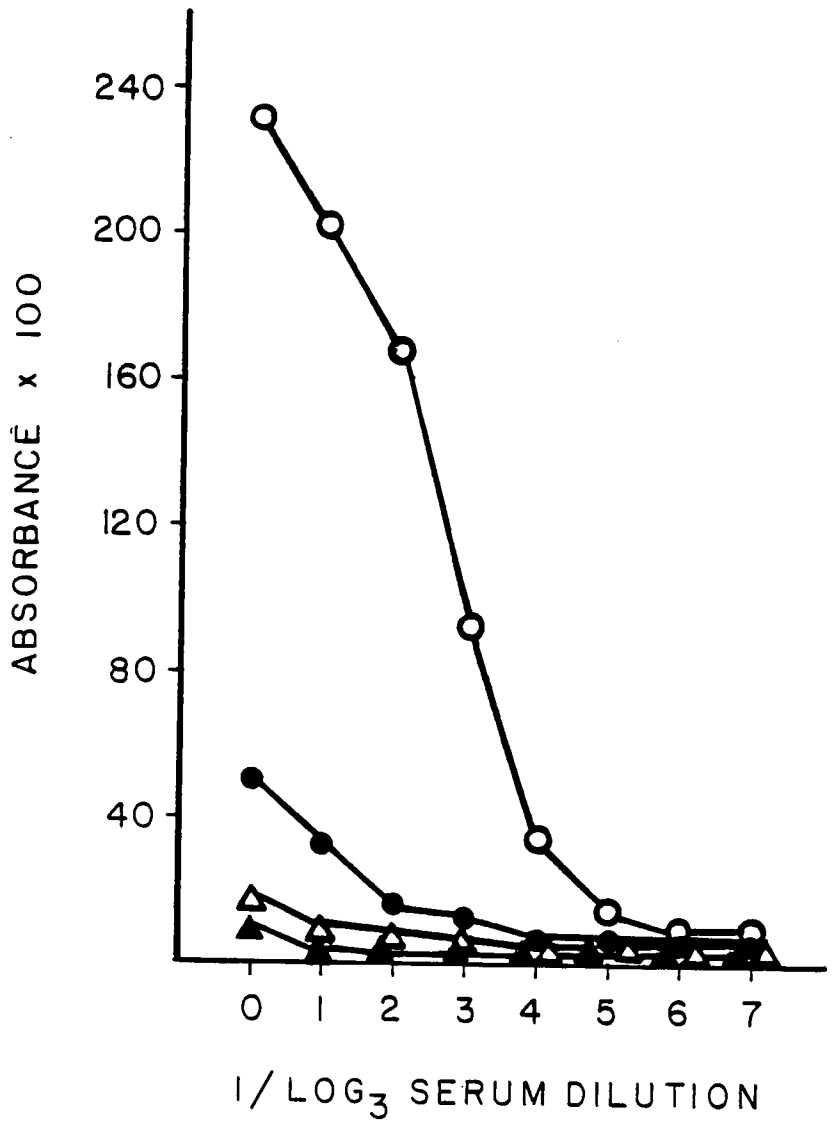

Figure 2.2. Titration of anti-id D using MoAb D4.2, MoAb D4.1, and normal mouse serum. MoAb D4.2 (O), MoAb D4.1 (●), or NMS (Δ), 0.8 ug per well; or carbonate buffer (▲) were absorbed to wells of microtiter plate. After absorption on NMS and MoAb D4.1 columns, serial 3-fold dilutions of anti-id D (initial concentration 5 ug) were added, followed by peroxidase labeled goat anti-rabbit antibody. Absorbances were determined at 488 nm.

absorption or by the presence of cross-reactive idiotypic determinants shared by MoAb D4.2 and MoAb D4.1.

In order to demonstrate that anti-id D would recognize and bind the antigen-combining site of MoAb D4.2, an ELISA inhibition assay was used in which the ability of anti-id D to inhibit the binding of affinity purified native gD to MoAb D4.2 was measured. Anti-id D preincubated with MoAb D4.2 totally inhibited the binding of MoAb D4.2 to gD (Figure 2.3), but preimmune rabbit serum, MoAb D4.1, or normal mouse serum had no effect on the MoAb D4.2/gD binding. Anti-id D was also assayed by ELISA against gD to assure that the inhibition was not the result of anti-id D binding directly to gD. Anti-id D showed no reactivity with gD (data not shown).

To better characterize the binding of anti-id D with MoAb D4.2, MoAb D4.2 was allowed to bind to a gD coated ELISA plate, then anti-id D was added, followed by anti-rabbit IgG labeled with peroxidase. Anti-id D was unable to bind MoAb D4.2 if the paratope was occupied by a gD epitope. Under these conditions MoAb D4.2 remained bound to gD (Figure 2.4). These data, together with the inhibition data presented above, imply that anti-id D is binding within the paratope of MoAb D4.2 and may mimic the epitope on gD recognized by MoAb D4.2.

If anti-id D does mimic an epitope of gD, then immunization with anti-id should stimulate a virus specific immune response. To investigate this possibility, mice were immunized with either anti-id D IgG, pre-immune IgG, or HSV. Eight weeks after the initial injection, spleen cells from mice in each group were tested <u>in vitro</u> for their ability to proliferate in response to HSV. Positive antigen specific

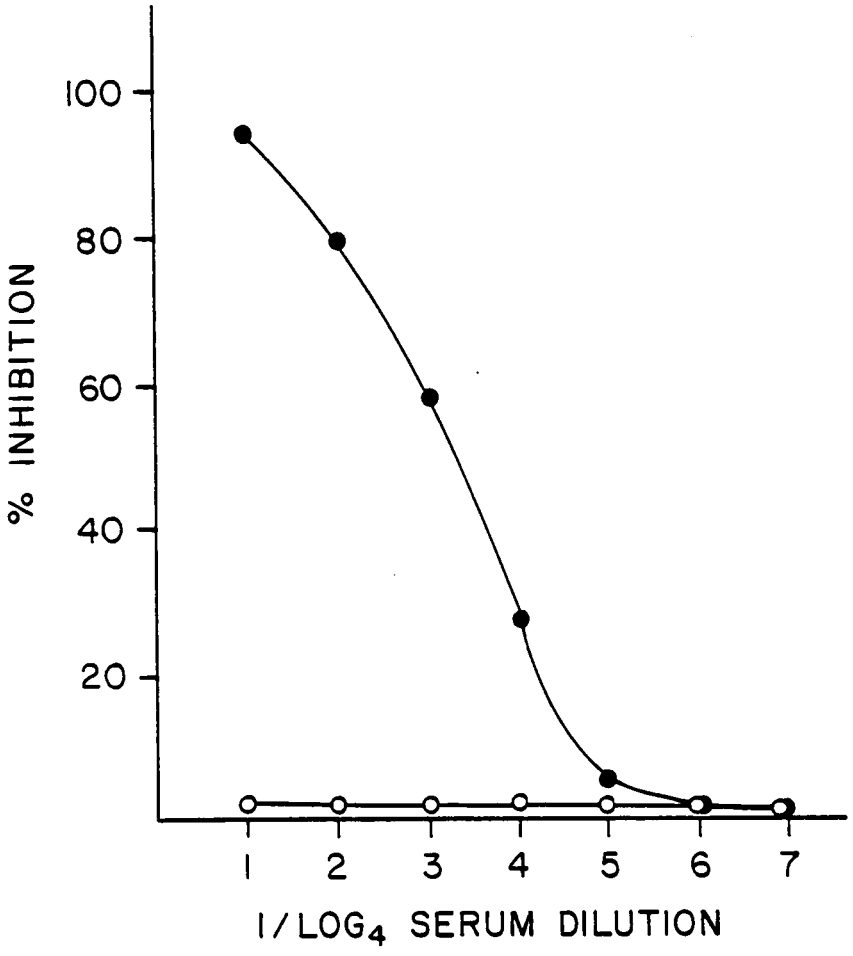

Figure 2.3. Inhibition of the binding of MoAb D4.2 to gD by anti-id D. A pretitrated amount of MoAb D4.2 conjugated with peroxidase was incubated with 4-fold serial dilutions of anti-id D (●) or control sera (○) (see below) with an initial protein concentration of 20 ug per well. After 30 minutes, this mixture was added to microtiter plates coated with gD. Absorbances were measured at 488 nm and percent inhibition calculated as described in Material and Methods. Control sera consisted of pre-immune rabbit serum, MoAb D4.1 IgG, and normal mouse IgG.

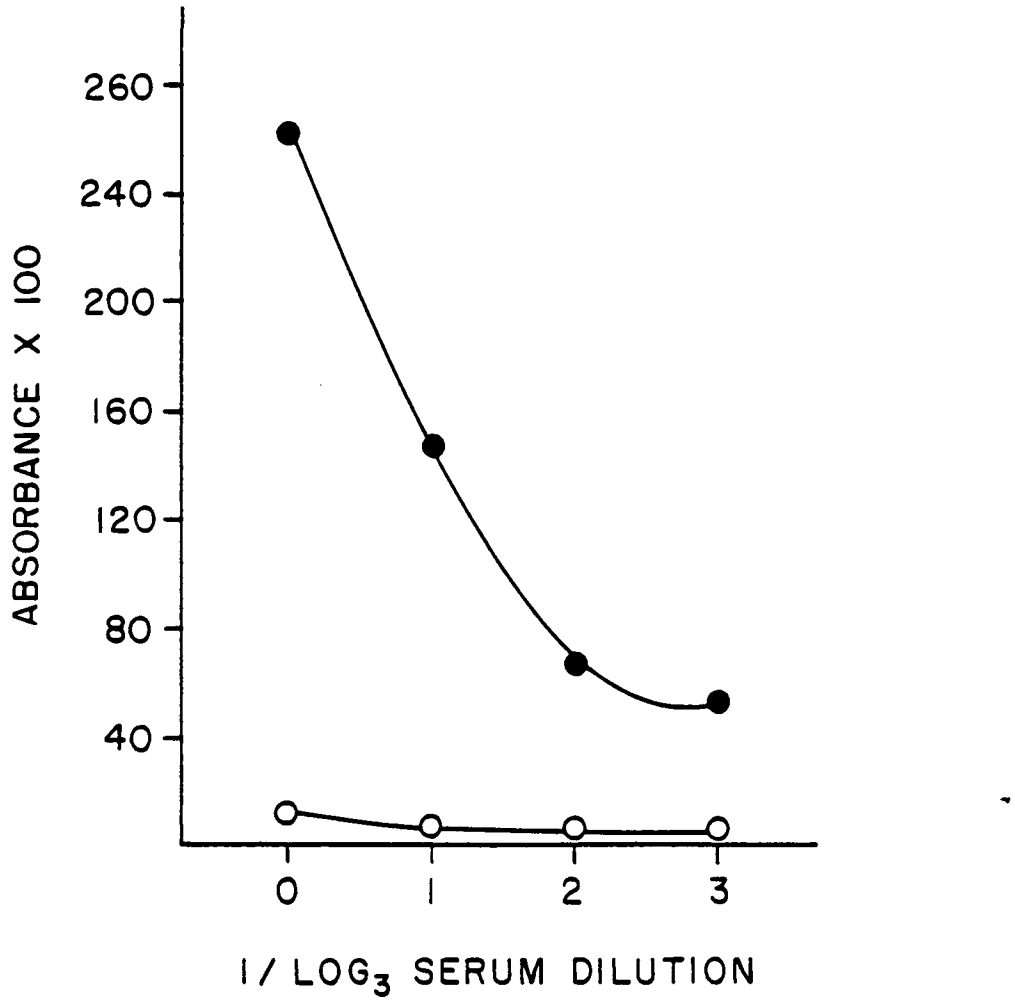

Figure 2.4. Inhibition of anti-id D binding to MoAb D4.2 by MoAb D4.2 binding gD. An optimal dilution of gD was absorbed to wells of a microtiter plate. MoAb D4.2 diluted 1:100 was allowed to bind gD. After one hour at 37°C various dilutions of anti-id D (O), initial dilution 5 ug/ml were added followed an hour later by anti-rabbit IgG conjugated with peroxidase. As a control MoAb D4.2 was absorbed to wells of a microtiter plate. Anti-id D was added directly to MoAb D4.2 followed by anti-rabbit IgG conjugated with peroxidase (●). Absorbances were measured at 488 nm. In this same experiment anti-gD, diluted 1:100, bound to gD was measured with anti-mouse IgG conjugated with peroxidase. This absorbance (x100) at 488 nm was 47, demonstrating MoAb D4.2 was bound to the gD absorbed to the plate.

proliferative responses were obtained with both the HSV and anti-id stimulated groups. The level of proliferation detected in the anti-id immunized mice was 72% of that obtained with the virus stimulated animals. This level of stimulation was significantly increased over that observed in nonimmunized or pre-immune IgG immunized mice (Figure 2.5). Although immunization with anti-id D primed spleen cells for HSV specific lymphoproliferation in vitro, virus specific or gD specific antibodies could not be detected either by ELISA or by viral neutralization.

Discussion

We have developed an anti-idiotypic antibody in rabbits against a mouse monoclonal anti-glycoprotein D (MoAb D4.2) of herpes simplex virus. Our results indicate that the anti-id was reacting with the paratope of the monoclone and, in addition, may be mimicking an epitope found on gD. Thus, binding of anti-id to the monoclone inhibited the subsequent reaction with gD. We interpret this to mean that both anti-id and gD bind to the same region of MoAb D4.2 (i.e. the paratope). However, other explanations include the induction of a conformational change in MoAb D4.2 after anti-id binds to an idiotopic determinant, this would affect paratopic conformation. Another possibility is that the binding of anti-id to the monoclone creates a steric hindrance which prevents gD from associating with the antigen-combining site of MoAb D4.2. This explanation was considered unlikely since binding anti-gD to the glycoprotein antigen (on a solid phase) prohibited subsequent binding by anti-id.

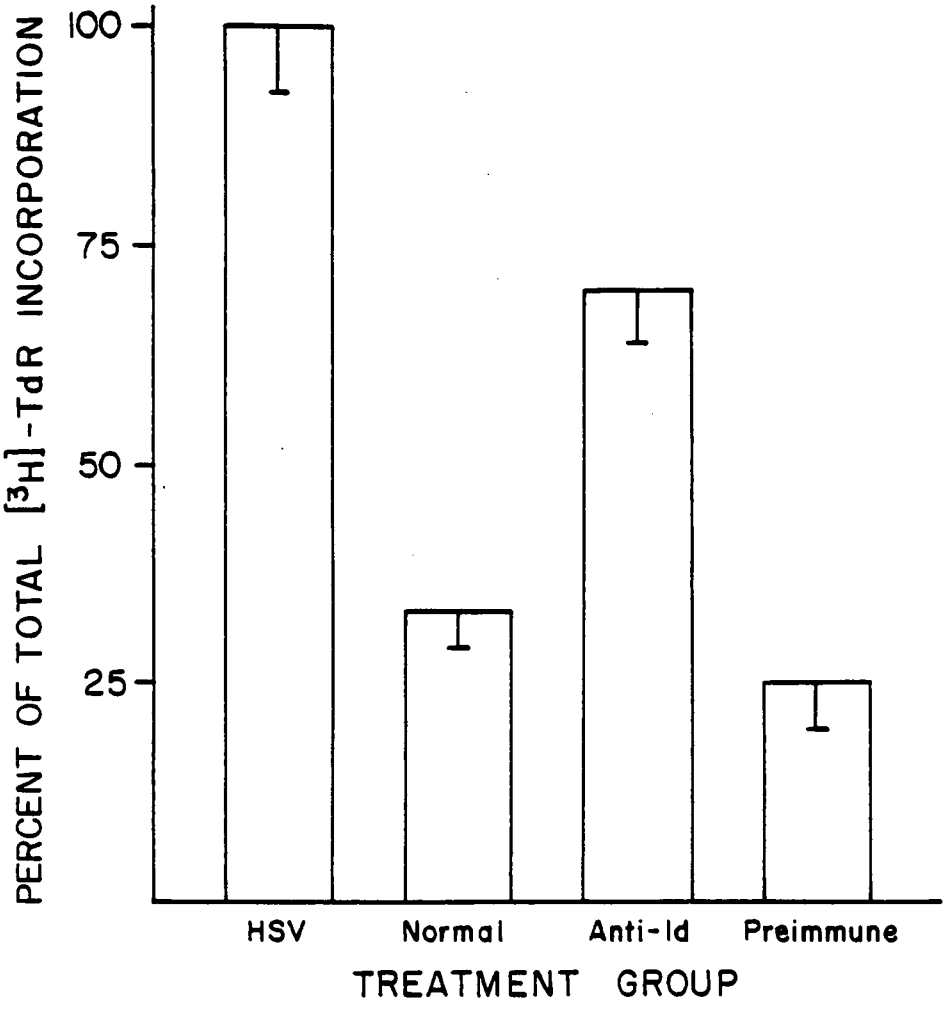

Figure 2.5. In vitro proliferation of anti-id D primed spleen cells in response to HSV antigen. Splenocytes from mice immunized with HSV, anti-id D IgG, pre-immune IgG, or nonimmunized mice were incubated with UV-inactivated HSV. On the fifth day the cells were tested for [^{3}H]-TdR incorporation. Each treatment group consisted of four mice and the results represent the mean for twelve replicates. The 100% value is based on the [^{3}H]-TdR incorporated by the HSV immune splenocytes (1768.2 $\pm$ 175.5 CPM). The differences among the four groups were shown to be significant by analysis of variance at $p < 0.001$. The [^{3}H]-TdR incorporation by anti-id D primed splenocytes (1274.4 $\pm$ 138.2 cpm) was significantly different from both pre-immune IgG primed splenocytes (433.2 $\pm$ 78.1 cpm) and unprimed normal splenocytes (577.3 $\pm$ 59.6) as determined by a Welsch Step-up procedure ($p < 0.05$). Spleen cells from immune BALB/c mice unstimulated with antigen in vitro have demonstrated levels of [^{3}H]-TdR incorporation similar to that shown here for both normal and pre-immune IgG primed spleen cells which were in vitro stimulated with antigen (data not shown).

In many systems previously studied, paratope-binding anti-id reagents, mimicking epitopes on appropriate antigens, could be substituted for antigen to induce antigen specific immune responses in vivo (reviewed in Kennedy and Dreesman, 1985). Following immunization of mice with our heterologous anti-id, animals became primed to generate a subsequent virus-specific in vitro lymphoproliferative response indicating that the anti-id possessed antigen mimicking properties. However, we failed to demonstrate anti-HSV or anti-gD antibody production in the anti-id immunized mice. This failure to induce detectable antibodies in vivo could mean that the mimicking epitope was not recognized by B cells. Alternatively, B cell recognition could have occurred but such cells failed to expand and produce antibody in vivo, perhaps because of inadequate stimulus or because of simultaneous induction of regulating suppressor mechanisms. In hapten systems anti-id immunization may not stimulate specific antibody production but does sensitize for a subsequent secondary response to antigen (Sacks et al., 1983). It is also possible that in vivo immunization with anti-id may activate B cells but such cells fail to fully differentiate unless further activated in vitro by antigen or mitogen. We have preliminary evidence that this may be occurring in our system. Thus in some cases we have observed by limiting dilution analysis of gD specific B cell precursors, an expansion in frequency following anti-id immunization (Lathey and Rouse, unpublished observations). It remains to be seen if suppressor cells are induced which regulate the in vivo differentiation of B and T cell reactions with gD.

It is important to ascertain if anti-id immunization affects the outcome of subsequent infection by HSV. Studies done by others in the HSV system have not produced protective responses. Thus, Dreesman and co-workers using anti-ids to IgM monoclonal antibodies to HSV-2, showed that recipients of the immunization became more susceptible to subsequent HSV-2 infection (Kennedy et al., 1984d). Such studies could imply that the anti-ids mimic suppressogenic determinants on viral antigens. In contrast, in the rabies virus system, anti-ids against monoclonal anti-glycoprotein G antibodies induced protective immunity (Reagan et al., 1983). Against rabies, as well as against Hepatitis B virus, anti-ids are considered possible vaccine candidates (Reagan et al., 1983; Kennedy et al., 1984b).

With respect to HSV, it is likely that an anti-id reagent produced against MoAb D4.2 will be a useful vaccine candidate to increase protective immunity. Thus, immunization with gD and passive administration of anti-gD monoclonal antibodies induce protection against subsequent challenge by both HSV-1 and HSV-2 (Balachandran et al., 1982; Dix et al., 1981; Long et al., 1984; Schrier et al., 1983). Furthermore, immunization with gD and a synthetic peptide prepared from gD induced neutralizing antibody (Cohen et al., 1984; Eisenberg et al., 1982). Since our anti-id reagent may mimic a conformational determinant on gD, expressed at the infected cell surface, seemingly involved in viral neutralization, and able to stimulate antibody production in humans, rabbits, and mice, we consider anti-id D a likely candidate for inducing protective immunity in vivo. Studies are

currently being conducted to investigate the specific immunomodulatory effect of anti-id D administered *in vivo*.

CHAPTER III

PRODUCTION, BINDING CHARACTERISTICS, AND IMMUNOGENICITY OF HETEROLOGOUS ANTI-IDIOTYPIC ANTIBODY TO HERPES SIMPLEX VIRUS GLYCOPROTEIN C

Introduction

Immunization with anti-idiotypic reagents may mimic the response to antigen (reviewed in Kennedy and Dreesman, 1985; Sacks et al., 1983; Rajewsky and Takemori, 1983). This form of immunization represents an alternative to traditional vaccines used against infectious agents. The outcome of anti-id immunization has been variable both in terms of the type of immune response induced and the influence on immune status. In some instances, responses to anti-id immunization have protected against challenge infection, whereas, in others heightened susceptibility occurred as reported for mice immunized with an anti-id derived with a monoclonal antibody to herpes simplex virus (HSV) (Kennedy et al., 1984d). Although anti-id against HSV monoclonals has elicited HSV specific cell-mediated immunity, these were of insufficient magnitude to influence the outcome of infection (Gell and Moss, 1985; Lathey et al., 1986). The factors responsible for the varied response to anti-id immunization are not fully understood, but the fine specificity of anti-id as well as the dose used for immunization play a role in some systems (Sacks et al., 1983; Rajewsky and Takemori, 1983). Ideally, anti-idiotypic reagents would be most useful as vaccines if they could be used to induce a desired type of

immune response. For example, against recrudescent HSV infection (Shore and Nahmias, 1981) this could be a T-helper and T-cytotoxic response without the concommitant induction of suppressor cells.

As a prelude to the development of anti-idiotypic reagents which may prove useful to tailor immunity to HSV in vivo, we have developed heterologous anti-idiotypic antibodies (Chapter 2). In this report we have characterized the binding activity of one such reagent prepared against a monoclonal antibody to glycoprotein C. Unexpectedly anti-id C also cross-reacted in vitro with monoclonal antibodies specific for glycoprotein B and glycoprotein D. Immunization of mice with anti-id C sensitized spleen cells to proliferate in response to HSV antigen in vitro. The cross-reactive nature of the idiotope recognized by anti-id C may indicate its overall importance in the immune regulation of the HSV system.

Materials and Methods

Monoclonal Antibodies and Antisera

The production, purification, and specificities of monoclonal antibodies (MoAb) D4.1, D4.2, and D4.8 were described elsewhere (Chapter 2). Monoclonal antibody D4.1 is of the IgG2a subclass and recognizes glycoprotein C (gC) of herpes simplex virus (HSV); D4.2 (subclass IgG2a) recognizes glycoprotein D (gD) of HSV; and D4.8 (subclass IgG2b) recognizes glycoprotein B (gB) of HSV. The ELISA, immunoprecipitation, immunofluorescence, and neutralization procedures used to characterize D4.1 were described elsewhere (Chapter 2).

Monoclonal antibodies D4.12, D4.11, and D4.25, specific for HSV and products of the same fusion were obtained from Dr. Dru Wiley, City of Hope Medical Center, Duarte, California. Several monoclonal antibodies (C3, C4, C8, C11, C12, C13, D2, D4, B3, B4, B6, and B9) which were specific for HSV glycoproteins were obtained from Dr. Joseph C. Glorioso, University of Michigan, Ann Arbor, Michigan. Other monoclonals specific for HSV (G83, D8A3, and H7E) were received from Dr. Robert Lausch, University of South Alabama, Mobile, Alabama. The anti-Thy 1.2 monoclonal, HO13.4 was from Dr. Mark E. Greene, Tufts University, Boston, Massachusetts. Anti H-$2K^k$ (11-4.1) was purchased from the American Type Culture Collection, Rockville, Maryland. The influenza specific monoclonal antibodies (78/2, 55/4, 219/3, 69/1, 125/5, 96/4, 11/4, 199/4, 171/5, 26/4, 78/6, 19/2, 139/1, 86/3, 83/2, 274/4) were received from Dr. Robert G. Webster, St. Jude's Children's Hospital, Memphis, Tennessee.

Human HSV sera used in this study were donated by volunteers with a history of HSV infection. These sera were not differentiated between HSV-1 and HSV-2 antibody positive. Control sera were from volunteers with no history of HSV infection. Mice and rabbit sera were obtained by hyperimmunization with either live HSV-1 or HSV-2. Control sera consisted of pre-immune mouse or rabbit sera.

Viruses

The KOS strain of HSV-1 and the 186 strain of HSV-2 were used throughout these studies. The production and titration of these stocks was previously described by Eberle and Courtney (1980). UV-inactivated

virus was prepared by exposing 0.5 ml HSV stock virus to a germicidal lamp at a distance of 3 cm for two minutes. This procedure reduced infectivity titers from 10^8 to 10^1 pfu/ml.

Affinity Chromatography

MoAb D4.1, MoAb D4.2, MoAb D4.8, and normal mouse immunoglobulin G (IgG) were individually bound to cyanogen-bromide activated Sepharose 4B (Pharmacia Fine Chemicals, Inc., New Jersey). Anti-id C was added to the gel mixture and agitated overnight at 4°C. Subsequently, the column was washed with 0.1M Tris HCl-0.5 M NaCl buffer, pH 7.6, followed by elution with 0.1M citrate buffer, pH 2.5. The eluted immunoglobulin was neutralized in 2 M Tris, then concentrated by ultrafiltration against phosphate-buffered saline, pH 7.4 (PBS). Protein concentrations were determined by Bio Rad protein assay (Bio Rad, Rockville Centre, NY).

Production of Anti-id C

Two New Zealand white rabbits were injected subcutaneously with 300 ug of protein A Sepharose-purified MoAb D4.1 emulsified in Freund's complete adjuvant. Two intramuscular boosters of 200 ug of IgG in Freund's incomplete adjuvant were given on days 14 and 28, respectively, and sera were collected 6 days later. Anti-id C was absorbed by 3 passages over a Sepharose 4B column coupled with normal mouse IgG. The effluent from the third passage was bound to a second column coupled with MoAb D4.1 IgG and eluted with 0.1 M citrate buffer, pH 2.5. Eluent was neutralized in 2 M Tris, then concentrated by ultrafiltration against PBS.

ELISA

Details of the method used were described previously (Chapter 2). Briefly, for the anti-id inhibition ELISA, polyvinyl chloride microtiter plates were coated with affinity purified gC, gD, or gB. Thirty minutes before addition to the above plate, 4-fold dilutions of anti-id C or pre-immune serum were combined with equal volumes of MoAb D4.1 conjugated with peroxidase, MoAb D4.2 conjugated with peroxidase, or MoAb D4.8. After a one hour incubation, plates were developed with O-phenylene diamine (MoAb 4.8 was first reacted with anti-mouse IgG conjugated with peroxidase). The reaction was stopped with 2.5M H_2SO_4 and absorbance determined on a Bio Tek ELISA reader at 490 nm. Results are expressed as percent inhibition of MoAb activity which was calculated as follows:

$$\frac{\text{(Absorbance of MoAb Control) - (Absorbance of sample)}}{\text{Absorbance of MoAb control}} \times 100$$

where sample is either anti-id C or control serum. The serum idiotype inhibition assay contained the following modification. The test serum and MoAb D4.1 labeled with peroxidase were added to gC coated microtiter plates without a thirty minute pre-incubation.

Mouse Immunizations

Six week old male C3H/HeJ mice were obtained from the University of Tennessee Medical Research Hospital. Four groups, anti-id C IgG, preimmune IgG, HSV, and normal were used for the lymphoproliferation assay. This was performed on splenocyte cultures from groups of 2 mice using methods described elsewhere (Chapter 2). Mice immunized with IgG received 4 intraperatoneal (IP) injections of either anti-id C IgG or

preimmune IgG on days 0, 13, 28, and 54. These injections contained 4 ug, 4 ug, 4 ug, and 10 ug of IgG, respectively, diluted in PBS and heated for 30 minutes at 56°C before injection. HSV immune mice were injected with 4 x 10^6 PFU of KOS on day 0. Normal mice received no injection. Mice were bled from the retroorbital sinus on day 63, then sacrificed and spleens removed for the lymphoproliferation assay.

Results

Characteristics of Monoclonal Antibody D4.1

The monoclonal antibody (D4.1) to glycoprotein C used to prepare anti-id C was selected because of its type-specific binding characteristics. Monoclonal antibody D4.1 immunopreciptated gC from HSV-1, but not HSV-2 infected cell lysates and was reactive by ELISA with only HSV-1 cell lysates. By means of immunofluorescence, MoAb D4.1 was shown to bind to the surface of HSV-1 infected HEp-2 cells but not to cells infected with HSV-2. However, the type 1 specific gC epitope bound by MoAb D4.1 was not a neutralizing epitope, since preincubation of HSV with MoAb D4.1 failed to neutralize the infectivity for Vero cells (Table 3.1).

To determine if the gC epitope recognized by MoAb D4.1 induced antibody production, human anti-HSV sera and sera collected from rabbits and mice hyperimmunized with HSV-1 or HSV-2 were tested for the presence of antibodies which bound the same epitope as MoAb D4.1. An ELISA inhibition approach was used which measured the ability of these sera to inhibit the binding of MoAb D4.1 to gC. Human, rabbit, and

Table 3.1. Characteristics of Monoclonal Anti-gC (D4.1)

Test evaluated	Virus type	
	HSV-1	HSV-2
ELISA Titers[b]		
Infected cell extract	≥2700	<100
Native gC	900	NT[a]
Neutralization titer[c]	<10	NT
Immunoprecipitation[d]	gC	Negative
Surface immunofluorescence[e]	Positive	Negative

[a]NT, not tested.

[b]Determined by standard ELISA procedure. Plates were coated with an optimum dilution of HSV-1 or HSV-2 infected cell extracts, or native gC. Titers were determined as the reciprocal of the dilution with an absorbance (490 nm) of at least 0.1 and 2-fold above negative control values.

[c]The endpoint was determined as the highest antibody dilution which reduced the number of plaques by 50%.

[d]HSV-1 or HSV-2 infected MRC-5 cell extracts labeled with ^{3}H glucosamine were reacted with MoAb D4.1 and then analyzed by SDS-PAGE.

[e]HSV-1 or HSV-2 infected HEp-2 cells were reacted with MoAb D4.1 ascites followed by fluorescein-conjugated anti-mouse serum.

mouse (BALB/c and C3H/HeJ) sera, anti-HSV-1 antibody positive by ELISA inhibited the binding of MoAb D4.1 to gC (Table 3.2). In contrast, inhibition was not obtained with anti-HSV-2 sera or seronegative samples tested. Consequently, our results show that MoAb D4.1 recognizes an epitope on the surface of HSV-1 infected cells and antibodies with similar binding specificities were present in anti-HSV-1 sera of all species tested.

Generation and Activity of Anti-idiotype Antiserum

To generate anti-idiotypic antibodies, rabbits were immunized with an affinity purified IgG fraction of MoAb D4.1. To remove zenotypic reactivity, the antiserum obtained was passed over an affinity column of normal mouse IgG (same allotype as MoAb D4.1) and the effluent bound to a MoAb D4.1 column. The bound material eluted from the last column was assayed by ELISA for anti-idiotypic activity against NMS, MoAb D4.1, MoAb D4.2, and MoAb D4.8. As shown in Figure 3.1, NMS binding was removed, but reactivity to MoAb D4.1, MoAb D4.2, and MoAb D4.8 was retained. Ascites fluid containing MoAb D4.8 was later shown to contain a larger amount of antigen-specific antibody than Moab D4.1, which may have resulted in the elevated binding activity of anti-id C to the gB monoclonal. Therefore, all additional assays were performed with Stapholococcus protein A affinity purified immunoglobulin, rather than ascites fluid. In all subsequent assays anti-id C demonstrated a higher level of binding to MoAb D4.1, the immunogen, than MoAb D4.8 (Figure 3.3 and Table 3.3).

Table 3.2. Inhibition of Binding of MoAb D4.1 to gC by Anti-HSV Sera of Different Origins.

Sera Tested[a]	Anti-gC/gC Inhibition[b]
Human	
HSV Seropositive	12/14[e]
HSV Seronegative	0/10
Rabbit	
HSV-1 Immune[c]	4/4
HSV-2 Immune[d]	0/3
HSV Normal	0/3
Mouse	
BALB/c	
HSV Immune	5/5
HSV Normal	0/5
C3H/HeJ	
HSV Immune	6/6
HSV Normal	0/6

[a]Determined by standard ELISA procedures. Plates were coated with optimum dilution of HSV-1-infected cell extracts, followed by test serum, then anti-species IgG conjugated with peroxidase.

[b]Determined by inhibition ELISA procedure. Plates were coated with optimum dilution of affinity purified gC, followed by test serum and a pretitrated amount of MoAb D4.1 conjugated with peroxidase. Absorbances were measured at 490 nm, and the percentage inhibition calculated as described in Materials and Methods. Values of 10% or greater were considered positive. Positive values ranged from 48 to 100% inhibition. Negative values ranged from 0 to 3% inhibition.

[c]Rabbits immunized with HSV-1 antigen only.

[d]Rabbits immunized with HSV-2 antigen only.

[e]Sera were not differentiated as HSV-1 or HSV-2 positive.

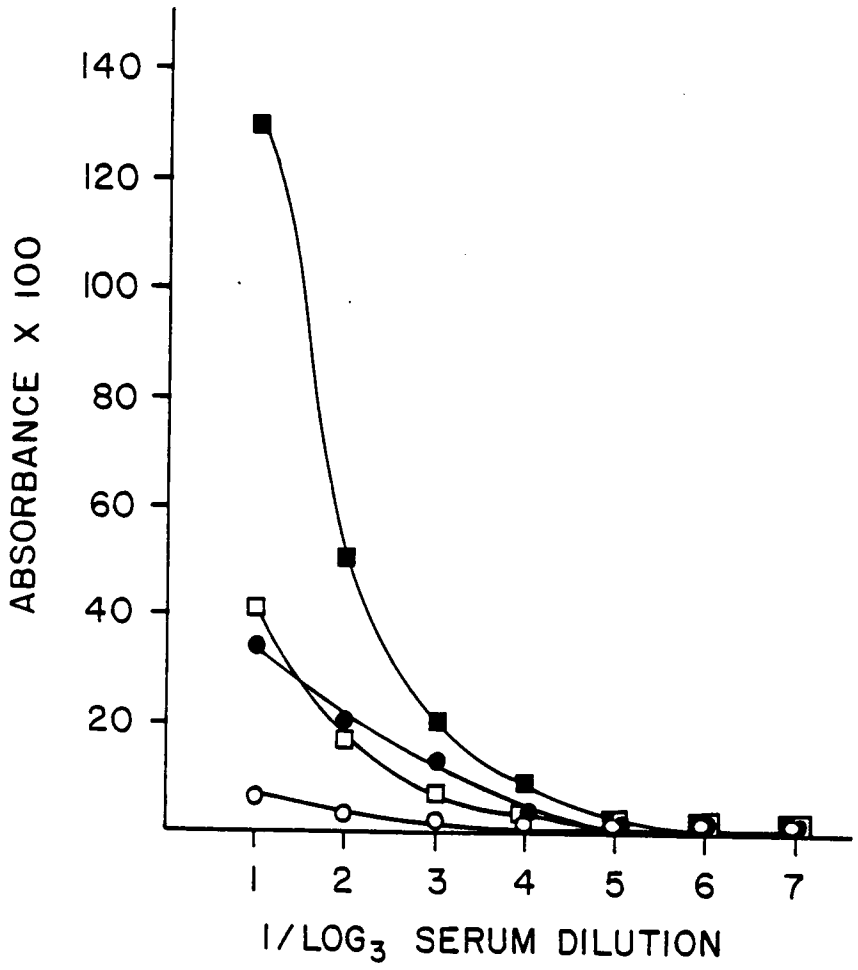

Figure 3.1. Binding of anti-id C using MoAb D4.1, MoAb D4.2, MoAb D4.8, and NMS. Ascites fluid containing MoAb D4.1 (●), or MoAb D4.2 (□), MoAb D4.8 (■), and NMS (O) were adsorbed to wells of a polyvinyl chloride microtiter plate. After absorption on a NMS column, followed by adsorption to and elution from a MoAb D4.1 column, serial 3-fold dilutions of anti-id C (initial concentration 2 ug) were added, followed by peroxidase labeled goat anti-rabbit antibody. Absorbances were determined at 490 nm.

To further examine the binding specificity of anti-id C, the antibody was tested by ELISA for its ability to bind to 36 other monoclonal antibodies. These included monoclonal antibodies to HSV-1 produced during the same fusion, other HSV-specific monoclonal antibodies, influenza virus specific monoclonal antibodies as well as anti-Thy 1.2 and anti-H-$2K^k$ monoclonals. Anti-id C did not recognize any of the antibodies tested (data not shown).

To evaluate the cross-reactive binding activity of anti-id C (eluent from MoAb D4.1 column), the anti-idiotypic antibody was adsorbed to then eluted from sepharose columns bound with either MoAb D4.8 or MoAb D4.2. Eluent from each of these columns was tested by ELISA against MoAb's D4.8, D4.2, and D4.1. Anti-id C, which had been adsorbed to and eluted from either column, retained binding activity for all three monoclonal antibodies. This activity was proportional to the activity of the original anti-id C against MoAb's D4.8, D4.2, and D4.1 (Table 3.3). In addition, Anti-id C, eluted from a MoAb D4.8 column then adsorbed to and eluted from a MoAb D4.2 column, retained binding activity for idiotopes present on all three monoclonal antibodies (data not shown). Thus, it appears that anti-id C recognizes a cross-reactive idiotope that MoAb D4.2 and MoAb D4.8 share with MoAb D4.1.

Binding Characteristics of Anti-idiotypic Serum

To determine if anti-id C could recognize and bind the antigen-combining site of MoAb D4.1, MoAb D4.2, and MoAb D4.8, an ELISA inhibition assay was used. This measured the ability of anti-id C to

Table 3.3. Binding Specificity of Anti-id C After Affinity Chromatography.

MoAb tested[b]	Affinity columns[a]		
	MoAb D4.1[c] only	MoAb D4.1[d] MoAb D4.2	MoAb D4.1[d] MoAb D4.8
MoAb D4.1	73000	36	36
MoAb D4.8	300	4	4
MoAb D4.2	100	4	4

[a]Anti-id C was adsorbed to, then eluted from the following series of affinity columns: MoAb D4.1; MoAb 4.1 followed by MoAb 4.2; and MoAb D4.1 followed by MoAb 4.8. The final eluents were collected separately, concentrated, and assayed by ELISA against MoAb D4.1, MoAb D4.8, and MoAb D4.2

[b]Titers were determined by standard ELISA procedure. Plates were coated with optimum dilutions of MoAb D4.1, MoAb D4.2, and MoAb D4.8. Anti-id C was added, followed by anti-rabbit IgG conjugated with peroxidase. Titers were determined as the reciprocal of the dilution with an absorbance (490 nm) of at least 0.1 and two-fold above negative control values.

[c]Eluent tested by ELISA contained 1.1 mg/ml of protein.

[d]Eluent tested by ELISA contained <10 ug/ml of protein.

inhibit the binding of MoAb D4.1, MoAb D4.2, and MoAb D4.8 to affinity purified gC, gD, or gB, respectively. As shown in Figure 3.2, the binding of all three monoclonals to their corresponding glycoprotein epitopes was significantly inhibited in each instance by preincubation of anti-id C with the monoclonals. In contrast, pre-incubation of anti-id C with either pre-immune rabbit serum or four other HSV monoclonals (D4.11, D4.25, B4, and B6 with binding specificities for gD, gD, gB, gB, respectively) failed to inhibit the binding of any of the monoclonals to their complementary antigens. Thus, the anti-id C recognized a crossreactive idiotope on MoAb D4.1, MoAb D4.2, and MoAb D4.8 which was antigen-binding-site associated.

To further define the idiotope recognized by anti-id C, a modified direct binding ELISA was performed. Affinity purified gC, gD, or gB was adsorbed to a microtiter plate after which MoAb's D4.1, D4.2, or D4.8 were allowed to bind to their complementary glycoproteins. Subsequently, anti-id C was added to determine if the anti-id could recognize the monoclonal idiotope already complexed with antigen. When MoAb D4.1 and MoAb D4.2 were bound to gC and gD, respectively, anti-id C was unable to recognize the crossreactive idiotope. However, anti-id C was still able to bind MoAb D4.8 after it was complexed with gB (Figure 3.3). Our results indicate that the determinant recognized by anti-id C may be located inside the antigen combining site of MoAb D4.1 and MoAb D4.2 (paratopic) and outside of this region on MoAb D4.8.

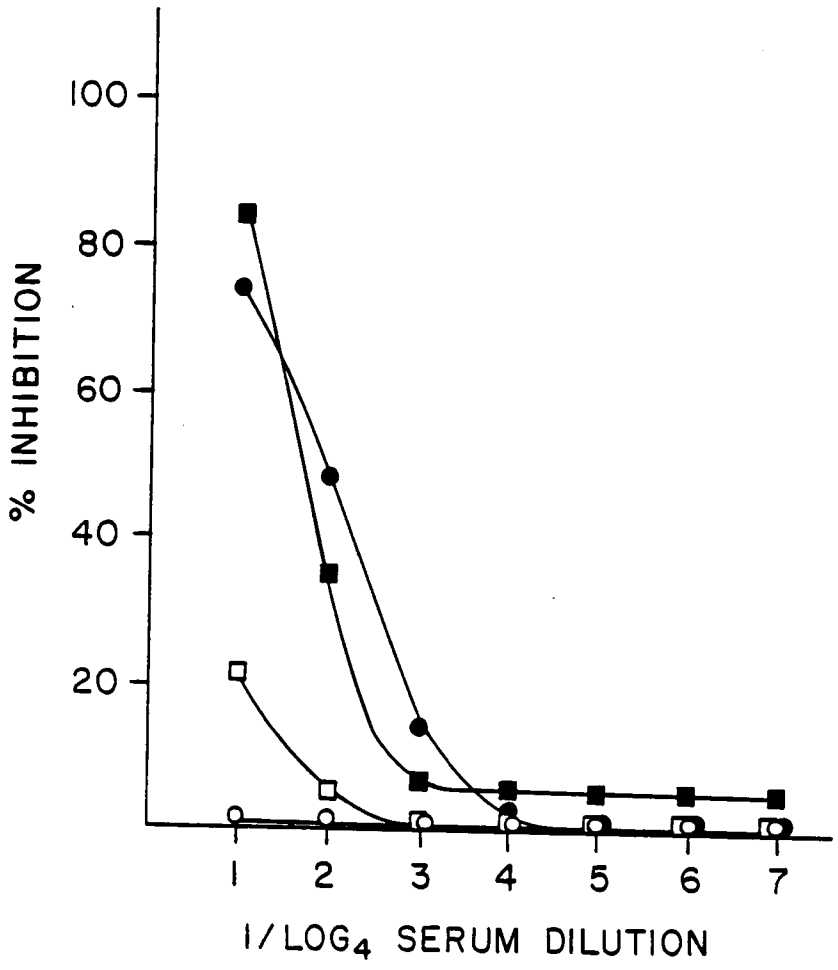

Figure 3.2. Inhibition of binding by anti-id C. A pretitrated amount of MoAb D4.1 (●), MoAb D4.2 (□), or MoAb D4.8 (■) was incubated with 4-fold serial dilutions of anti-id C or pre-immune serum (O) with initial protein concentration of 20 ug per well. After 30 minutes, this mixture was added to microtiter plates coated with gC, gD, or gB, respectively. Absorbances were measured at 490 nm and percent inhibition calculated as described in Materials and Methods. Anti-mouse IgG could not inhibit binding.

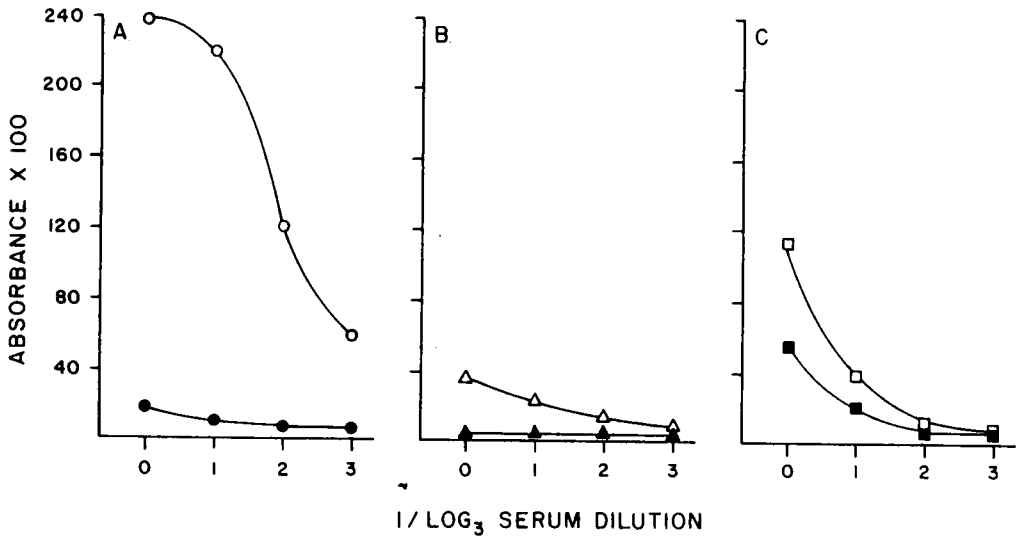

Figure 3.3. Inhibition of anti-id C binding to MoAb by MoAb binding protein. An optimal dilution of gC, gD, or gB was absorbed to wells of a microtiter plate. MoAb D4.1 (●), MoAb D4.2 (▲), or MoAb D4.8 (■), diluted 1:100, was allowed to bind to the glycoproteins. After one hour at 37°C, various dilutions of anti-id C, initial dilution 5 ug/well, were added. This was followed one hour later by anti-rabbit IgG conjugated with peroxidase. As a control MoAb D4.1 (O), MoAb D4.2 (△), and MoAb D4.8 (□) were absorbed to wells of a microtiter plate. Anti-id C was added directly to MoAb's followed by anti-rabbit IgG conjugated with peroxidase. There was no binding of anti-id C if gC, gD, or gB was followed by NMS instead of MoAb. Panel A, MoAb D4.1/gC; Panel B, MoAb D4.2/gD; Panel C, MoAb D4.8/gB.

Proliferation of Anti-id C Primed Spleen Cells

Mice were immunized with anti-id C to determine if the cross-reactive idiotope defined by anti-id C played a role in the immune response to HSV infection. Two weeks after the fourth injection, spleen cells from these mice, as well as cells from normal mice or mice immunized with pre-immune IgG or HSV, were tested *in vitro* for their proliferative response to HSV antigen stimulation. Positive antigen specific proliferative responses were obtained with both HSV and anti-id immunized mice (Figure 3.4). However, responses in the latter group were markedly less than those in the HSV immunized group. These results show that anti-id C could prime spleen cells *in vivo* to proliferate in response to HSV *in vitro*. Antibodies specific for HSV or gC, however, could not be detected by ELISA in sera from any of the anti-id immunized mice.

Discussion

Our report describes the development of a heterologous anti-idiotypic antibody (anti-id C) against monoclonal anti-glycoprotein C (MoAb D4.1) of HSV. Anti-id C reacted with an idiotope of MoAb D4.1 (anti-gC) shared with MoAb D4.2 (anti-gD) and MoAb D4.8 (anti-gB), but not 36 other herpes virus, influenza virus, or anti-H-2 monoclonal antibodies. The cross-reactive idiotope detected by anti-id C appeared to be within or close to the paratope of the monoclonals, since anti-id C inhibited the binding of each of the monoclonal antibodies to their complementary protein epitopes. The actual location of the idiotope on each monoclonal, however, appeared

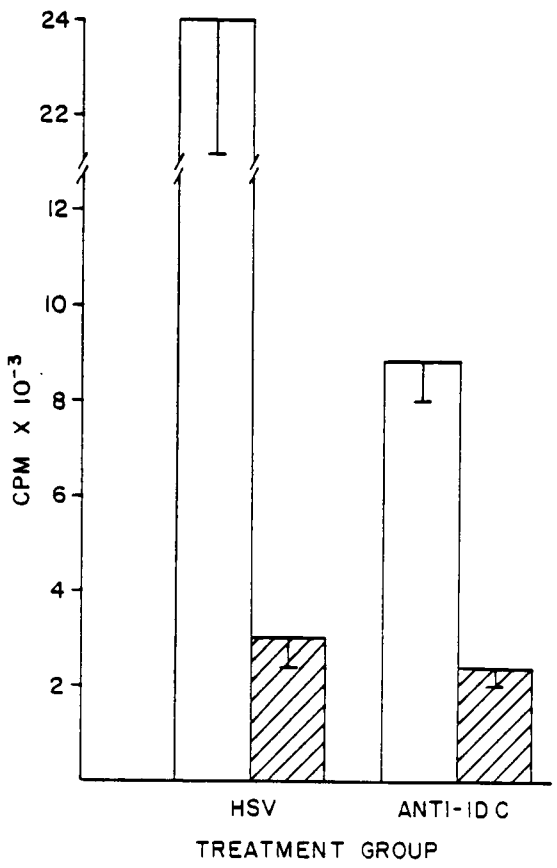

Figure 3.4. In vitro proliferation of anti-id C primed spleen cells in response to HSV antigen. Splenocytes from mice immunized with HSV, anti-id C, preimmune IgG, or non-immunized mice were incubated with UV-inactivated HSV. On the fifth day the cells were tested for [^{3}H]Thymidine(TdR) incorporation. Each treatment group consisted of two mice and the results are the mean for 11 replicates. There was no significant increase in the level of [^{3}H]TdR incorporation in the HSV stimulated group as compared to the unstimulated group for the pre-immune or normal treatment groups. The HSV and anti-id C stimulated groups' response were both significantly higher than their respective unstimulated group ($p < .01$, t-test). HSV stimulated in vitro (☐), unstimulated control (▨).

different. Whereas, the binding of MoAb D4.1 to gC and MoAb D4.2 to gD inhibited the subsequent binding of anti-id C to the idiotope, this did not occur in the case of MoAb 4.8 reacting with gB. This could mean that the cross-reactive idiotope is positioned inside the antigen combining site on MoAb D4.1 and MoAb D4.2 but outside the site on MoAb D4.8. However, another explanation includes the induction of a conformational change in MoAb D4.1 and MoAb D4.2 after their binding to gC or gD, respectively, which acts on the idiotope recognized by anti-id C.

The crossreactivity detected by anti-id C was confined to 3 of 6 monoclonals tested from the same fusion and was not found on 33 other monoclonal antibodies tested. However, since only 14 of these were specific for HSV glycoproteins, the sample size may have been too small to determine if other HSV monoclonals contained the cross-reactive idiotope. It is also possible that fine specificity differences of the sample idiotopes may have lowered the binding efficiency of anti-id C below the detection level of our ELISA assay. The fact that anti-id C bound three monoclonals with different antigen specificities could be explained if regions of the heavy or light chain variable region were coded for by identical gene sequences. Cases of cross-reactive idiotopes being mapped to identical genes have previously been reported in hapten systems (Dzierzak et al., 1985; Victor-Kobrin et al., 1985; Legrain and Buttin et al., 1985). The cross-reactive idiotope, CRI^{AD8}, which is the dominant idiotope in the anti-arsonate (ABA) system, is also present on antibodies which react with chicken gamma globulin, trinitrophenyl, azobenzenephosphonate, and dansyl (Hornbeck

and Lewis, 1985). In the lysozyme system, there is a common idiotope found on antibodies that bind to different lysozyme epitopes (Benjamin et al., 1980). In addition to the ABA system, other hapten systems have cross-reactive idiotopes. These include NP, A5A, and T15 (reviewed in Sacks et al., 1983; Rajewsky and Takemori, 1983). In these systems cross-reactive idiotopes were suggested to be regulatory, either stimulating or suppressing idiotype production.

It will be important to determine if the crossreactive idiotope we have described plays any immunoregulatory role in the response to HSV. As a prelude to such investigations, we have immunized mice with anti-id C and measured certain aspects of cellular and humoral immunity as well as their resistance to viral infection. As observed in previous reports in the HSV system (Kennedy et al., 1984d; Gell and Moss, 1985; Lathey et al., 1986), our animals failed to develop detectable anti-HSV antibodies and were unchanged in their resistance pattern. However, weak yet positive cellular responses occurred, as splenocytes from anti-id C immunized mice expressed HSV-specific proliferation responses in vitro. These results indicate that spleen cells with receptors recognizing anti-id C were capable of responding to HSV antigens. Since C3H/HeJ mice are a different Igh type (1^j) than the Balb/C mice (1^a) in which the monoclonal D4.1 was generated, the immune response to anti-id C immunization is not Igh restricted. A similar lack of restriction for a cellular response to anti-id immunization has been previously reported in the reovirus system (Sharpe et al., 1984). Considering the idiotype MoAb D4.1 was present in C3H/HeJ and BALB/c mouse sera, as well as rabbit and human sera, it

was no surprise that the immune response to anti-id C which was produced against MoAb D4.1 would be Igh unrestricted.

More extensive studies using various routes of exposure as well as the use of adjuvants and carriers must be done before we can conclude that the anti-id C reagent fails to induce antibody reactive with HSV. However, it is well-known that certain antigen epitopes only induce some components of immunity and it is possible that anti-id C mimics a T cell inducing epitope. Experiments are underway to further identify the functional nature of cells primed by anti-id C to proliferate in vitro upon antigen stimulation. Thus, it could be that the proliferating cells represent suppressor cells that act as negative idiotope regulators, resulting in the suppression of HSV-specific idiotopes. The ability to stimulate cellular proliferation yet suppress idiotype production was demonstrated previously with a crossreactive anti-idiotypic antibody that reacts with rheumatoid factor (Fong et al., 1984).

CHAPTER IV

SUPPRESSION OF DELAYED TYPE HYPERSENSITIVITY TO HERPES SIMPLEX TYPE ONE FOLLOWING IMMUNIZATION WITH ANTI-IDIOTYPIC ANTIBODY - AN EXAMPLE OF SPLIT TOLERANCE

Introduction

The ability of anti-idiotypic (anti-id) antisera to induce positive and immunoregulatory aspects of the immune response has been well-documented particularly in hapten systems (Sacks et al., 1983; Kennedy and Dreesman, 1985). Since anti-ids may mimic antigenic epitopes, they represent an alternative to traditional vaccines and their use has been advocated against certain viruses (Kaprowski, 1985; Dreesman and Kennedy, 1985). Nevertheless, despite some encouraging observations, no anti-id can yet be considered as an acceptable anti-viral vaccine.

In some instances, recipients of anti-id vaccines have become more susceptible to infection following immunization (Kennedy et al., 1984). This effect could result from the induction of an immunoregulatory suppressor circuit that impedes immunity. Precedence for this notion comes from hapten systems where exposure of mice to certain anti-id antibodies induces suppressor cells that inhibit aspects of immunity such as the delayed type hypersensitivity (DTH) response (Sy et al., 1979; Thomas et al., 1983).

Recently, we have reported on the in vitro binding characteristics of two heterologous anti-id antibodies produced against monoclonal

antibodies to herpes simplex virus type 1 (HSV-1) glycoproteins (Chapters 2 and 3). However, mice immunized with these reagents failed to mount more than minimal anti-HSV responses. Furthermore, as is reported in the present communication, one of the anti-id reagents effectively activated an immunoregulatory suppressor cell(s) that selectively prevented a DTH response to HSV-1. The response was antigen-specific, long lasting, mediated by T cells and was adoptively transferable. The effect appears to be identical to split tolerance that occurs in mice following intraveneous or intraperitoneal exposure of mice to HSV-1 (Nash, 1985; Larsen et al., 1984). The implications of our observations to the possible manipulation of immunopathological situations are discussed.

Materials and Methods

Anti-idiotypic Antibodies

The production and characterization of the anti-idiotypic antibodies (anti-id) has been described previously (Chapters 2 and 3). Briefly, rabbits were immunized with protein A purified monoclonal antibodies specific for glycoprotein C (gC) or glycoprotein D (gD) of HSV-1, producing anti-id C and anti-id D, respectively. Activity against allotypic determinants was removed by absorption with normal mouse immunoglobulin (NMS). Each anti-id was next adsorbed to and then eluted from an affinity column bound with the immunizing monoclonal. Anti-id C recognized a cross-reactive idiotope found on three HSV-specific monoclonal antibodies and interfered with the binding of each to its complementary protein epitope (Chapter 3). Conversely,

anti-id D was specific for the anti-gD immunogen and could inhibit the binding of anti-gD to gD (Chapter 2).

Viruses

The KOS strain of HSV-1, the 186 strain of HSV-2 and the WR strain of vaccinia were used throughout these studies. The production and titration of the HSV stocks was previously described (Eberle and Courtney, 1980). The vaccinia virus was purified from the cytoplasm of infected HEp-2 cells as described previously (Joklik, 1962). Ultraviolet light (UV)-inactivated virus was prepared by exposing 0.5 ml of virus stock to a germicidal lamp at a distance of 3 cm for two minutes. This reduced infectivity titers from 10^8 to 10^1 plaque forming units (PFU)/ml.

Mouse Immunizations

Six-week-old male C3H/HeJ mice were obtained from the University of Tennessee Memorial Research Hospital. Five groups of mice inoculated with anti-id C, anti-id D, preimmune immunoglobulin (IgG), HSV-1, and saline were used for the DTH studies. Mice immunized with IgG received 3 intraperitoneal (IP) injections, biweekly. These injections consisted of 5 ug of IgG diluted in phosphate-buffered saline pH 7.4 (PBS). HSV immunized mice were given two biweekly IP injections of 5×10^6 PFU of HSV-1. Saline mice received 0.1 ml IP of PBS 3 times biweekly. A further group of control mice received two biweekly subcutaneous (SC) injections of 5×10^6 PFU of HSV-1. Mice were bled from the retroorbital sinus 6 weeks after the initial injection, followed by SC priming with HSV-1 in the left pinna (4 x

10^6 PFU). Mice primed with HSV-2 and vaccinia received 5 x 10^5 PFU and 1 x 10^5 PFU, respectively, in the pinna.

Hypersensitivity Procedure

Six days to two months after priming, or simultaneously with priming, mice were challenged in the rear footpad. Ultraviolet light inactivated virus (HSV-1, 3 x 10^6 PFU; HSV-2, 5 x 10^5 PFU; or vaccinia, 1 x 10^5 PFU, before inactivation) was injected in one hind footpad and an equal volume (20-30 ul) of saline was injected into the alternate footpad. Footpad swelling was measured with an Olditest micrometer at 3, 24, 48, and 72 hours, post challenge. Swellings at 3 hours were considered immediate hypersensitivity reactions which were T-cell independent (data not shown) and those of 24 hour and after, DTH. Data are reported as mean differences in footpad swelling between the virus challenged footpad minus the saline challenged footpad. Specific details of each experiment are included in figure legends.

Adoptive Transfer

Donor mice tolerized for HSV-1, or control mice, were bled from the retroorbital sinus, then sacrificed and spleens removed. Spleen cells processed by the procedure of Lawman et al. (1980), were diluted in saline to 6 x 10^7 cells/ml and transferred intraveneously (IV), 0.5 ml per mouse. Recipient mice were x-irradiated (150R) immediately before IV injection of donor cells. Twenty-four hours post transfer, recipient mice were primed in the left pinna (SC) with 4 x 10^6 PFU of infectious HSV-1. The first hypersensitivity challenge was performed

14 days later. Spleen cells used in the cell depletion assay were treated with antibody plus complement (C) to remove specific cell populations before adoptive transfer. Antibody treatment was accomplished by incubating spleen cells with pre-titered amount of monoclonal anti-mouse Thy 1.2 (a gift from Dr. Mark I. Greene, Tufts University, Boston, MA) or goat anti-mouse immunoglobulins (IgA + IgG + IgM) (Cappel Laboratories, West Chester, PA) at 4°C for one hour. The cells were then washed, and Low-Tox-M rabbit C (Accurate Chemical and Scientific Corp., Westbury, NY) was added for one hour at 37°C. After C treatment, the cells were washed and injected. Each mouse received 0.9 of a spleen equivalent determined before antibody treatment and C depletion.

ELISA

The ELISA using infected cell extracts was performed as previously described (Chapter 2).

Lymphocyte Assays

Mice which were the recipients of spleen cells from preimmune IgG, anti-id C, or HSV-1 IP immunized mice or normal mice were given 4 x 10^6 PFU of HSV-1 in each pinna (10 ul) 24 hours post adoptive transfer. One week later, footpad DTH was performed. One month following DTH priming, spleens were removed for lymphocyte assays. For the cytotoxic T cell (CTL) assay spleen cells, processed as described by Lawman et al. (1980), were incubated with UV-inactivated HSV-1 (MOI = 2) in a small volume for 15 minutes at 37°C. After enumeration, 5 x 10^5 cells were mixed with 5 x 10^5 viral stimulator cells for 5

days. Culture was performed in U bottom 96 well microtiter plates in a 200 ul volume of RPMI-1640, 10% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 ug/ml), gentamicin (50 ug/ml), and 5×10^{-5} mM 2-mercaptoethanol. Before the four hour CTL assay on day 5 cells were washed one time with PBS. The CTL assay was performed as described previously (Horohov et al., 1985b). Specific release was determined by the following formula:

$$\frac{\text{Experimental - Spontaneous}}{\text{Total - Spontaneous}} \times 100$$

Viral stimulator cells were prepared by incubating spleen cells from normal mice with UV-inactivated HSV-1 (MOI = 2) for two hours. Infected cells were X-irradiated with 2400 rads. The lymphocyte proliferation assay was performed as previously described (Chapter 2).

Statistical Analysis

Each treatment group for DTH contained 4 to 6 mice. The mean differences in footpad swelling between the virus challenged footpad and the opposite pad treated similarly with saline was analyzed by the Student's t test. The mean differences in footpad swelling among the treatment groups was analyzed by a combined t test (Swinscow, 1978).

Results

Abrogation of DTH Induction by Anti-id Immunization

C3H/HeJ mice were immunized IP with anti-id C, anti-id D, preimmune IgG, HSV-1, or saline. An additional group was immunized

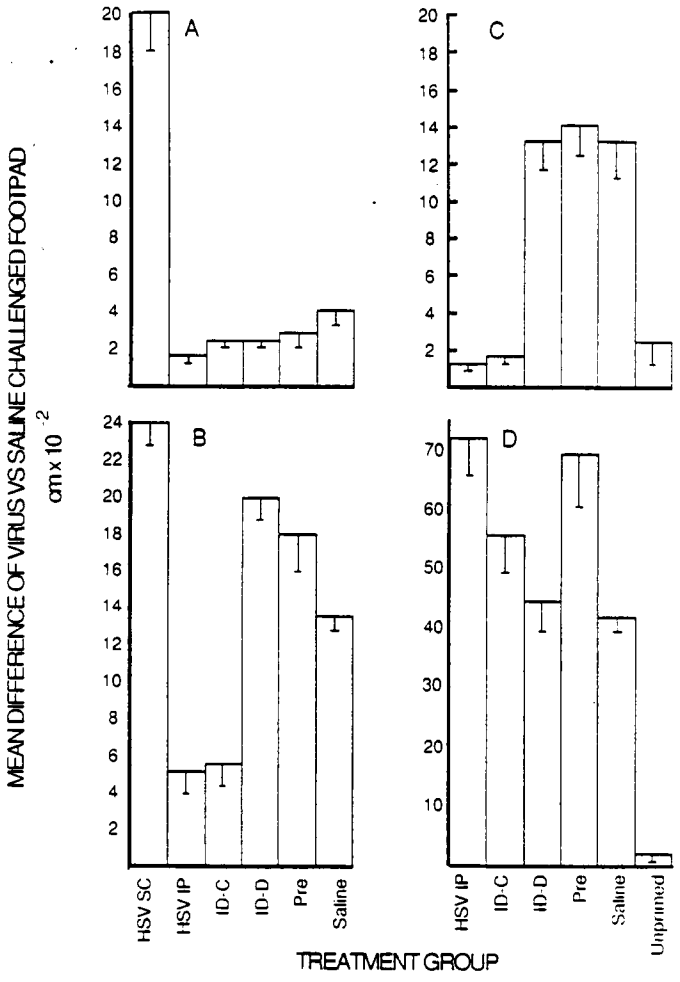

Figure 4.1. HSV-1 DTH response following IP immunization. Groups of 4-6 C3H/HeJ mice were immunized biweekly, as described in Materials and Methods with HSV-1 subcutaneously (HSV SC); or HSV-1 (HSV IP), anti-id C (ID-C), anti-id D (ID-D), pre-immune IgG (Pre), or saline intraperitoneally. Two weeks following the last immunization each mouse was primed in the left pinna with 3 x 10^6 PFU of HSV-1 then challenged in a hind footpad with UV-inactivated HSV-1 simultaneously, panel A; or 7 days later, panels B and C. Values represent mean differences $\pm$ SEM between virus challenged minus saline challenged footpad at 24 hours post challenge. In experiments B and C HSV-IP and ID-C were significantly less than all other groups $p < .05$ (combined t test). Mice described in panel C were primed with live vaccinia after HSV-1 challenge then challenged with UV inactivated vaccinia 7 days later (panel D). There was no significant increase in the footpad swelling of unprimed mice challenged with vaccinia or HSV-1 ($p < .05$, t-test). At 48 hours post challenge all vaccinia primed mice groups demonstrated equivalent footpad swelling (mean differences 30.4 $\pm$ 1.0).

subcutaneously with HSV-1. Six weeks after the initial injections, mice were challenged in the footpad with UV-inactivated HSV-1. None of the mice that received IP immunization demonstrated a significant DTH response, but those mice immunized subcutaneously with HSV-1 generated prominent HSV-specific DTH reactions (Figure 4.1A).

Intraveneous (IV) and IP exposure to infectious HSV-1 is known to result in tolerance for the DTH response (Nash, 1985; Larsen et al., 1984). To determine if the same occurred in mice immunized with the anti-id reagents, animals in these and control groups, which received saline or preimmune rabbit IgG, were sensitized SC with infectious HSV-1 at the time of initial DTH testing. After a further seven days, mice in all groups were retested for HSV-1 specific DTH. Whereas, mice in groups which received anti-id D, saline or preimmune IgG became DTH positive, mice given anti-id C or HSV-1 IP remained unresponsive (Figure 4.1B and 4.1C). Mice in both these latter groups remained DTH unresponsive for at least 5 months (data not shown). The failure to develop DTH was specific to HSV-1 since significant DTH responses were observed in mice that received anti-id C or HSV-1 IP and were subsequently sensitized with vaccinia virus and tested for vaccinia DTH 7 days later (Figure 4.1D). These mice remained DTH tolerant for HSV-1 after vaccinia challenge (data not shown).

Suppression of HSV DTH by Adoptive Transfer

To further investigate if the state of DTH tolerance to HSV-1 induced by anti-id C immunization was similar to that which resulted from IP immunization with HSV-1, an adoptive spleen cell transfer

approach was used to measure DTH suppression. In these experiments, mice in the various groups described in Figure 4.1 were killed after immunization, HSV priming and DTH testing and their splenocytes transferred to X-irradiated (150R), syngeneic, naive mice. Recipient mice were primed in the ear 24 hours later with HSV-1 and then DTH tested by footpad injection of UV-irradiated HSV-1 after a further two weeks. As is shown in Figure 4.2, mice in all groups developed HSV-1 specific immediate hypersensitivity reactions and mice in all but the anti-id C and anti-HSV (IP) immunized groups developed DTH responses when measured at 24, 48 and 72 hours post-challenge. Thus, splenocytes from both anti-id C and HSV-1 tolerized mice prevented the DTH response in recipients sensitized with HSV-1. All donor mice used in this adoptive transfer had HSV-1 antibodies with titers of $\geq$16,000 by ELISA (data not shown).

Phenotype of Cell Involved in HSV-1 DTH Suppression

To further elucidate the cellular mechanism of suppression, splenocytes from mice that received anti-id C, HSV-1 (IP), or preimmune IgG were obtained and 0.9 splenic equivalents were either treated with anti-Thy 1.2 + C, anti-Ig + C, or were treated with C alone. Treated cell populations were transferred to X-irradiated (150R) recipients and the effects on subsequent sensitization with HSV-1 assessed 7 days after virus priming. As before, splenocytes from mice immunized with pre-immune IgG had no effect on the generation of a DTH response in recipients. Furthermore, whereas C treated and Ig + C treated splenocytes, from anti-id C and HSV-1 (IP) immunized mice, could

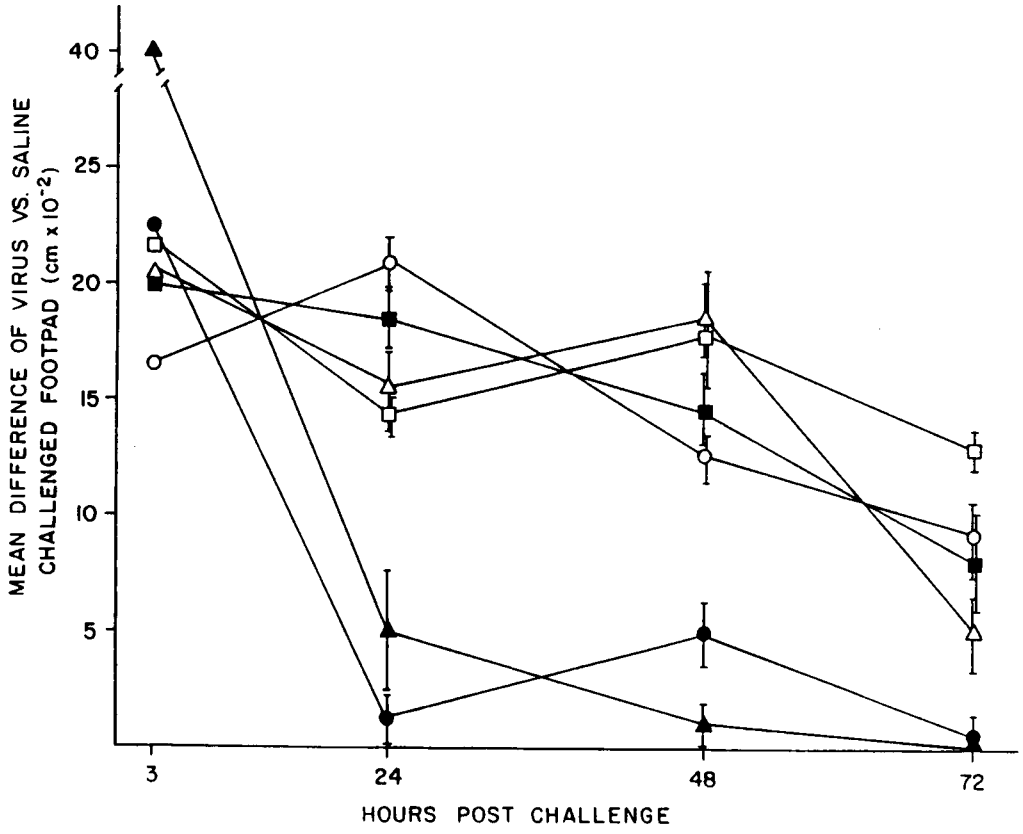

Figure 4.2. Suppression of HSV-1 DTH by adoptively transferred spleen cells. Spleen cells from mice immunized IP with HSV-1 (▲) or anti-id C (●), and shown to be tolerant to an HSV-1 DTH response; spleen cells from mice immunized IP with anti-id D (□), pre-immune IgG (○), or saline (△) which responded with a positive HSV-1 DTH and spleen cells from normal mice (■) were adoptive transferred to X-irradiated (150R) syngeneic mice. 3×10^7 cells were injected IV followed 24 hours later with 4×10^6 PFU of HSV-1 in the left pinna. Recipient mice were challenged in the hind footpad 14 days later. Each group consisted of four mice. HSV-1 and anti-id C groups demonstrated significantly reduced footpad swelling compared to all other groups, $p < .05$ (combined t test).

suppress DTH, this effect was abrogated following treatment with anti-Thy1.2 + C. Thus, in both instances the suppression of DTH was mediated by a T cell (Figure 4.3).

To test if the suppression of DTH was specific to HSV-1, mice unresponsive to HSV-1 were subsequently sensitized with either HSV-2 or vaccinia virus then challenged with the appropriate virus. Mice in all groups develop DTH responses to the second virus, while unresponsive to HSV-1 (Table 4.1).

Split Tolerance in Anti-id C and HSV-1 DTH Suppressed Mice

To determine if the failure of mice adoptively transferred with anti-id C and HSV-1 tolerized splenocytes was restricted to the DTH response, selected mice were bled and killed 3 weeks after SC priming with HSV-1 and subsequent DTH testing. Recipients of splenocytes from non DTH tolerized mice were also investigated. Sera from all groups of mice contained specific anti-HSV-1 antibody (titers $\geq$5500 by ELISA) and their *in vitro* stimulated (5 days) splenocytes lysed HSV-1 infected target cells in a MHC restricted manner (Table 4.2). Splenocytes from the same groups demonstrated antigen-specific proliferation in response to HSV-1 stimulation (Table 4.2). Consequently, the suppression induced by IP immunization of HSV-1 or anti-id C was confined to the DTH response.

Discussion

We demonstrate in this report that immunization of mice with a heterologous anti-idiotypic antibody to monoclonal anti-glycoprotein C

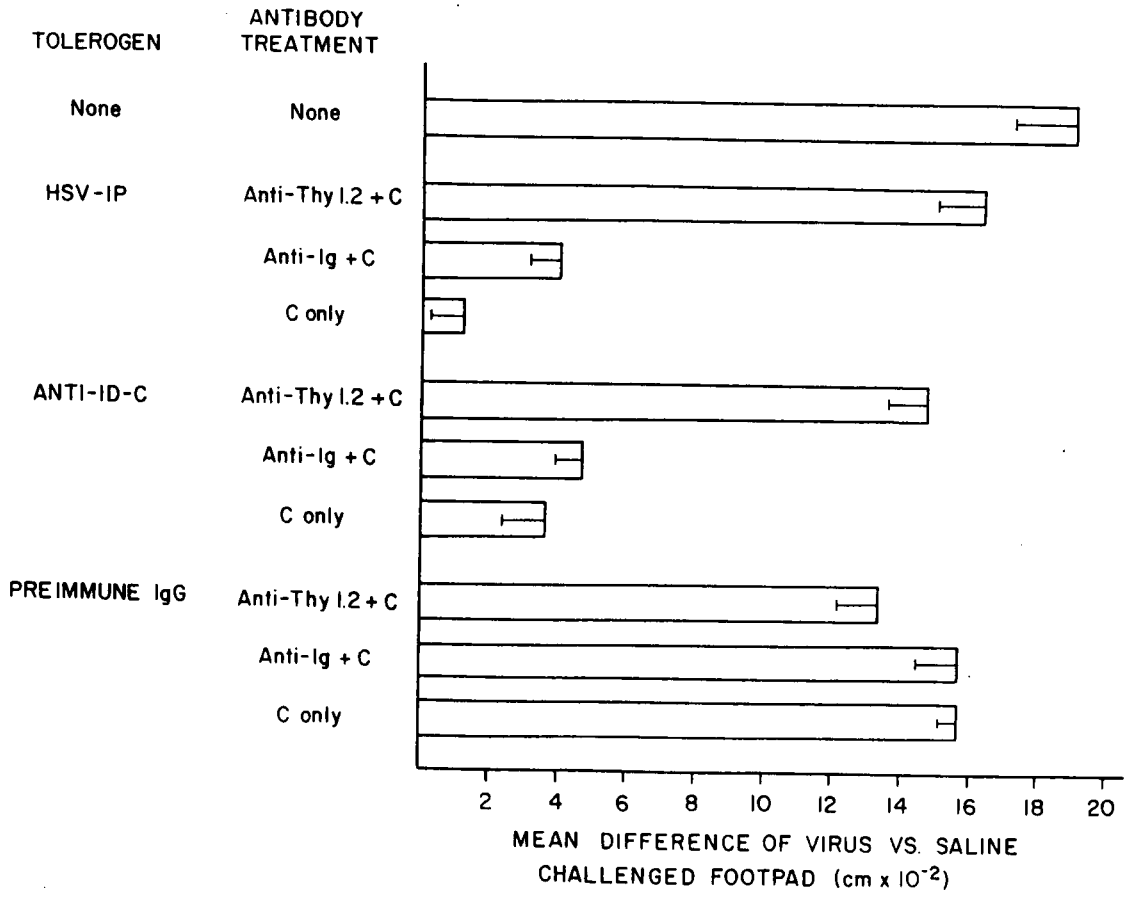

Figure 4.3. Phenotype of DTH suppressor cell. Spleen cells from mice previously positive for HSV-1 DTH tolerance (Figure 4C) were divided into three equal parts and treated with antibody plus complement or complement alone (equivalent to 5 x 10^7 spleen cells per recipient mouse per treatment group). Depleted cell populations were injected IV into X-irradiated syngeneic mice followed 24 hours later with 4 x 10^6 PFU of HSV-1 injected in both pinnae. Recipient mice were challenged 7 days later. Each group contained four mice. The anti-immunoglobulin (anti-Ig) and complement (C) only treatment groups receiving HSV-IP and anti-id C tolerized spleen cells showed significantly reduced footpad swelling compared to all other groups tested, $p < 0.05$ (combined t test).

Table 4.1. Virus Specificity of HSV-1 and Anti-id C DTH Tolerization

Challenge Virus	Tolerogen[a]		
	Anti-id C	HSV-IP	Preimmune IgG
HSV-2[b]	19.3 ± 1.9	18.0 ± 0.8	19.5 ± 1.0
HSV-1[b]	0.8 ± 1.0[d]	1.5 ± 0.3[d]	9.0 ± 0.8
Vaccinia[c]	53.3 ± 1.0	40.5 ± 1.8	46.8 ± 2.7

[a]Mice described in Figure 4.3 which had received spleen cells treated with anti-Ig plus C.

[b]Mice were primed with 5 x 10^5 PFU of HSV-2 in the right pinna two weeks following HSV-1 challenge (Figure 4.3). Two weeks later, the right hind footpad was challenged with UV-inactivated HSV-2. Seven days later the left hind footpad was challenged with UV-inactivated HSV-1. All values are the mean difference ± SEM between the virus challenged minus the saline challenged footpad at 24 hours post challenge.

[c]Mice were primed with 1 x 10^5 PFU of vaccinia 7 days post HSV-1 (b) challenge, then challenged 7 days later with UV-inactivated vaccinia in the footpad. All values are the mean difference ± SEM between the virus challenged minus the saline challenged footpad at 24 hours post challenge.

[d]Mean differences in footpad swelling of these groups were significantly lower than all other groups tested, $p < .05$ (combined t test). Also, in contrast to all other groups, the swelling seen in the test footpad compared to the saline control foot was not significant at $p < .05$ (Student's t test).

Table 4.2. Split Tolerance Induced by HSV-1 and Anti-id C Immunization

Assay	Tolerogen[a]			
	None	HSV-IP	Anti-id C	Preimmune IgG
Cytotoxicity[b] (specific release)				
L cells ($H\text{-}2^k$)	6.5 ± 1.6	3.2 ± 0.3	4.4 ± 0.8	5.5 ± 1.6
HSV-1 L cells ($H\text{-}2^k$)	32.2 ± 1.5	51.4 ± 2.4	62.5 ± 2.0	50.6 ± 0.9
HSV-1 A cells ($H\text{-}2^d$)	0.1 ± 0.1	1.0 ± 0.3	2.8 ± 0.5	2.1 ± 0.2
Proliferation[c] (cpm)				
HSV-1	20,300 ± 213	12,619 ± 158	36,735 ± 1,157	17,808 ± 355
Media	6,832 ± 78	5,711 ± 42	8,871 ± 59	6,280 ± 126
Antibody[d]	≥5,500	≥5,500	≥5,500	≥5,500

[a]Two C3H/HeJ ($H\text{-}2^k$) mice from each C only treatment group described in Figure 4.3 were bled from the retroorbital sinus, sacrificed and spleens removed three weeks after HSV-1 challenge. Mice receiving adoptively transferred cells at the same time demonstrated DTH tolerance during this period (Table 4.1).

[b]Data represent the average ± SEM of three replicates. The L targets were L929 cells either uninfected (L cells) or infected with HSV-1 (HSV-1 L cells). The A targets were BALB/c 3T3 A31 cells infected with HSV-1 (HSV-1 A cells).

[c]Splenocytes were incubated *in vitro* with UV-inactivated HSV-1 or media only. On the fifth day the cells were tested for [^{3}H]-TdR incorporation. Data represent the mean for 12 replicates ± SEM.

[d]Titer determined by a standard ELISA procedure. Plates were coated with an optimum dilution of HSV-1 infected or uninfected cell extracts. Titers were determined as the reciprocal of the dilution with an absorbance (490 nm) of at least 0.1 and two-fold above negative control values.

selectively tolerizes recipients for a HSV-1 specific DTH response. The effect mimics split tolerance that occurs following intravenous, or intraperitoneal, exposure of mice to infectious virus (Nash, 1985; Larsen et al., 1984). Thus, in both cases the lack of responsiveness involves only DTH and tolerized mice still produce antibody, express immediate hypersensitivity and their splenocytes generate antigen-specific CTL and lymphoproliferative responses upon in vitro stimulation. The suppressed DTH response was antigen-specific and mediated by T cells. Accordingly, suppression was transferrable with splenocytes and splenic T cells from anti-id C immunized mice. Moreover, whereas recipients were incapable of mounting DTH responses following immunization with HSV-1, they could generate DTH responses after immunization with vaccinia virus and even cross-reacting herpes simplex virus type 2.

Although split tolerance following certain routes of immunization with viruses has repeatedly been demonstrated and shown to be mediated by suppressor cells (Schrier et al., 1983; Nash and Ashford, 1982; Leung et al., 1980), a mechanistic explanation for the selective effect on DTH is not at hand. Some have suggested that split tolerance involves induction of idiotype-anti-idiotype regulatory networks (Nash and Gell, 1981). Our observation of split tolerance following immunization with an anti-id reagent is in accord with the hypothesis. It could be that the anti-id we describe either elicits or passively acts as a component of the postulated regulatory network that prevents the development of DTH. Since the effect lasted for at least 5 months and was transferable with cells, the immunization seemingly plays an

active role and induces antigen-specific regulatory cells. Such cells could directly inhibit DTH after subsequent exposure to a specific HSV-1 epitope, or stimulate a component of a DTH suppressor cell cascade. Alternatively, anti-id C could prime a T suppressor cell, initiating proliferation and factor production. This primed cell would then release suppressor factor only when activated by HSV-1 antigen. There is evidence for existence of a primed pre T suppressor cell in the 4-hydroxy-3-nitrophenyl-acetyl (NP) system (Minami et al., 1983).

The apparent failure of anti-id C to regulate other aspects of immunity requires explanation. It could be that immune networks involved in humoral antibody and CTL induction are distinct and are controlled by different regulatory idiotopes. Accordingly, the selective effect on DTH by IV or IP exposure to virus could result from preferential induction of suppressor cells directly by antigen or by subsets of antigen presenting cells that differ functionally from those found in subcutaneous locations. Alternatively, networks involved in antibody and CTL induction may have auxiliary mechanisms that compensate for idiotope specific suppression. This latter effect has been demonstrated for antibody production in the hapten system. For example, a normal anti-NP response can be observed in anti-id suppressed mice, but none of the antibodies express the idiotopes recognized by the anti-id (Rajewsky and Takemori, 1983). Further experiments are clearly needed to identify the mechanisms of suppression as well as to investigate if anti-id C immunization can induce DTH tolerance when given by routes other than the intraperitoneal route.

Our observation of anti-id C induced DTH suppression was unexpected although it is not the first report of anti-id immunization inducing suppression of a DTH response. A well defined suppressor-cell mediated mechanism active against DTH and induced by anti-id immunization has been described in the anti-azobenzenearsonate system (Monroe et al., 1985). However, our findings represent the first in the virus field and are of interest since suppression was obtained against a highly complex set of antigens following exposure to antibody against a monoclonal antibody produced against a single epitope on only one of many glycoproteins found in the envelope of HSV-1. Elsewhere we have described the spectrum of reactivity of the anti-id C reagent (Chapter 3). Whereas, it does detect idiotopes on monoclonals reactive with some other HSV glycoproteins the idiotope was detectable on very few reagents and was neither detectable on other monoclonal antibodies to gC nor on polyclonal anti-HSV antibodies. Thus, the anti-id C apparently does not react with an immunodominant cross-reactive idiotope present on HSV antibodies. We must presume, however, that anti-id C recognizes a dominant idiotope of HSV-1 (but notably not HSV-2) of the immune network that induces DTH suppression. Alternatively, the anti-id C may elicit suppressor cells which upon activation by specific antigens induce nonspecifically acting mechanisms that modulate DTH responses to other antigens. If the latter effect occurs, we might expect that whereas suppression of DTH induction is confined to HSV-1 if animals were simultaneously primed and challenged with HSV-2, or even vaccinia, at the same time as HSV-1 inoculation, then modulation of the DTH response to HSV-2 and vaccinia

would also occur. Support for this latter idea comes from observations that the suppressor factor involved in anti-id stimulated suppression of the ABA DTH response is antigen nonspecific in its activity. However, the T suppressor cell that produces this factor has to be activated by ABA (Monroe et al., 1985).

Anti-idiotypic antibodies have been advocated as alternatives to traditional vaccines (Koprowski, 1985; Dreesman and Kennedy, 1985) but in viral systems so far without notable success. In our own investigations with anti-idiotypic reagents against monoclonal anti-HSV-1 glycoproteins, we have observed only weak cellular responses and no measurable antibody following in vivo administration to mice (Chapters 2 and 3). Furthermore, anti-id immunized mice were not more resistant to infection. One group has even observed the contrary result and noted that mice immunized with an anti-id to a monoclonal anti-HSV glycoprotein were more susceptible to infection (Kennedy et al., 1984). Our present observation of split-tolerance following anti-id immunization suggests a clinical use of anti-ids in certain situations where the immune response plays an immunopathological role. This may be pertinent to HSV infections since the DTH response is implicated as the cause of demyelination within the murine central nervous system (CNS) during an acute infection. Adoptive transfer of efferent suppressor cells, which abrogate the HSV specific DTH response, protected recipient mice from demyelination with the CNS. Additionally, these mice were protected against lethal herpes encephalitis (Altmann and Blyth, 1984; 1985). A treatment which could induce similarly acting T suppressor cells would presumably lead to a

reduction in viral pathology without a concomitant increase in the pathogenicity of the virus. Such a treatment could prove useful for HSV stromal keratitis, where in mice scarring of the cornea has been associated with an immunopathological T cell response (Metcalf et al., 1979). Hence we are currently assessing models of HSV immunopathology to determine if anti-id C immunization can favorably influence HSV pathogenesis.

CHAPTER V

SUMMARY AND SPECULATION

When this project was initiated, there were little published data relating to anti-idiotypic antibodies in the herpes virus system. The general expectation of the experiments outlined in the previous chapters was to produce anti-idiotypic antibodies and determine their role in herpes virus immune regulation in mice. Thus, two such antibodies were produced in rabbits against BALB/c monoclonal antibodies specific for HSV-1 glycoproteins. Monoclonal antibody (MoAb) D4.2, which was used to produce anti-idiotypic antibody D (anti-id D), is a type-common, neutralizing antibody specific for glycoprotein D of HSV. Conversely, monoclonal antibody D4.1, which was used to produce anti-idiotypic antibody C (anti-id C), is a type-specific, nonneutralizing antibody specific for glycoprotein C of HSV.

Anti-id D and anti-id C were made specific for their respective immunogen by affinity chromatography procedures using normal mouse immunoglobulin (NMS). Activity against NMS was adsorbed out, while activity for the immunizing monoclonal was retained. Anti-id D was specific for MoAb D4.2 and could inhibit the binding of MoAb D4.2 to gD as determined by an ELISA inhibition assay. Conversely, anti-id C bound MoAb D4.2 and MoAb D4.8, specific for gB, in addition to MoAb D4.1 the immunogen. Consequently, anti-id C could also inhibit the binding of each monoclonal antibody to its complementary protein epitope. Thus, anti-id D defines a specific idiotope on MoAb D4.2 and

anti-id C recognizes a cross reactive idiotope present on MoAb D4.2 and MoAb D4.8, as well as MoAb D4.1.

In order to investigate the immunoregulatory ability of anti-id D and anti-id C each was used to immunize mice. Splenocytes from BALB/c mice immunized with anti-id D could proliferate in vitro in response to HSV-1 antigens. C3H/HeJ splenocytes primed in vivo with anti-id C also demonstrated in vitro HSV specific lymphoproliferation. Thus, spleen cells primed in vivo with either anti-id D or anti-id C immunization possess receptors which recognize HSV antigen in vitro.

To elucidate the function of the immune cells primed in vivo by anti-id immunization a HSV DTH model was utilized. Mice immunized IP with anti-ids C and D, preimmune IgG, or HSV or SC with HSV were primed in the pinna and simultaneously challenged in the footpad with HSV. Only mice immunized SC with HSV were capable of a positive DTH response. In order to determine if the absence of a DTH response in the other groups was due to lack of sensitization or active suppression, all mice were rechallenged 7 days later. After priming with HSV, all groups were DTH positive except HSV-IP and anti-id C. Thus, IP immunization with anti-id C or HSV set up a state of DTH tolerance in these mice. This tolerance was specific for the DTH response, since tolerized mice demonstrated functional HSV antibody, CTL, and lymphoproliferative responses in vitro.

Anti-id C and HSV IP tolerization was the result of the activation of a suppressor T cell (Ts). Consequently, suppression could be adoptively transferred with whole spleen and anti-immunoglobulin depleted cell populations, but anti-Thy 1.2 depletion abrogated the

suppressor ability of transferred cells. This DTH suppression was specific for HSV-1, since tolerized mice were still capable of a vaccinia and HSV-2 specific DTH responses after priming with the specific virus.

The specific cell population involved in anti-id C and HSV-IP DTH suppression, or the type of suppression, afferent or efferent have not been determined. In order to identify the effector cells adoptive transfers from tolerized mice should be performed with splenic cell subset populations. Reagents such as anti-Lyt 2.1, anti-L3T4 (Lyt 1) and anti-I-J^k antibodies could be used in either cell depletions or cell panning before adoptive transfer into naive mice. Experiments mixing primed DTH competent cells with tolerized cells would be useful in determining the kinetics of the suppression. Only efferent acting cells could suppress under these conditions. Alternatively, primed DTH competent cells could be adoptively transferred into tolerized mice. Efferent acting suppressor cells would also be necessary to abrogate the DTH response. To determine the time after virus priming the suppressor cells were effective, adoptive transfer of tolerized cells could be performed before or on successive days after priming, before challenge. If the target cell of anti-id C and HSV IP tolerization blocks only the induction of DTH then suppression of DTH would only be observed in mice receiving tolerized cells before or soon after priming (afferent suppression). Efferent suppression should be effective through the entire period between priming and challenge. Since the data reported in Chapter 4 resembles Nash's IV suppression model (Nash, 1985) we may or may not expect parallel results in the anti-id C

system. At 28 days post IV tolerization with HSV-1, a population of spleen cells exists that is capable of specifically suppressing HSV-1 DTH. This population contained a Lyt 2^+, I-J^+ afferent or efferent suppressor cell subset depending on whether virus or virus infected cells, respectively were used to tolerize. The cell was not present in the spleen at earlier times (Nash and Gell, 1983; Schrier et al., 1983). Unfortunately, the mechanism of action of either form of DTH suppression was not investigated.

The mechanism by which anti-id C suppresses the HSV-1 DTH response has not been delineated at this point. In all experiments the results obtained with IP anti-id C immunization parallel those of HSV-1 from IP or IV immunization. One could speculate that one mechanism of viral DTH tolerization utilizes a network of idiotype-anti-idiotype interactions. In the ABA hapten system, a role for idiotype control of DTH suppression has been demonstrated (Sy et al., 1979; Thomas et al., 1983). Suppressor cells from mice immunized with a monoclonal ABA anti-id were shown to possess receptors for ABA and/or anti-id. Thus, in this system DTH suppression could be adoptively transferred with anti-id immune spleen cells which bound to anti-id or ABA coated plates (Thomas et al., 1983). In the same system (Monroe et al., 1985) Lyt 2^+ T cells from mice immunized with anti-CRI, a heterologous ABA anti-id, could transfer suppression of ABA specific DTH and CTL to ABA primed recipients (efferent acting). The cell lysate from these cells could also transfer DTH suppression to cyclophosphamide treated or untreated IgH matched mice. The latter suppression, however, was antigen non-specific. Thus, the generation of this suppressor factor

by anti-id immunization was antigen specific, but the activity of the factor was antigen non-specific. This anti-id induced factor mimics the activity of the third-order suppressor factor (TsF_3) induced by IV immunization of ABA (Sy et al., 1981). Therefore, Monroe et al. (1985) postulate that anti-id immunization activates a Ts_3, effector suppressor cell in the presence of ABA to release a Ts_3 suppressor factor with antigen nonspecific efferent suppressor activity.

Unfortunately, there are no DTH suppressor circuits defined in the HSV system. However, a CTL suppressor circuit has been postulated in our laboratory by Horohov et al. (1985b) which shares some similarities to the ABA system. In the HSV CTL suppressor circuit, a Lyt 1^+ Ts_1 cell is activated by the presentation of viral antigens to release a Ts_1 factor. This factor, stimulates a Lyt 2^+ Ts_2 cell to produce an anti-idiotypic factor (TsF_2) which is $I\text{-}J^+$. TsF_2 then causes an HSV immune Ts_3 cell to produce a soluble antigen nonspecific factor which mediates suppression either on its own or through an auxiliary cell.

It is possible that anti-id C is involved in a specific suppressor circuit similar to the ones described above. Anti-id C immunization stimulates a suppressor T cell which abrogates the entire HSV-1 DTH response but has no effect on HSV-2 or vaccinia DTH. The anti-id Ts is specific for the DTH response, not acting upon other arms of the immune response. DTH suppression has been observed from 36 days to a minimum of 5 months post initial anti-id C immunization. A similar acting Ts described by Monroe et al. (1985) was a Lyt 2^+, IJ^+, efferent acting suppressor cell. If anti-id Ts has the same phenotype and

efferent activity it could be a Ts_3-like cell as described in the ABA or HSV_{CTL} system. Conversely, in the HSV_{DTH} system an afferent and efferent acting cell have been described with the same phenotype (Nash, 1985). Experiments to identify the phenotype and action of anti-id Ts have been described in previous paragraphs.

To further support the idea of anti-id Ts stimulating a Ts_3-like cell, one must look at the requirements for its stimulation. Anti-id C Ts (Ts_{Id}) has to be stimulated by antigen as well as anti-id. Accordingly, suppression of DTH only occurs when HSV-1 antigens are present during challenge in anti-id C immune mice. The suppression observed after virus specific stimulation, however, appears to be nonspecific. Anti-id C is specific for only one idiotope, yet it can prime Ts_{Id} which suppresses the response to an entire virus when further activated by antigen. An alternative to nonspecific suppressor activity could be the presence of the cross reactive idiotope on all HSV-1 DTH suppressor cells. The Ts_3 in both systems described above requires antigen specific activation which results in the release of an antigen nonspecific Ts factor which in turn mediates suppression. Additionally, activation of the Ts_3 cell in the NP system requires in vitro both antigen and B cells bearing anti-idiotypic molecules (Hausman et al., 1986).

The following experiments would be helpful in analyzing the Ts_3-like activity of Ts_{Id}. If the activity of Ts_{Id} is antigen nonspecific, then tolerized mice primed with HSV-1 and another virus, when challenged simultaneously with both viruses should show suppressed DTH to both viruses. Also, factor production of the

Ts_{Id} could be examined. Soluble extracts from Ts_3 cells usually mediate nonspecific suppression (Dorf and Benacerraf, 1984). Thus, the soluble portion of tolerized Ts_{Id} after freeze-thaw could be transferred to naive mice. The recipient mice would then be primed and challenged with HSV-1, as well as other viruses to test the virus specificity of the suppression.

Based on the previous discussion I propose the following model for anti-id C suppression (Figure 5.1). After immunization, anti-id C binds a cross reactive idiotope present on DTH specific suppressor cells. Anti-id binding primes the Ts_3-like cell and may stimulate proliferation and factor production without releasing the factor from the cell. The primed Ts_3-like cell remains inactive until exposed to HSV-1 antigens. HSV-1 antigen binds a specific antigen receptor on the Ts_3-like cell signaling factor release. The factor then blocks, by an unknown mechanism, any DTH cells in the local environment. A similar pre Ts_3 cell has been described in the NP system. Minami et al. (1983) have described a primed Ts_3 cell which contains Ts_3F that is only released when the Ts_3 cell is activated.

To determine if there is a suppressor cell containing a dual specificity for both anti-id C and antigen in anti-id C immune mice, adoptive transfer experiments must be performed with specific cell populations. Panning with both anti-id C and antigen will be necessary to determine if the cell mediating suppression possesses one or both receptors.

Although the mechanism of specific DTH tolerance has not been delineated, Nash and Gell (1981) have speculated about possible

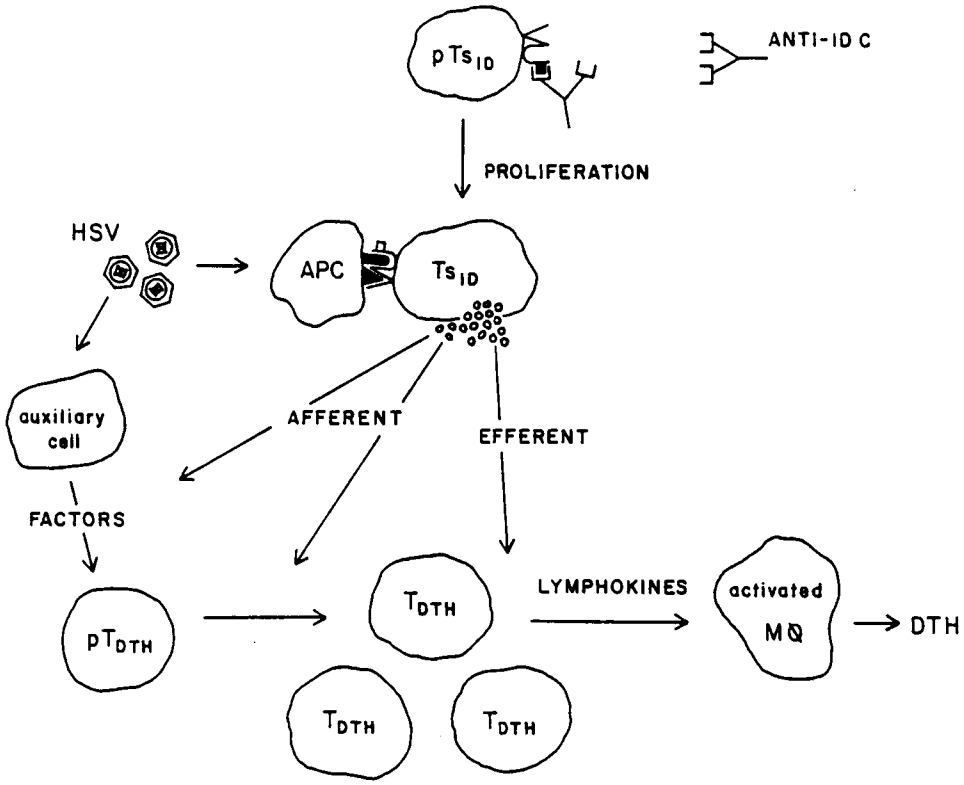

Figure 5.1. Mechanism of anti-id C suppression.

mechanisms. Since the suppression is specific for the DTH response, there may be a specific membrane antigen that is peculiar to the DTH cell which allows it to be regulated independently. Similarly, there could be a specific DTH helper cell which could be modulated without affecting other immune responses. There is evidence in the influenza system for a DTH helper cell (Leung and Ada, 1981). Thus, the suppressor cell could interact directly with the DTH cell or act indirectly through the DTH helper cell. Since DTH suppression can either be afferent or efferent, the maturation level of the DTH cell determines whether suppression will be effective. In general, a pre DTH cell when stimulated by antigen proliferates and differentiates into a mature DTH cell. The mature DTH cell is capable of increased lymphokine production and release and a subset of these cells become memory DTH cells. Thus, there are several points at which a DTH suppressor cell could block the DTH response. An afferent Ts which blocks induction of DTH could either interfere with the activation or differentiation of the pre DTH cell. An efferent cell, on the other hand, could inhibit lymphokine production or release by the mature DTH cell. Either afferent or efferent Ts could inhibit the development of a DTH memory cell.

Anti-id C suppression could be operating by any of above described mechanisms. A summary of the possibilities is presented in Figure 5.1. Ts_{Id} may be mediating suppression by inhibiting the interaction between the DTH helper cell and the pre DTH cell. Thus, the signal to proliferate and/or differentiate may not be received by the pre DTH cell. Ts_{Id} may interact directly with the pre DTH cell

to inhibit proliferation and/or differentiation. In either case the action of Ts_{Id} could be described as afferent, since the induction of DTH is blocked. Conversely, if Ts_{Id} is efferent acting inhibition of lymphokine production and/or release may be responsible for DTH suppression.

It will not be an easy task to determine the mode of action of Ts_{Id}, particularly since there are no good in vitro DTH models. Research in this area is also hindered, because there are no defined marker antigens to allow differentiation between pre and mature DTH cells, or between DTH cells and other similar immune cells. Obviously, one can determine if Ts_{Id} is an afferent or efferent acting cell. Such experiments have been described in preceeding paragraphs. The effect of growth factors like interleukin 2 (IL-2) could also be investigated. If the suppression is mediated through inhibition of a DTH specific helper cell, local production of IL-2 may be inhibited. This could be evaluated by local administration of IL-2 during priming and/or challenge or by in vitro culture of tolerized cells with exogenous IL-2 before adoptive transfer. There is evidence that HSV IP tolerized DTH can be abrogated with a five day in vitro incubation period with HSV antigen, before adoptive transfer (Larsen et al., 1984).

The long immunization period used in this study could predispose the discovery of a Ts_3-like suppressor cell. Nash (1985) has described different suppressor cell populations in the spleen at 7 days and 28 days following IV HSV tolerization. It is possible that the initial target of anti-id C may be a cell which occurs early in the Ts

circuit and develops into the proposed Ts_3-like cell during the 36 day immunization period. To test this possibility, various shorter immunization regimens should be tested for their ability to tolerize the DTH response. If tolerization occurs, then the previously described experiments should be performed to determine phenotype, specificity, factor activity, and kinetics of suppression.

The preceeding discussion is based on DTH tolerance by intraperitoneal immunization of anti-id C. It is yet to be determined if the suppression induced is a result of route of immunization or the binding specificity of anti-id C. It has been shown previously with herpes virus that the route of immunization can determine whether an animal responds to virus challenge with a DTH response. Following subcutaneous or intradermal inoculation, a DTH response is observed whereas, intravenous or intraperitoneal inoculation results in DTH tolerance (Nash et al., 1981a; Schrier et al., 1983; Larsen et al., 1984). The route of immunization with an anti-id reagent has also been shown to be important. In the ABA system, subcutaneous exposure leads to a DTH response, whereas IV inoculation results in tolerance, which was similar to the response observed with HSV antigen (Sy et al., 1979; 1980). Thus, it will be important to try different routes of immunization with anti-id C. If route, rather than binding specificity determines the regulatory activity of anti-id C, then one might postulate a role for antigen presenting cells (APC). Different subsets of APC appear to be involved in stimulatory responses as compared to suppressive responses (Dorf and Benacerraf, 1984). One could speculate that the APC population varies throughout the body and different routes

of immunization expose antigens to different subsets, results in either stimulation or suppression of the immune response. There is no evidence to date that anti-id is presented to a regulatory cell by APC. However, anti-id could bind the Fc receptor of an APC, allowing the binding portion of the antibody to recognize its target cell. In the ABA system only whole antibody molecules tolerize, Fab fragments have no effect (Sy et al., 1979). This effect, however, may only indicate a need for cross-linking receptors on the regulatory cell. In any case it will be necessary to examine the effect of varying the route of anti-id C immunization, as well as using antibody fragments and whole antibody molecules.

A reagent like anti-id C could be of medical importance in treating herpes keratitis since immunopathological damage is associated with recrudescent disease (Kaufman, 1978). In stromal keratitis, viral antigens may persist at least a month in keratocytes in the absence of infectious virus (Meyers-Elliot and Chitjian, 1976). In the murine model stromal disease and permanent scarring occur in BALB/c mice, but not in athymic nude mice. Thus, the disease state is associated with a T cell response. Athymic mice, however, eventually die from HSV encephalitis whereas normal mice do not. Therefore, T cell immunity is necessary to protect against HSV systemic infection (Metcalf et al., 1979). Accordingly, any treatment used to reduce immunopathology, must not totally block immune T cell responses. The efficacy of inducing split T cell tolerance in mice for reducing stromal damage was tested using Nash's IV tolerance model (Nash, 1985). Mice with suppressed DTH responses, but competent CTL and antibody responses, demonstrated

diminished stromal reactions (Metcalf et al., 1979). Since, in all aspects tested anti-id C mimics the IV split tolerance model it may prove to be effective prophylaxis or perhaps even treatment against HSV stromal keratitis.

A variety of experiments in the mouse model must be performed to determine the utility of anti-id C in treating HSV stromal keratitis. First, an anti-id C immunization regimen proven to induce HSV split tolerance, should be tested for its ability to reduce or prevent HSV stromal keratitis. Assuming success, varying doses, routes of injection, and time courses must be tested to determine the most efficacious anti-id C prophylaxis. It will also be important to observe histologically the progression of the disease in the cornea in anti-id C tolerized mice. Hopefully, the immunopathological response can be reduced, without increasing the rate of viral replication. Monitoring the infected, tolerized animals will be necessary to assure the reduced anti-viral immune response (lack of DTH) does not increase the animals susceptibility to HSV encephalitis.

As HSV stromal keratitis in humans involves recrudescence, permanent scarring of the cornea can occur (Meyers-Elliott et al., 1980). Thus, it will be important to determine the effectiveness of anti-id C treatment after HSV infection has been initiated. Unfortunately, mouse keratitis is a poor model for human recrudescence, since spontaneous HSV reactivation does not occur. However, the efficacy of anti-id C treatment in reducing immunopathological disease after mechanically induced recrudescence can be evaluated in mice.

Obviously, many modifications will have to be made before a reagent like anti-id C can be used in humans. However, a working mouse model would demonstrate the reagent's utility in treating HSV stromal keratitis, thus paving the way for successful keratitis prophylaxis in humans.

There is one major drawback to continuing this project as described in the preceeding pages. The reagent is a rabbit antibody, thus in limited supply. It would be advantageous to produce a monoclonal antibody which mimicks the activity of anti-id C. In the reovirus system a monoclonal anti-id was produced with activity identical to the original heterologous anti-id (Noseworthy et al., 1983). I would suggest immunizing BALB/c origin (RBF) mice with MoAb D4.1 IgG coupled to Staph-A sepharose. Hybridoma supernatant fluids could be screened with peroxidase-conjugated MoAb D4.1. Next, positive cultures could be screened by an inhibition ELISA for their ability to inhibit the binding of gC to MoAb D4.1, gB to MoAb D4.8, and gD to MoAb D4.2. Finally, mice would be immunized with inhibition positive antibodies and tested for HSV-1 DTH tolerance. Assuming a monoclonal antibody could be selected with the same activity as anti-id C, there would be a continuous supply of specific reagent for all subsequent evaluations.

It is unfortunate that an immunoregulatory role for anti-id D could not be discovered. It should be noted, however, that most experiments were performed with C3H/HeJ mice ($H\text{-}2^k$, $IgH\text{-}1^j$). The anti-id D reagent reacted with a BALB/c idiotype ($H\text{-}2^d$, $IgH\text{-}1^a$) and primed for a HSV lymphoproliferative response in the same strain of

mice. It is possible that the activity and/or recognition of anti-id D is haplotype restricted, as opposed to anti-id C which is unrestricted at least in these two haplotypes. Thus, similar experiments as described in the dissertation must be performed in BALB/c mice, before the possibility of an immunoregulatory role for anti-id D can be ruled out.

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